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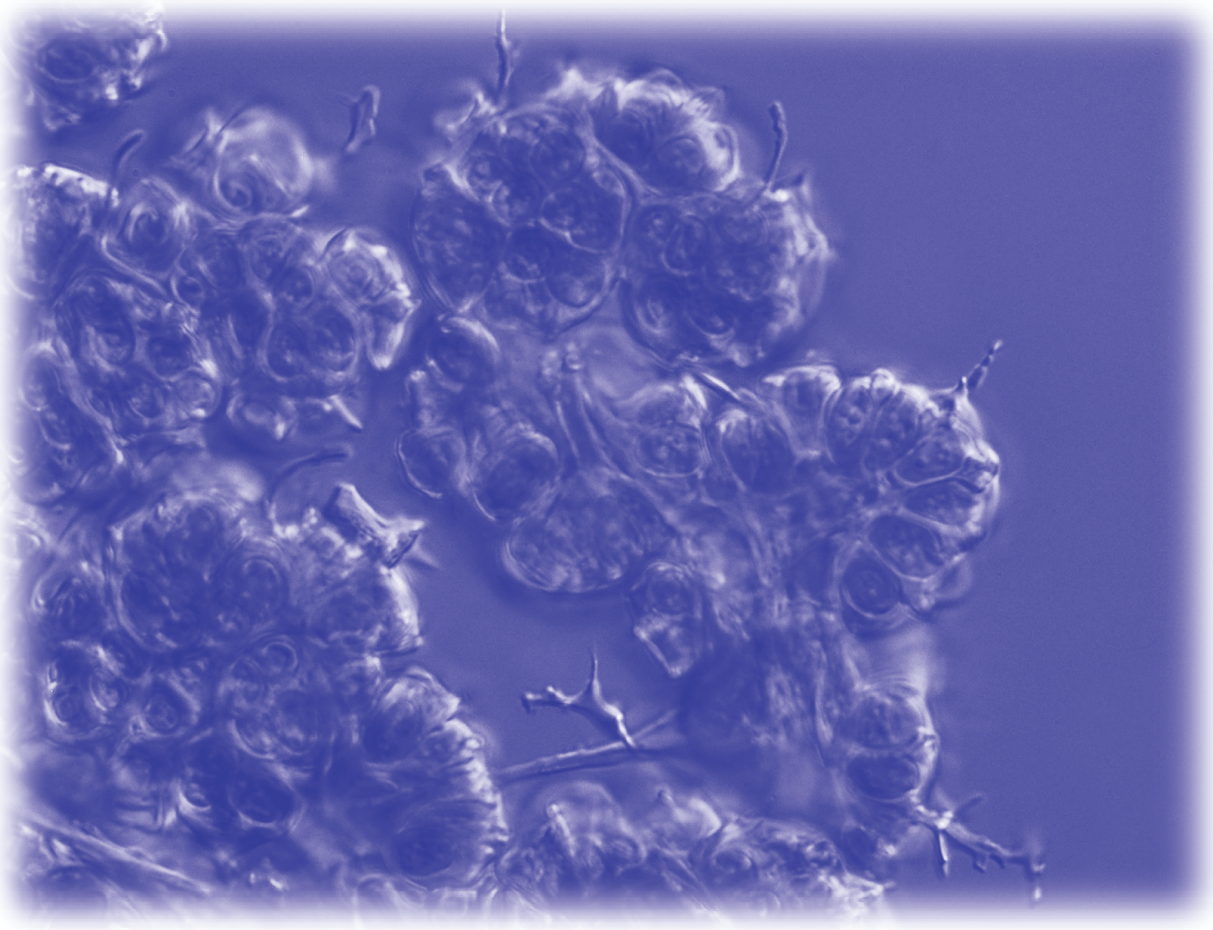

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All authors are responsible for submitting manuscripts in comprehensible US or UK English and ensuring scientific accuracy.

Original pictures on front cover:

Colony of *Botryococcus terribilis* AICB 870 connected by mucilaginous strands and showing different types of mucilaginous processes © Tiberiu Szőke-Nagy

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, 1, 2015 (p. 5-22)

Bacterial diversity in a microbial mat colonizing a man-made geothermal spring from Romania

Cecilia M. Chiriac[1,2], Lucian Barbu-Tudoran[3], Andreea Baricz[1,2], Edina Szekeres[1,2], Tiberiu Szoke-Nagy[1,2], Nicolae Dragoş[1,2] and Cristian Coman[1,2, ✉]

SUMMARY. Some of the oldest evidence of life on Earth comes from microbialites, or biologically induced carbonate deposits. Modern lithified microbial mats are considered analogues to some of the earliest Archaean ecosystems. This study investigated the bacterial diversity in a microbial mat developed on the surface of a hot spring carbonate deposit from Romania. A clone library was constructed and more than 200 partial 16S rRNA gene sequences were obtained. Phylogenetic analysis showed the existence of nine major groups. Gammaproteobacteria, Cyanobacteria and Betaproteobacteria were dominant, comprising 75% of the clone library. Verrucomicrobia, some Cyanobacteria (*Phormidium, Oscillatoria* and *Leptolyngbya*), Chloroflexi, Firmicutes and Deltaproteobacteria taxa observed in the investigated mat are common inhabitants of this type of environments. *Arthrospira platensis* and *Desertifilum tharense* (Cyanobacteria) were described for the first time in association with a geothermal habitat. Also, the representatives of Gammaproteobacteria, Betaproteobacteria, Bacteroidetes and Chrysiogenetes identified in the mat have not been described in geothermal habitats, but are known to prevail in saline, neutral to alkaline environments.

Keywords: bacterial diversity, community structure, cyanobacterial mat, hot springs.

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Introduction

Modern microbial mats, especially those dominated by cyanobacteria, are often considered analogues to some of the earliest communities on Earth (Wacey, 2009). They have been present on our planet for at least 3 billion years and are examples of self-sustainability (Noffke *et al.,* 2008, Allwood *et al.,* 2009). They had a substantial impact on the evolution of life forms as we see them today, especially due to oxygenic photosynthesis of cyanobacteria (Kasting and Howard, 2006). One of the features of microbial mats is their laminary structure in which certain groups of microorganisms are distributed in different layers. In rare cases, mineral precipitation (mainly calcite) can be observed, the process leading, in time, to the formation of stratified rocks (microbialites), thus trapping the microorganisms between layers. These microbialites are considered modern analogues of ancient stromatolites and are of great importance for studies regarding the evolution of life on Earth.

In natural aquatic settings, microorganisms form benthic biofilms that may develop into thick microbial mats (Pagaling *et al.,* 2012). Initially, the biofilm is composed of cells appertaining to few microbial groups and their extracellular polymeric substances (EPS). Over time, the biofilm becomes highly diverse with more and more microorganisms migrating into the consortium. Mature microbial mats include photosynthetic microorganisms (e.g., cyanobacteria, diatoms) and a wealth of chemoorganotrophic and chemolithotrophic bacteria (Konhauser, 2007).

Cyanobacterial communities of coccoidal and/or filamentous groups constitute major microbial mat builders (Chacon, 2010). Hot spring cyanobacterial mats are excellent model systems for biodiversity studies and are intensively surveyed worldwide (Dupraz and Visscher, 2005; Couradeau *et al.,* 2011).

As contemporary microbial mats are believed to hold the key to the past and to provide insight into the role of microbes in mineral precipitation, this paper focuses on the description of the microstructure and the bacterial diversity of a non-mineralised microbial mat that colonizes the surface of a hot spring carbonate deposit near the village of Ciocaia (Bihor County, Romania). A culture-independent approach was undertaken, as it was proven successful in other similar studies (Huang *et al.,* 2011; Pagaling *et al.,* 2012). Currently, there are very few studies on the microbial diversity in the thermophilic mats from the Western Plain of Romania where sedimentary structures were observed (Coman *et al.,* 2011; 2012). Therefore, this study further increases our knowledge of microbial diversity in this geothermal region.

Materials and methods

Sampling

Microbial mat samples were obtained from Ciocaia village (Bihor County, Romania) (470 19’ 97” N; 220 03’ 09” E). The samples were collected from the

blue-green layer directly in contact with the surface of the geothermal water flow from the drilling situated in the vicinity of the village and immediately frozen in liquid nitrogen. One sample was used for SEM (Scanning Electron Microscopy) and another for DNA extraction and clone library construction.

Optical and electronic microscopy

Microbialites lamellar structure was investigated by optical microscopy performed using a Nikon TE-2000 apparatus with a Nikon D90 digital camera. For electronic microscopy the samples were fractured in liquid nitrogen, fixed on copper holders, covered with a 10 nm gold layer and observed with a Jeol JSM 5510LV electron microscope.

DNA extraction

DNA was purified from fresh samples using the ZR Soil Microbe DNA Kit (ZymoResearch, Orange, CA, USA) according to the manufacturer's instructions. Briefly, samples were added to lysis tubes and the microbes were rapidly lysed. After centrifugation, the supernatant was transferred to a spin column and the DNA was washed twice for contaminants removal. The DNA was eluted in 35µL DNase/RNase-Free Water.

16S rRNA gene clone library construction, sequencing and phylogenetic analysis

Bacterial 16S rRNA gene fragments were amplified using universal primers 27F-1492R (Lane, 1991). The PCR mix consisted of: 1X DreamTaq Buffer (Fermentas, Vilnius, Lithuania), 1µM dNTP mix (Fermentas, Vilnius, Lithuania), 0.25 µM of each primer (synthesized at Eurogentec - Liège, Belgium), 1.5 units of DreamTaq polymerase (Fermentas, Vilnius, Lithuania), and 50 ng of DNA in a final volume of 50 µl. The PCR program included 1 cycle of initial denaturation at 94°C for 3 min, followed by 30 cycles with a denaturing step of 45 sec at 94°C, an annealing step of 1 min at 53°C, and an elongation step of 2 min at 72°C. The final elongation was performed for 10 min at 72°C.

The PCR products were purified using the GeneJET Gel Extraction Kit (Fermentas, Vilnius, Lithuania) and a clone library (C3b) was constructed using the InsTAclone PCR cloning kit (Fermentas, Vilnius, Lithuania). The clones were partially sequenced at Macrogen (The Netherlands) using the M13F-pUC primer. The resulting 16S rRNA gene sequences, containing the V1-V4 variable regions, were tested for chimeras using Bellerophon (Huber *et al.,* 2004) and compared to sequences stored in the GenBank nucleotide database using the blastn algorithm (Altschul *et al.,* 1990, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Operational Taxonomic Units (OTUs) were designated based on the highest scores after Basic Local Alignment Search Tool (BLAST) interrogation, with a threshold for species delimitation (where possible) of 97% or higher identity between the 16S rRNA gene sequences.

A multiple alignment was performed using ClustalX algorithm in MEGA5.1 (Tamura *et al.,* 2011). JModelTest software (Guindon and Gascuel, 2003; Posada et al. 2003) was used to select an appropriate model of sequence evolution for phylogenetic inference. Generalized Time Reversible with gamma distribution (GTR+G) was found to be the best fit model that can be applied to our 16S rRNA gene sequences. A Maximum Likelihood tree was constructed with MEGA version 5. The bootstrap analysis included 500 replicates. The 16S rRNA gene sequence from *Methanosaeta thermophila* was used as outgroup.

Accession numbers of nucleotide sequences

Partial 16S rRNA gene sequences obtained from bacterial clones used for phylogenetic analyses as described above, have been deposited in GenBank under accession numbers JX575076- JX575101.

Results

Study site and material

The Ciocaia drilling site dates from 1970 and the well is part of the Lower Pontian thermal aquifer from Săcuieni. Generally, the thermal aquifer from Săcuieni is located at depths between 1250 and 1700 m. The surface water temperature varies between 50 and 85°C (Antics and Roșca, 2003). In situ, the measured temperature was between 55°C-60°C, and the pH was ~7.5.

Because the chemical characterization of the thermal water from Ciocaia was performed by Țenu *et al.* (1981) and, in time, its composition was proven relatively constant with only minor fluctuations (Romanian Waters Administration, personal communication), repeating the chemical analysis would be redundant. The chemical composition of the geothermal water is as follows (in $mg \cdot L^{-1}$): Cl^- - 812; HCO_3^- - 7,283; SO_4^{2-} - 27.9; NH_4^+ - 7.9; Na^+ - 3,525; K^+ - 30; Ca^{2+} - 790; Mg^{2+} - 2.6; Fe^{2+} - 0.2; total mineralization: 12,106; TDS - 1,600. A particularity of the geothermal water from Ciocaia is the increased HCO_3^- concentration, which is 3 to 4 times higher than in other similar springs from this area (Coman *et al.,* 2011; 2012).

The mat presented the typical three-layered structure, observation based on the ratio between filamentous and coccoid bacteria (Fig. 1): i) an upper layer of net-like arranged cyanobacterial filaments; ii) a middle layer of filaments and coccoid cells of possibly aerobic bacteria; iii) a third layer, with very few filaments and an increased number of round-shaped, probably anaerobic taxa. The mat structure resembles that of microbial mats described by Ward *et al.,* (1998) and Pagaling *et al.,* (2012).

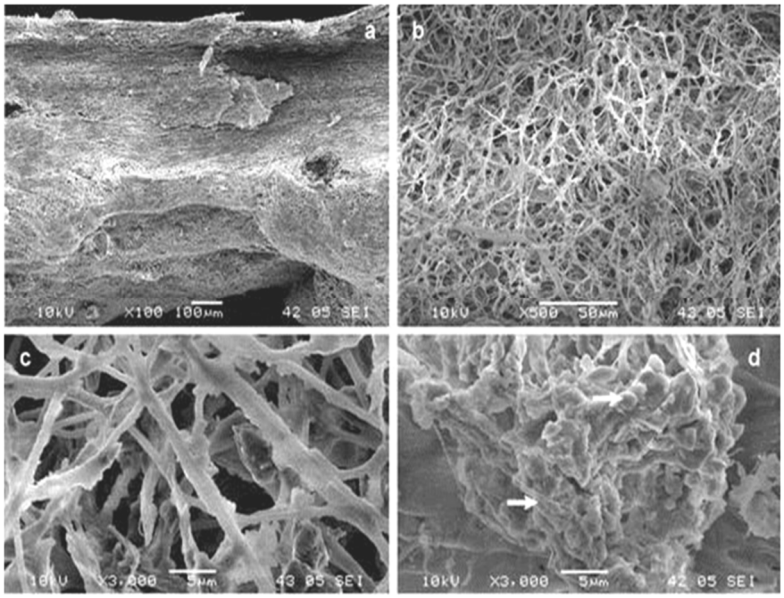

Figure 1. The structure of the Ciocaia microbial mat as observed by SEM. (a) cross-section showing the entire structure; (b-c) middle layer consisting in a tight net of bacterial filaments; (d) inner layer, with an increased number of bacteria (most-likely anaerobic) attached to the substrate.

Bacterial diversity

In order to assess the bacterial diversity in the Ciocaia mat, a 16S rRNA gene clone library (C3b) was constructed and a total of 200 partial sequences (~1000 bp) were obtained. After removing duplicates, the unique sequences were used for OTU identification using the BLAST feature in NCBI (Table 1). Both the rarefaction curve (Fig. 2), and the Chao1 index (26.0) (Yanan *et al*., 2006) showed that clones sampling was performed to saturation.

Bacterial diversity included nine major groups (Fig. 3A) of which Gammaproteobacteria, Betaproteobacteria, Cyanobacteria, Verrucomicrobia and Bacteroidetes accounted for 87% of the total clones in the 16S rRNA gene clone library. We compared OTUs from Ciocaia with OTUs from other microbial mats described in the literature and with nucleotide sequences stored in GenBank (NCBI).

Gammaproteobacteria is the dominant class in the C3b library, totalizing 41% of the sequences obtained (Fig. 3A). The three genera identified were *Ectothiorhodospira, Nitrincola* and *Aquimonas* (Table 1, Fig. 4) As far as we are aware, the last two heterotrophic genera were never encountered in association with carbonate deposits or with a geothermal habitat.

Table 1.

Bacterial OTUs detected in C3b library. The closest GenBank matches with accession numbers, percentage of identity and percentage of abundance in the clone library for each OTU are given.

	Phylum/ Class	Closest GenBank match	Accession no.	Ident. %	Abundance%
C3b-G5	Betaproteobacteria	*Azoarcus* sp. CR23	AF011328.1	96	2
C3b-A2	Betaproteobacteria	*Azoarcus* sp. KH32C	AP012304.1	98	2
C3b-B7	Betaproteobacteria	Rhodocyclaceae bact. 5BCVA	DQ343837.1	99	2
C3b-A4	Betaproteobacteria	Beta proteobacterium 2B2	HM587245.1	97	8
C3b-C5	Gammaproteobacteria	Uncult. gamma proteobact. ST5-34	DQ501349.1	98	1
C3b-D2	Gammaproteobacteria	*Ectothiorhodospira* sp. AM4	EU252492.1	99	4
C3b-A1	Gammaproteobacteria	*Nitrincola* sp. E-048	FJ764762.1	99	30
C3b-A3	Gammaproteobacteria	Uncult. *Aquimonas* sp. clone 26	JQ183097.1	99	2
C3b-G8	Gammaproteobacteria	*Ectothiorhodospira shaposhnikovii* strain DSM2111	FR733667.1	99	2
C3b-B11	Deltaproteobacteria	*Desulfovibrio alkalitolerans* strain RT2	NR_043069	99	1
C3b-V2	Cyanobacteria	*Arthrospira platensis* Sp-11	DQ279771.1	99	3
C3b-V1	Cyanobacteria	*Oscillatoria earlei* strain NTAP016	DQ308545.1	96	2
C3b-V4	Cyanobacteria	*Phormidium* sp. 195-A12	EU282429.1	98	5
C3b-B2	Cyanobacteria	*Desertifilum tharense* PD2001/TDC4	FJ158994.1	99	3
C3b-V3	Cyanobacteria	*Leptolyngbya* sp. LEGE 07319	HM217045.1	99	9
C3b-B3	Chloroflexi	Uncult. *Hydrogenophaga* sp. clone XJ64	EF648133.1	89	1
C3b-B4	Chloroflexi	Uncult. Chloroflexi bacterium clone TDNP_Bbc97_242_1_63	FJ516783.1	86	2
C3b-E8	Bacteroidetes	*Flexibacter ruber* IFO 16675	AB078064.1	99	1
C3b-F11	Bacteroidetes	Uncult. *Sphingobacteria* bacterium clone A831	EU283540.1	94	1
C3b-D12	Bacteroidetes	*Belliella pelovolcani* strain CC-SAL-25	EU685336.1	89	1
C3b-C1	Bacteroidetes	Uncult. bact. clone ambient_alkaline-120	GU455103.1	99	7
C3b-H2	Bacteroidetes	Uncult. bacterium clone Pb40	HQ857681.1	96	2
C3b-D7	Verrucomicrobia	Uncult. Verrucomicrobiales clone	FJ516831.1	92	1
C3b-F2	Verrucomicrobia	Uncult. *Verrucomicrobium* sp. clone 78 T12d+oil	FM242437.1	91	5
C3b-E2	Chrysiogenetes	*Desulfurispirillum indicum S5*	NR_074463	99	2
C3b-F4	Firmicutes	Uncult. Firmicutes bacterium clone x1	GQ848202.1	97	1

Interestingly, the C3b_C5 clone, showing a high identity score to an unidentified gammaproteobacterium (Table 1), clustered together in the phylogenetic tree with *Alishewanella jeotgali*, with a bootstrap value of 100 (Table 1; Fig. 4) and until now was not encountered in a thermophilic microbial mat.

In the C3b library, Cyanobacteria, representing 18% of the total clones sequenced (Fig. 3A), comprises five OTUs identified either at species level (*Arthrospira platensis* and *Desertifilum tharense*) or at the genus level (*Oscillatoria* sp., *Phormidium* sp. and *Leptolyngbya* sp.) (Table 1; Fig. 4). Species of *Oscillatoria*, *Phormidium* and *Leptolyngbya* genera are common inhabitants of thermophilic microbial mats and present a worldwide distribution (Bryanskaya *et al.,* 2006; Sompong *et al.,* 2008).

The class Betaproteobacteria encompasses purple nonsulfur bacteria with high metabolic versatility. It represents 16% of the C3b library (Fig. 3A), with clones affiliated to *Azoarcus* sp. and to an unidentified *Rhodocyclaceae* bacterium (Table 1; Fig. 4).

Verrucomicrobia covers 6% of the Ciocaia clone library (Fig. 3A), one OTU being identified at the genus level (*Verrucomicrobium* sp.) (Table 1; Fig. 4). It is a widespread phylum, inhabiting a wide range of habitats (Kanokratana *et al.,* 2004; Bohorquez *et al.,* 2012).

The Bacteroidetes group comprises 6% of the C3b clone library, several 16S rRNA gene sequences presenting a high degree of identity with *Flexibacter* sp., *Belliella* sp. and some uncultured taxa (Table 1; Fig. 4). *Flexibacter* sp. was weakly represented at Ciocaia thermo-mineral spring, with clones that were affiliated to *Flexibacter ruber* (99% sequence identity), described in a hot spring in Yellowstone National Park (USA).

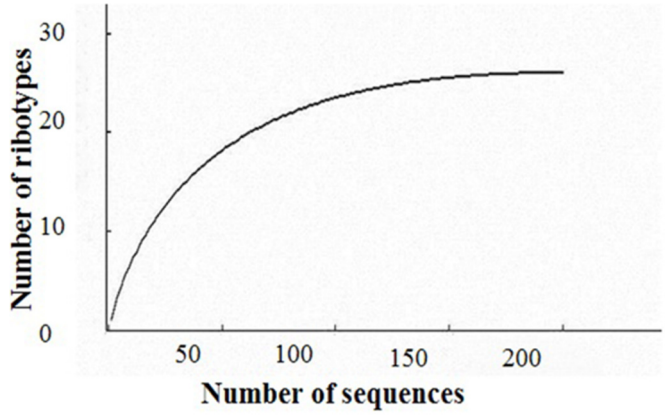

Figure 2. Rarefaction curve for the C2b library. The number of detected OTUs was plotted against the cumulative number of individuals (i.e., clones) analysed.

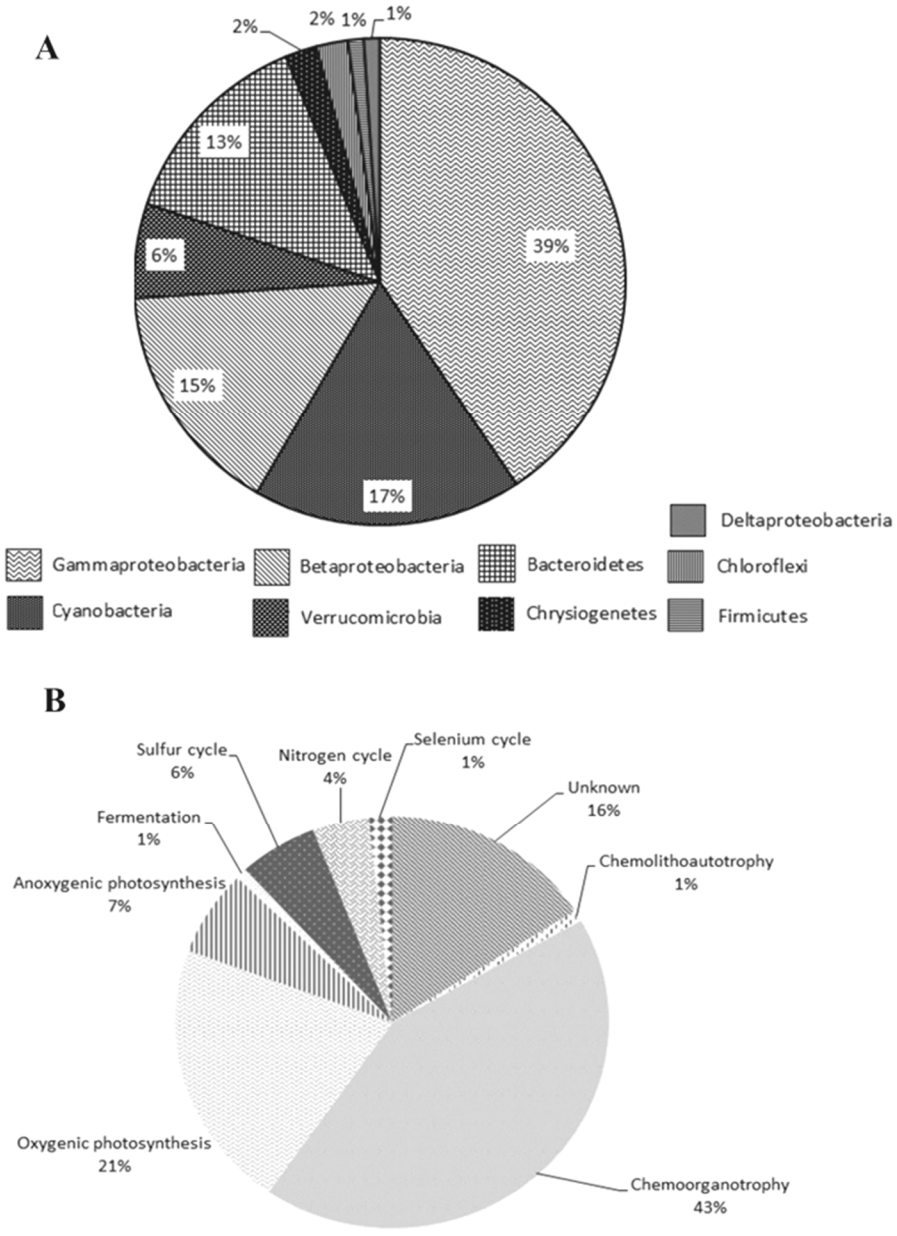

Figure 3. (A) Distribution of major groups in the Ciocaia bacterial clone library; (B) Putative functional groups encountered in the Ciocaia sample.

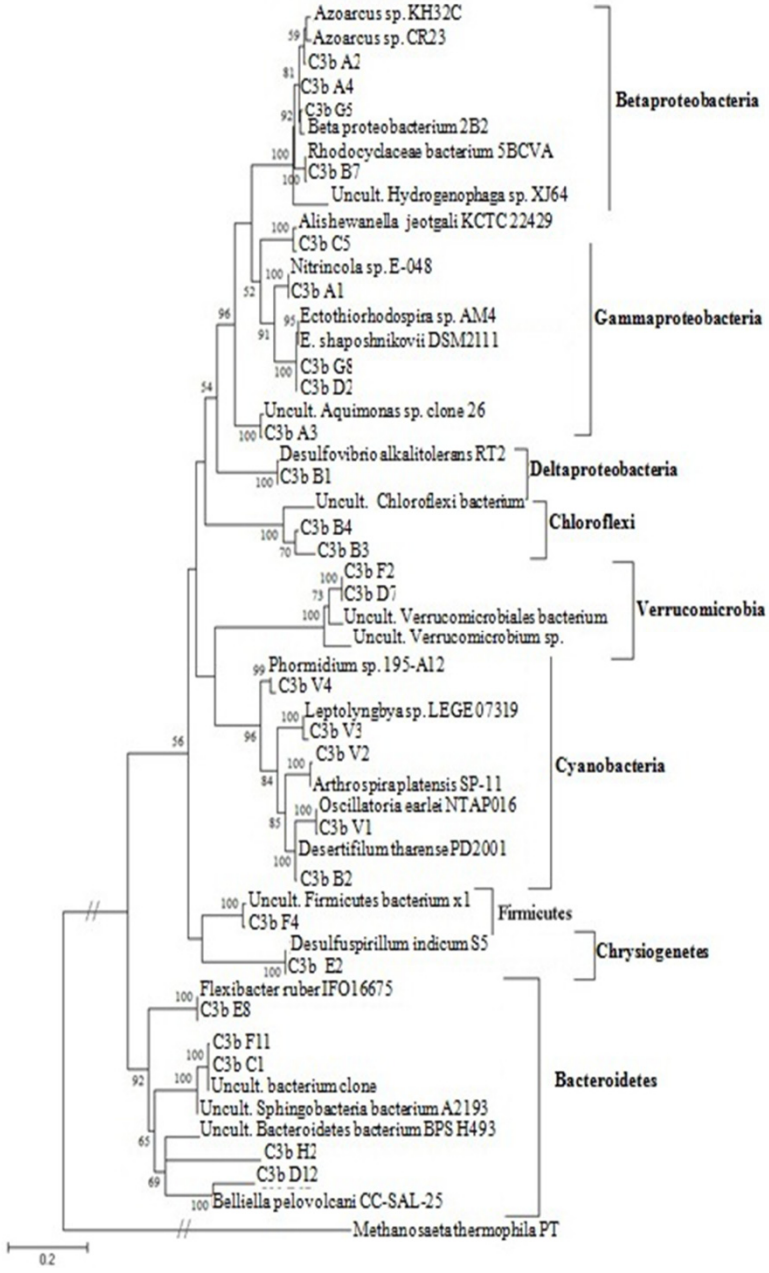

Figure 4. Maximum-likelihood tree (MEGA 5) showing the phylogenetic relationships of bacterial 16S rRNA gene sequences cloned from the microbial mat colonizing the geothermal spring near Ciocaia. Bootstrap values <50% are not shown.

Members of the *Belliella* genus are known to have a preference for carbonate-rich waters (Akhwale *et al*., 2015). Also, they were previously described as being in close association with *Nitrincola* sp. (Hamamura *et al.,* 2012), one of the most abundant proteobacterial taxon in our sample.

Around 11% of the C3b bacterial clone sequences were affiliated with minor groups, such as Chrysiogenetes, Chloroflexi, Firmicutes, Deltaproteobacteria and some unidentified bacteria (Fig. 3A and 4). The majority of these sequences are closely related to thermophilic OTUs isolated from thermal, alkaline environments (Kanokratana *et al.,* 2004; Bryanskaya *et al.,* 2006; Rauschenbach *et al.,* 2011).

Discussion

Putative functional role of bacterial OTUs within the Ciocaia microbial mat

Phylogenetic analysis performed in this study allowed classification of the majority of the community members at the species/genus level. Thus, a diverse range of putative metabolic pathways can be identified within the mat. Despite the fact that 16S rRNA gene-based analysis is not always completely correlated with similarity of metabolic pathways and caution in attributing functional roles within the microbial community is necessary, a possible scenario regarding the ecological interactions among the observed bacterial groups can be presented (Fig. 3B).

As in many other microbial mats belonging to different environments, cyanobacteria are a dominant group, being the primary producers and the major fraction involved in N_2 fixation (e.g. *Leptolyngbya*) along with *Azoarcus* (Reinhold-Hurek *et al.,* 1993; Charpy *et al.,* 2010). Because the organisms included in this phylum are light dependent, they are distributed at the surface of the carbonate deposit. On account of the death of the primary producers, a compact biomass is accumulated that supports the development of aerobic heterotrophs (e.g. *Belliella, Aquimonas, Verrucomicrobium, Nitrincola*) in the upper layers and anaerobic species in the lower layers (e.g. *Desulfovibrio, Desulfurispirillum*). It is known that Cyanobacteria are a key component of microbial mats and that they are responsible for early lithification of stromatolites, thus linking the studied microbial mat with the formation of the carbonate deposits (Konhauser, 2007).

Specific metabolic bacterial groups have vertical distribution based on the concentration of various gas-phase nutrients such as H_2S, O_2 or CO_2. They tend to be limited, and so, their consumption/production rates may dictate the distribution of various metabolic groups. Usually, under the cyanobacterial layer are found other groups that have photosynthetic or phototrophic nutrition, usually purple sulfur bacteria (e.g., *Ectothiorhodospiraceae*) and green non-sulfur bacteria (e.g., Chloroflexi). Although Cyanobacteria are the most commonly detected microbial group in thermophilic mats, they have been reported to dominate these mats at the functional level together with phototrophic Chloroflexi (Boomer *et al.,* 2000; Portillo *et al.,* 2009). The oxygenic photosynthesis of Cyanobacteria is considered to depend on the sulfide depletion by the anoxygenic *Chloroflexus* sp. (Jørgensen and Nelson, 1988).

Ectothiorhodospira, a genus of purple-sulfur bacteria, are able to perform photosynthesis under anoxic conditions, without O_2 production (Mobberley *et al.,* 2012). Thus, their upper border in the microbial mat is determined by the limit of H_2S diffusion and the lower limit by the light penetration into the mat. Chloroflexi phylum contains genera that are able to perform anoxygenic photosynthesis, but some species were found to exhibits metabolic diversity, growing either as aerobic chemoheterotrophs or as anaerobic photoheterotrophs. Using electrons extracted from H_2 or H_2S, they can fixate CO_2 through the 3-hydroxypropionate pathway instead of Calvin cycle (Konhauser, 2007; Bolhuis and Stal, 2011).

The organic matter synthesized by photosynthetic and chemolithoautotrophic (e.g. *Hydrogenophaga*) microorganisms can be used by various organotrophic (heterotrophic) bacteria in the Ciocaia mat: *Nitrincola* sp., *Aquimonas* sp., *Flexibacter* sp., *Belliella* sp., *Desulfovibrio alkalitolerans*, probably the Firmicutes taxa and *Azoarcus* sp (Reinhold-Hurek *et al.,* 1993; Brettar *et al.,* 2004; Saha *et al.,* 2005). Some heteroorganotrophs, such as *Desulfovibrio alkalitolerans,* are using sulfate, sulfite and thiosulfate as electron acceptors. The presence of autotrophic sulfur-oxidizing bacteria together with heterotrophic sulfate-reducing microbes may imply the coupling of carbon and sulfur cycles (Antony *et al.,* 2013).

An unexpected discovery was the presence of *Desulfurispirillum indicum* in our sample. These organisms are able to utilize selenite, nitrate and arsenate as electron acceptors. It was previously identified in an estuarine canal in Chepau, India (Rauschenbach *et al.,* 2011), but this is the first report of *Desulfurispirillum indicum* in thermal environments or in association with carbonate deposits. The ability of this species to reduce nitrate to ammonium illustrates that nitrogen and carbon cycles could be inter-connected in this microbialite (Antony *et al.,* 2013).

Overall, the Ciocaia mat harbors microbial taxa with diverse types of metabolism. As a possible consequence of the temperature and pH values in the Ciocaia spring, there was no absolute dominance by a single bacterial group. We can assume that there is an ecologically balanced community, with well-defined metabolic niches.

High-temperature and carbonate specific OTUs from Ciocaia sample

Even though the phylogenetic analysis revealed a low diversity of bacterial 16S rRNA gene sequences in the C3b clone library, this situation resembles that of other hot spring mats described in literature (Huang *et al.,* 2011; Pagaling *et al.,* 2012). Certain clone sequences were related to others of low-temperature origin, like soil or marine environments (Fig. 5A), but this situation was also observed in other hot spring biodiversity studies (Song *et al.,* 2009; Huang *et al.,* 2011). Besides contamination with DNA from the surrounding environment, another possible explanation could be that microorganisms with a significant level of 16S rRNA gene sequence similarity may have distinct physiological properties (Jaspers and Overmann, 2004), especially when other features except the temperature (e.g., alkalinity, salinity) create an environment suitable for colonization.

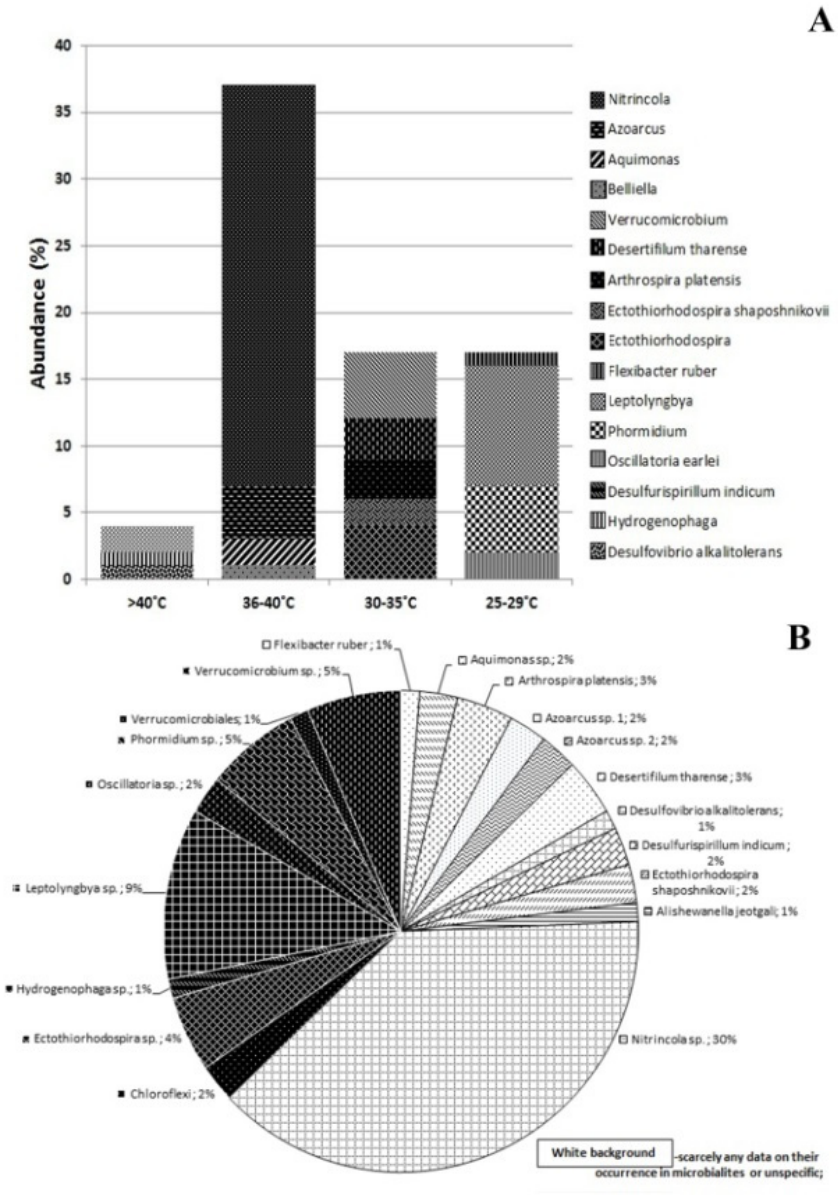

Figure 5. (A) OTUs abundances in relation with their optimum growth temperature. The majority of species have a lower optimum growth temperature than the water temperature which varies between 50 and 85°C; (B) Carbonate specific (black background) and carbonate non-specific (white background) bacterial taxa encountered in Ciocaia microbialite.

The dominant group in the C3b clone library is represented by the gammaproteobacterial OTUs (e.g. *Nitrincola*), a group that was not previously described in literature as being associated with geothermal environments. Nevertheless they have a tendency to colonize saline, alkaline environments (Mwirichia *et al.,* 2011; Hamamura *et al.,* 2012; Antony *et al.,* 2013). Organisms from *Aquimonas* genus were previously isolated from a warm water spring. Because the optimum physico-chemical parameters for the type species, *A. voraii*, are very similar to those in the Ciocaia spring, *Aquimonas* presence in our sample is not surprising (Saha *et al.,* 2005). This genus was found before in a microbial mat from Movile cave, Romania (Chen *et al.,* 2009), but was never encountered in association with carbonate deposits. In the microbial mat from the Ciocaia hot spring habitat, we also encountered the cyanobacteria *Arthrospira platensis*. Even though the harsh conditions that prevail in this environment may cause difficulties for colonization, the increased HCO_3^- concentration in the Ciocaia thermal water may favor the spread of *Arthrospira platensis*, its affinity to high bicarbonate levels and moderately termophilic waters being previously documented (Whitton and Potts, 2000; Fujisawa *et al.,* 2010).

Our study reports for the first time the presence of *Desertifilum tharense* in a hot spring microbial mat. This taxon is a desert cyanobacterium described by Dadheech *et al.* (2012) from an arid area in India. *Microcoleus steenstrupii*, observed in the hot spring mat from Marghita, Romania (Coman *et al.,* 2011), was initially considered a desert cyanobacterium (Garcia-Pichel, 2002), but it was later encountered in other hot spring mats (Boyer *et al.,* 2002; Coman *et al.,* 2011). The fact that 16S rRNA gene sequences belonging to these two cyanobacteria were observed in the C3b clone library does not necessarily imply an active role within the bacterial community. Thus, future culture-dependent studies should be undertaken in order to confirm their functionality in the Ciocaia microbial mat.

As it can be observed in Fig. 5B, the majority of hot spring OTUs could be assigned to carbonate specific or non-specific groups, based on literature data. In contrast to other microbialite communities, the unique character of Ciocaia community is given by the fact that approximately two-thirds of the bacterial community is included in OTUs that were never described in association with carbonate-impregnated structures. Nevertheless, these OTUs are known to have a preference for an alkaline pH that may favour precipitation of carbonate especially in waters with increased HCO_3^- concentration (Saha *et al.,* 2005; Antony *et al.,* 2013).

An alkaline microenvironment can be achieved within the mat most likely through the metabolic activity of different bacterial groups. In mesothermal environments, rich in HCO_3^-, the external pH can increase significantly, as inorganic carbon is consumed by cyanobacteria faster than it can be replaced from the geothermal water (Badger *et al.,* 2006). Sulfate-reducing bacteria (SRB), observed in the C3b clone library in both Deltaproteobacteria and Firmicutes groups, can take part in increasing the environment's alkalinity by generating

carbonate ions through sulfate reduction. Part of the H_2S consumed by anoxygenic photosynthesis may come from the activity of SRB as well. Heterotrophic types, observed in almost all bacterial groups from Ciocaia (e.g., Betaproteobacteria, Bacteroidetes, Chrysiogenetes, Verrucomicrobia), can also increase the environment pH towards alkalinity by decomposing organic residues (Baumgartner *et al.,* 2006; Konhauser, 2007).

The results of this study gave rise to the hypothesis that, in some cases, when a proper ecological niche is created, bacterial diversity can be influenced by abiotic factors (e.g., pH, temperature). Future studies, mainly culture-dependent, should be undertaken, in order to confirm this observation.

Conclusions

The discovery of new modern stromatolites and the characterization of their microbial diversity are very important in order to understand the microbe-mineral relationship in the formation of sedimentary structures. This study was focused on the structure and bacterial diversity investigation of a microbial mat that formed above a man-made geothermal spring from Romania. The mat presented a laminated structure similar to the models described in literature. Twenty-six OTUs were identified, grouped in nine major bacterial groups, Gammaproteobacteria, Cyanobacteria and Betaproteobacteria being dominant. Verrucomicrobia, some Cyanobacteria (*Phormidium, Oscillatoria* and *Leptolyngbya*), Chloroflexi, Firmicutes and Deltaproteobacteria observed in the Ciocaia mat are common inhabitants of these types of environments. *Atrhrospira platensis*, *Desertifilum tharense* and *Desulfurispirillum indicum* were reported for the first time in association with a geothermal habitat. The representatives of Gammaproteobacteria, Betaproteobacteria, Bacteroidetes and Chrysiogenetes identified are not typical for geothermal habitats, but are known to colonize saline, neutral to alkaline environments. Further cultivation and physiological studies should be undertaken in order to determine whether they actively inhabit the microbial mat around the geothermal spring from Ciocaia and to assess which is their putative functional role within the community. Overall, this study has provided valuable information about the diversity of microorganisms that inhabit the thermal environments from the Western Plain of Romania. In addition, due to the incipient sedimentary structures observed, the geothermal well from Ciocaia is an important site for future studies regarding the results of microbe-mineral interactions on the formation of modern stromatolites.

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Identification, isolation and bioinformatic analysis of squalene sinthase-like cDNA fragments in *Botryococcus terribilis* AICB 870 strain

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SUMMARY. *Botryococcus terribilis* is a freshwater colonial green microalga very similar to *B. braunii,* due to the hydrocarbon biosynthesis and accumulation of those biosynthetic products in the extracellular matrix. The hydrocarbon biosynthesis pathway was intensively studied, especially in differents strains of *B. braunii.* Recent studies revealed the presence of three squalene synthase-like (SSL) enzymes involved in the last steps of hydrocarbon biosynthesis from *B. braunii*. The aim of the study is to identify homologous SSL enzymes to *B. terribilis* AICB 870, a freshwater isolate from Bihor County (Romania), based on new isolated cDNA fragments and bioinformatics analysis of sequenced fragments. Light and fluorescence microscopy observation revealed that AICB 870 strain presents features similar to a *B. terribilis* species, especially simple or branched mucilaginous processes and a high number of lipids vesicles. PCR primers designed using SSL nucleotide sequences from *B. braunii* were successfully used to amplify homologous SSL cDNA fragment in the AICB 870 strain. Bioinformatic analysis of nucleotides and translated amino acid sequences including G+C content, nucleotide frequencies, amino acids frequencies, computed Mw/pI and transmembrane motif prediction showed a high degree of similarity between the SSL identified as pertaining to *Botryococcus braunii* and those generated in the present work. The results of the present study pointed out for the first time the presence of three squalene synthase-like enzymes in a strain of *B. terribilis* species.

Keywords: Bioinformatics analysis, *Botryococcus terribilis,* cDNA, hydrocarbons, squalene synthase-like.

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Introduction

Botryococcus genus comprises 16 species, of which 13 are currently accepted, including *Botryococcus braunii* Kutzing (Guiry and Guiry, 2015). *B. braunii*, especially strains belonging to race B, has been the most studied species of the genus in respect to hydrocarbon biosynthesis. Recently, beside *B. braunii*, another species pertaining to *Botryococcus* genus, named *B. terribili*s, was reported to accumulate hydrocarbons, mostly botryococcenes (Hegedűs *et al.*, 2014).

Botryococcus braunii is a freshwater, colonial, green microalga, which can synthesize high amounts of hydrocarbons within the cell that are accumulated in the extracellular colonial matrix. The hydrocarbons can constitute between 27 and 86% of its dry weight (Brown *et al.*, 1969).

The *B. braunii* strains can produce various types of hydrocarbons, which have been traditionally classified into three chemical races (A, B and L), according to the hydrocarbon oils synthesized: i) race A produce odd-numbered (C_{23} to C_{33}) n-alkadiene and triene (Largeau *et al.*, 1980; Metzger *et al.*, 1985), derived from fatty acids through the very long-chain fatty acids elongation pathway (Baba *et al.*, 2012); ii) race B produce unsaturated triterpene (squalenes, botryococcenes and their methylated derivatives), having general formula C_nH_{2n-10}, n=30-37 (Metzger *et al.*, 1987; Okada *et al.*, 1995); iii) race L which produce a single type of hydrocarbon known as lycopadiene ($C_{40}H_{78}$) (Metzger *et al.*, 1990). Moreover, recently, a new chemical race was described based on GC/MS analysis named race S, which produces epoxy-n-alkane and saturated n-alkane (Kawachi *et al.*, 2012).

Strains belonging to race B are intensively studied because their hydrocarbons have been converted by standard hydrocracking reactions to combustible fuels, including gasoline, kerosene and diesel (Hillen *et al.*, 1982).

Biosynthesis of hydrocarbons in race B takes place in the presence of isopentenyl diphosphate (IPP), supplied through the mevalonate-independent pathway (Sato *et al.*, 2003). Subsequently, farnesyl diphosphate (FPP) is produced from IPP and its isomer dimethyl diphosphate (DMPP). Further, the hydrocarbon biosynthesis can be described as a two-step reaction mechanism (Poulter, 1990): firstly, two molecules of the FPP form presqualene diphosphate (PSPP) through a head-to-head condensation (Sasiak and Rilling, 1988); secondly, in the presence of NADPH, farnesyl moieties are rearranged in order to form squalene with a C1`-1 linkage or botryococcenes with a C1`-3 linkage, between the farnesyl moieties (Blagg *et al.*, 2002).

The enzymes associated with hydrocarbon biosynthesis have been mostly unknown, until Niehaus *et al.* (2011) isolated and characterized three unique squalene synthase-like (SSL-1, SSL-2 and SSL-3) genes by screening cDNA libraries under low stringency hybridization and by computational screening of *B. braunii* transcriptomic data. According to Niehaus *et al.* (2011) the yeast expression experiments of single

SSL enzymes revealed that the SSL-1 is involved in the biosynthesis of PSPP from two moieties of FPP, the SSL-2 produce especially bisfarnesyl ether and low amounts of squalene, while SSL-3 did not lead to the accumulation of any major product. Subsequently, the coexpression of SSL-1 and SSL-2 revealed the biosynthesis of squalene and significant amounts of bisfarnesyl ether, but interestingly, when SSL-1 and SSL-3 were coexpressed together, botryococcenes biosynthesis occurred. These new findings are contradictory to the hypothesis that only squalene synthase (SQS) catalyzed both reactions from the last steps of the hydrocarbon biosynthesis pathway (Okada *et al.*, 2004).

Although the SQS enzymes were described among different taxa (a short list is detailed in the Material and Methods section), including some species of algae, e.g. *Chlamydomonas reinhardtii* (Merchant *et al*., 2007), *Auxenochlorella protothecoides* (Gao *et al*., 2014), *Bathycoccus prasinos* (XP_007512409.1), the SSL enzymes were solely described and studied in *Botryococcus braunii* (Niehaus *et al*., 2011; Bell *et al*., 2014). Thus, the aim of this study was to determine the presence of homologous squalene synthase-like enzymes in *B. terribilis* AICB 870, an algal strain isolated from Bihor County (Romania), study based on direct sequencing of cDNA fragments, synthesized from purified algal RNA and bioinformatics analyses. The importance of searching for SSL enzymes in other (*Botryococcus*) species is given by the possibility to compare the hydrocarbons biosynthesis pathways in order to: i) find one strain with a higher growth rate which can produce large amounts of hydrocarbons; ii) compare the SSL gene expression levels and correlate them with the amount of hydrocarbons biosynthesized; and iii) based on the previously mentioned reasons, it is possible to identify a *Botryococcus* strains with a particular set of SSL gene, which can be further overexpressed in bacteria, yeast or other organisms with higher growth rate in order to obtain large amounts thus efficiently increasing the production of hydrocarbons.

Material and methods

Strain culture

The AICB 870 strain was isolated from Cristur fishpond, Bihor County and AICB 874 strain was isolated from Tăureni fishpond, Mureş County, Romania, both algal strains are deposited in the Algal and Cyanobacterial Culture Collection (AICB) at the Institute of Biological Research, Cluj-Napoca, Romania (Dragoş *et al.,* 1997). The algal cultures of *B. terribilis* AICB 870 and AICB 874 were grown on BBM medium, under continuous irradiation of approximately 150 µmol photons $m^{-2}s^{-1}$, a temperature of 25°C±2°C, in 500 ml conical bubbler using a continuous airlift system. The biomass was harvested in the exponential growth phase and aliquoted for subsequent molecular analysis.

Light and fluorescence microscopy

Light and fluorescence microscopy was performed using a Nikon TE-2000 Eclipse microscope equipped with a Nikon D90 photo camera. For hydrocarbon visualization, cells were collected by centrifugation and stained using 1 µl of Nile red (1 mg/ml stock solution dissolved in acetone). Samples were kept in the dark for 5 min, diluted with 1 ml BBM medium and centrifuged 1 min at 10,000 rpm. The rinsing process was repeated three times, in order to remove the excess of dye, followed by fluorescence microscopy analysis (Weiss *et al*., 2012).

RNA Isolation and cDNA synthesis

In order to obtain sufficient quantities of high quality RNA, seven commercially available kits (*Innu PREP RNA Kit* - Analytik Jena, Germany; *Direct-zol™ RNA MiniPrep* - Zymo Research, USA; *ZR RNA MicroPrep™* - ZymoResearch, USA; *ZR Plant RNA MiniPrep™* - Zymo Research, USA; *SV Total RNA Isolation System* - Promega, USA; *TRIzol®* - Ambion-Life Technologies, USA; *Isolate Plant RNA/RNA Kit* - Bioline, UK) and two protocols (Kim *et al.,* 2012; Ghawana *et al.,* 2011), were tested on the AICB 874 strain (chemical race A), and then the best protocol was used on the AICB 870 strain. This RNA extraction strategy was approached due to the following reasons: i) the AICB 874 strain has a simplified cellular organization because colonies rarely occur and cell walls are less rigid; ii) the doubling time of AICB 874 is much higher, thereby cell yields is higher than at the AICB 870; iii) the AICB 874 strain do not have the extracellular matrix, thereby RNA lysis/extraction buffer can disrupt the cells much better than the other strain. All tested kits and protocols were performed according to manufacturer`s and authors instructions. Subsequently, the most efficient protocol was used to extract total RNA from the AICB 870 strain.

RNA electrophoresis was performed using 1.2% formaldehyde denaturing agarose gel in order to verify the integrity of RNA (Farrell, 2010). RNA quantification was performed on a NanoDrop 2000 spectrophotometer (ThermoScientific, USA).

Before cDNA synthesis, RNA samples were treated with DNase. 1U of Turbo DNase Free, (Ambion Europe, UK) was used in order to remove the residual genomic DNA. The cDNA was obtained with a First Strand cDNA Synthesis Kit (ThermoScientific, SUA), using oligo $d(T)_{18}$ primers.

PCR Amplification, Cloning and Sequencing

In order to amplify the SSL cDNA fragments, three pairs of primers were designed for each SSL using Primer-BLAST (Ye *et al.,* 2012) and tested *in silico* with FastPCR 6.4 (Kalendar *et al.,* 2011).

The PCR was carried out with the newly designed primers as follows: each 50 µl reaction volumes containing 1U of Thermo Scientific™ DreamTaq™ DNA Polymerase in 5 µl of the manufacturer`s buffer, 0.25 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, and approximately 300 ng of cDNA template. The Touchdown PCR (TD-PCR)

was performed in a TProfessional TRIO Termocycler (Biometra, Germany). TD-PCR cycling condition were: initial denaturation at 94 °C for 2 min followed by 19 cycles of 94 °C for 50 s, T_a+10 °C for 55 s (with an increment of -0.5 °C / cycle), 72 °C for 60 s, followed by another 19 cycles of 94 °C for 50 s, T_a for 55 s, 72 °C for 60 s and a final extension at 72 °C for 2 min.

The PCR products were verified by electrophoresis on a 1% agarose gel in 1 X TAE running buffer, stained with ethidium bromide (1 µg/ml), and visualized on an UVP transilluminator. Subsequently, the PCR products were purified with GeneJet™ Gel Extraction Kit (Fermentas, Canada) and cloned into the pGEM®-T vector (Promega, USA) following the manufacturer's instructions. Plasmids were isolated with GeneJet™ Gel Plasmid MiniPrep Kit (Fermentas, Canada), and sequenced by a commercial company (Macrogen, The Netherlands), with both forward and reverse M13 primers.

Bioinformatic analysis of nucleotides and translated amino acid sequences

The SSL nucleotide sequences obtained in this study were manually corrected for mismatches and ambiguous nucleotides using Chromas Lite 2.1.1 software (Technelysium, Australia). Nucleotide distribution and frequency between our sequences and those from *B. braunii* (HQ585060.1-3) were analyzed with CLC MainWorkbench 7.6 (CLCbio, Danemark).

Using the SSL nucleotide sequences obtained, amino acid sequences were generated using Translate, from ExPaSy (Gasteiger *et al.,* 2005). In order to identify the similarities or differences between SSL sequences from this study and those deposited in the GenBank database (National Center for Biotechnology Information), multiple amino acid sequences alignments were performed using MEGA 6.06 (Tamura *et al.,* 2013). The following homologous and biochemically characterized SQS sequences from GenBank were used for the multiple sequences alignment: *Homo sapiens* - AAA36645.1 (McKenzie *et al*., 1992), *Rattus norvegicus* - AAA42179.1 (Shechter *et al*., 1992), *Mus musculus* - NP_034321.2 (Schechter *et al*., 1994), *Arabidopsis thaliana* - P53799.1 (Nakashima *et al*., 1995), *Solanum chacoense* - AEX26932.1 (Ginzberg *et al*., 2012), *Nicotiana tabacum* - AAB08578.1 (Devarenne *et al*., 1998), *Saccharamyces cerevisiae* - AAA34597.1 (Jennings *et al*., 1991), *Auxenochlorella protothecoides* - KFM22694.1 (Gao *et al*., 2014), *Bathycoccus prasinos* (XP_007512409.1), *Chlamydomonas reinhardtii* - EDP06129.1 (Merchant *et al*., 2007), *Botryococcus braunii* – SSL-1 - G0Y286.1 (Niehaus *et al*., 2011), *Botryococcus braunii* – SSL-2 - G0Y287.1 (Niehaus *et al*., 2011), and *Botryococcus braunii* – SSL-3 - G0Y288.1 (Niehaus *et al*., 2011).

Isoelectric point and molecular weight were predicted using Compute pI/Mw from ExPaSy (Gasteiger *et al.,* 2005). Frequencies of hydrophobic and hydrophilic residues as well as charged residues, were performed with CLC MainWorkbench 7.6 (CLCbio, Danemark). The protein secondary transmembrane domains were identified using TMHMM (Sonnhammer *et al.,* 1998), using default settings.

Results and Discussions

Light microscopy observations of the studied strain showed that *Botryococcus* sp. AICB 870 presents the features of a *Botryococcus terribilis* species, as was described by Komárek and Marvan (1992), FanésTreviño *et al.* (2010), de Queiroz Mendes *et al.* (2012) and Hegedűs *et al.* (2015).

Colonies of AICB 870 strain are ellipsoid or spheroid in shape and sometimes subcolonies can be clearly seen, connected by mucilaginous strands (Fig.1 a-c, black arrow). Simple or branched mucilaginous processes were observed at the periphery of the colony (Fig.1 a-c, white arrow). Occasionally, small lipid droplets can be seen at the surface of the mucilaginous processes. Pyriform-shape cells, more or less radially oriented, are usually completely embedded in a hydrocarbon-rich colonial matrix or slightly emerged from it (Fig. 2 c).

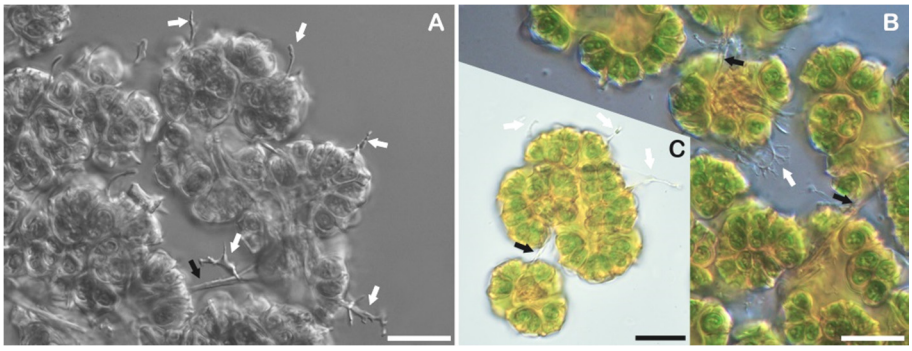

Figure 1. Light micrograph of *Botryococcus terribilis* AICB 870. (A-C) Subcolonies connected by mucilaginous strands (black arrow) and different types of mucilaginous processes (white arrow). Bars = 20 µm.

Colonies stained with a Nile red dye (Fig.2 a-d) showed numerous lipid bodies within the cells (Fig. 2 c-d). Lipid bodies play a key role in hydrocarbon secretion, finally resulting in the accumulation of hydrocarbons in the colonial matrix.

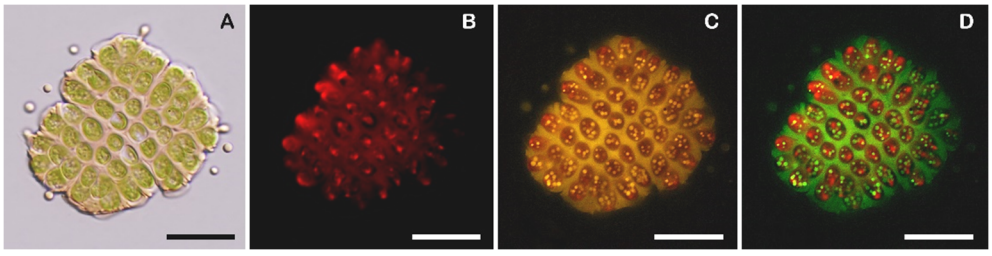

Figure 2. Colony of *Botryococcus terribilis* AICB 870 stained with Nile red dye. (A) Color DIC microscopy image. (B) Chlorophyll autofluorescence - channel. (C) Nile red stained colony. (D) Merged chlorophyll autofluorescence and Nile red stained colony (false colored green). Bar = 20 µm.

RNA Isolation

The results of RNA extraction are presented in Table 1. The RNA yield obtained with the available kits (sample A-G) varied between 9.4 and 175.9 ng/μL, with a very high $A_{260/280}$ ratio but with a low $A_{260/230}$ ratio. Quantitatively, the highest RNA concentration was obtained in the samples H (867.2 ng/μL) and I (3228.4 ng/μL) from AICB 874, further protocol H and I were tested at the AICB 870 (samples H* and I*).

RNA electrophoresis yielded faint bands in samples A-G (except sample D and F) and highly strong bands in samples H, I, and I*. RNA isolated from sample H* was partially or totally degraded. DNA contamination was confirmed in all samples.

The low quality and quantity of the extracted RNA performed with the commercially available kits may be due to the lacking of lysis buffer in order to disrupt the cells and extract the total RNA. This disadvantage was removed from the tested protocols because phenol was used as lysis and extraction buffer combined with silica beads and freeze/thaw.

Table 1.

The RNA yield (ng/μL), $A_{260/280}$ and $A_{260/230}$ ratios of all tested protocols.

Kit / Protocol	***Sample***	***ng/μL***	***$A_{260/280}$***	***$A_{260/230}$***
Innu PREP RNA Kit	A	16.0	2.18	0.92
Direct-zol™ RNA MiniPrep	B	57.9	1.94	1.57
ZR RNA MicroPrep™	C	129.3	1.88	0.78
ZR Plant RNA MiniPrep™	D	175.9	2.02	1.54
SV Total RNA Isolation System	E	9.4	1.65	0.35
TRIzol®	F	162.4	2.00	1.29
IsolatePlant RNA/RNA Kit	G	18.7	1.99	0.82
Kim *et al.*, (2012)	H	867.2	2.04	2.45
Ghawana *et al.*, (2011)	I	3228.4	1.98	1.92
Kim *et al.*, (2012)	H*	229.6	2.02	2.28
Ghawana *et al.*, (2011)	I*	966.1	1.99	1.51

The great advantage of the last tested protocol (Ghawana *et al.*, 2011) is the ability to extract very large amounts of RNA, but there are several disadvantages: residual DNA present in RNA probes and toxicity of the phenol used as extraction agent.

PCR Amplification of cDNA fragments

Starting from the SSL sequences published by Niehaus *et al.*, (2011), for each SSL three pairs of primers were designed in this study using Primer-BLAST (Ye *et al.,* 2012). The primers are detailed in Table 2, with regards to their 5`-3` sequence, T_m and expected length of amplicons. PCR products expected lengths were predicted *in silico* using FastPCR 6.4 (Kalendar *et al.,* 2011).

Using the newly designed primers, four SSL fragments were successfully amplified (Fig.3) using the cDNA as template. For SSL1, a 1200 bp fragment was amplified with the primer pair no. 2 and a 1000 bp fragment with the primer pair no. 3. For SSL2 only one fragment was amplified using the primer pair no. 6 with an approx. length of 1300 bp. A single fragment of approx. 1000 bp in length was amplified for SSL3 using the primer pair no. 9.

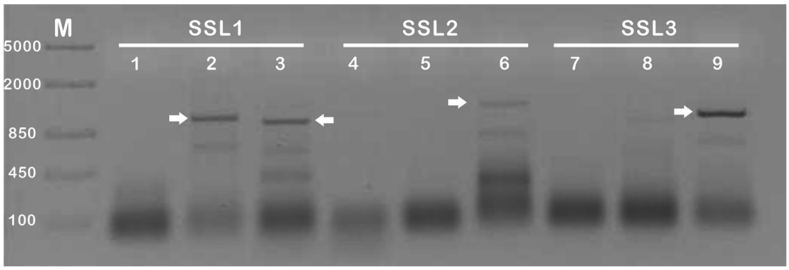

Figure 3. 1% agarose gel electrophoresis for the PCR products obtained through touchdown PCR (white arrow). Marker: FastRuler Middle Range.

Table 2.

The primers pairs used in this study to amplify squalene synthase-like 1-3 cDNA fragments from *B. terribilis* AICB 870.

Primer Pair	Primer name	5`-3` Sequence	T_m (°C)	Expected length (bp)
1	SSL1-F	ATGACTATGCACCAAGACCACG	54.8	1212
	SSL1-R	TCACTTGGTGGGAGTTGGGG	55.9	
2	SSL1-F2	ATGACTATGCACCAAGACCACGG	57.1	1169
	SSL1-R3	GAGGGTGTGTCATACTTGGC	53.8	
3	SSL1-70F	CTCCAACTAGCTGCAACAACGT	54.8	1100
	SSL1-R3	GAGGGTGTGTCATACTTGGC	53.8	
4	SSL2-F	ATGGTGAAACTCGTCGAGGTTT	53.0	1398
	SSL2-R	CTACTGCTTGGAAGAAGCAGAG	54.8	
5	SSL2-F2	CAGATGTTGCATAAGACCTACC	53.0	1334
	SSL2-R2	GAAGAAGCAGAGGTGAGCAAGG	56.7	
6	SSL2-50F	ATGTTGCATAAGACCTACCGCG	54.8	1326
	SSL2-R3	AGCAGAGGTGAGCAAGGGAAGG	56.7	
7	SSL3-F	ATGAAACTTCGGGAAGTCTTGC	53.0	1152
	SSL3-R	CTAAGCACCCTTAGCTGAAACC	54.8	
8	SSL3-F2	ATGAAACTTCGGGAAGTCTTGCAGC	57.7	1149
	SSL3-R2	AGCACCCTTAGCTGAAACCTTTCC	57.4	
9	SSL3-40F	CCCTCTCCTGCAAATGATGGTC	56.7	1058
	SSL3-1099R	ATAACGCCTGGACATCCTGAAG	54.8	

The PCR reaction was repeated using the PCR products obtained in the first reaction as templates, purified from agarose gel, cloned in pGEM-T vector and sequenced.

Bioinformatic analysis of nucleotides and translated amino acid sequences

The bioinformatic analysis was difficult due to a very low number of homologous sequences deposited in the GenBank database and related to *B. braunii* or *B. terribilis*.

The nucleotide sequences generated in the present work were identified based on BLAST report. Our sequences showed a percentage of identity with the SSL-1-3 from *B. braunii* (HQ585060.1-3) that varies between 96% (SSL-2) and 98% for SSL-1 and SSL-3, respectively.

The nucleotide frequencies showed few differences between the SSLs in the two different strain analyzed. The G+C content of the analyzed SSL fragments was: 49.7% in *B. braunii,* 49.5% in AICB 870, for SSL-1; 54.0% in *B. braunii,* 54.2% in AICB 870, for SSL-2 and 50.4% in *B. braunii,* 50.9% in AICB 870, for SSL-3. Overall, from this point of view the CDS fragments from the AICB 870 strain, show high similarity to sequences from *B. braunii*.

The nucleotide sequences were translated into amino acid sequences with the Translate tool from ExPaSy (Gasteiger *et al*., 2005) using the standard genetic code.

The amino acids sequences showed high similarity to SSL from *B. braunii*, as it follows: 97% (399 aa out of 410 aa) with SSL-2 (G0Y287.1) and 99% (398 aa out of 403 aa and 346 aa out of 348 aa) with SSL-1 (G0Y286.1) and SSL-3 (G0Y288.1).

Based on amino acids frequencies (Table 3) it can be observed that the frequencies of hydrophobic amino acids residues are at least two times higher than the hydrophilic ones, varying between 0.474 in SSL-1 and 0.52 in SSL-2 and 3.

The calculated Mw, detailed in Table 3, showed slightly few differences for all six analyzed SSLs. The pI showed differences only in the case of SSL-1 (7.12 at SSL-1 from *B. braunii* and 7.96 at SSL-1 from AICB 870). Using sequence alignments, two possible amino acids substitutions were identified which can result in changing of isoelectric point: i) Glu256 from *B. braunii* SSL-1 is replaced by Lys in SSL-1 from *B. terribilis* AICB 870; and ii) Lys263 is replaced by Arg.

Table 3.

The amino acids frequencies, molecular weight (Mw) and isoelectric point (pI) of all SSL fragments from *B. braunii* Showa (G0Y286-8) and AICB 870 (*complete aa sequence, **partial aa sequence, H^+- hydrophilic, H^- - hydrophobic).

Strain	Fragment	Length (aa)	AA frequencies			Mw (kDa)	pI
			H^-	H^+	Others		
B. braunii Showa	SSL-1	403*	0.474	0.268	0.258	45.95	7.12
AICB 870	SSL-1	403*	0.474	0.268	0.258	45.96	7.96
B. braunii Showa	SSL-2	410**	0.527	0.222	0.251	45.81	6.11
AICB 870	SSL-2	410**	0.529	0.224	0.246	45.84	6.11
B. braunii Showa	SSL-3	348**	0.520	0.201	0.279	40.49	5.95
AICB 870	SSL-3	348**	0.520	0.201	0.279	40.49	5.95

Multiple sequence alignments between our sequences and the sequences retrieved from GenBank, revealed four conserved domains, two aspartate-rich motifs and one NADPH binding domain (Fig. 4), a similar situation being previously described by Lee and Poulter (2008) in the case of the squalene synthase from *Thermosynechococcus elongatus* strain BP-1.The most conserved motif was observed in domain I. The motif (CVF[YL]V[LR]AL[DT]VE[DD]) consisted of 16 amino acids residues of which eight residues are perfectly conserved (brackets). Domain I presented the first aspartate-rich motif [DT]VE[DD]. Domain II had the consensus sequences [D]L[Y]CHY VA[G]LVGIG and presented a partial degree of conservation. The second aspartate-rich motif [D]YL[ED] was observed in domain III. Domains I and III are possibly involved in the binding of substrate (FPP in the case of the first 14 sequences and PSPP in the last four sequences) via Mg^{2+} bridging (Lee and Poulter, 2008; Pandit *et al.*, 2000; Gu *et al.*, 1998). Domain IV presents the highest degree of variability from all four domains and could be involved in rearrangement of PSPP to squalenes or botryococcenes. Downstream of domain IV, all sequences present the domain VKIRK which has role in binding of NADPH (Lee and Poulter, 2008).

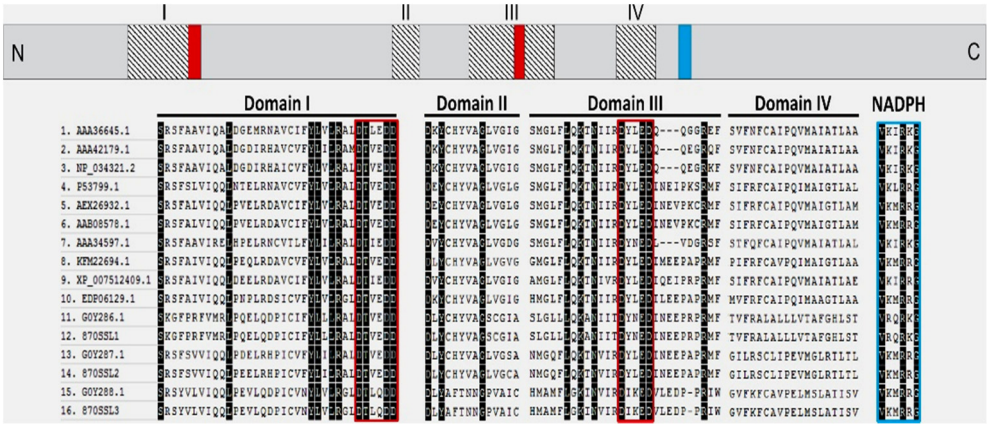

Figure 4. Amino acid sequence alignments of SQS. The conserved domain I-IV, aspartate-rich motifs (red rectangle) and putative NADPH binding domain (blue rectangle) are also depicted.

The 16-aa sequences were checked (mentioned in the Material and Methods section) in order to predict transmembrane motifs (TMM) using TMHMM program which are depicted in Fig. 5 (the identical TMHMM plots are not shown).

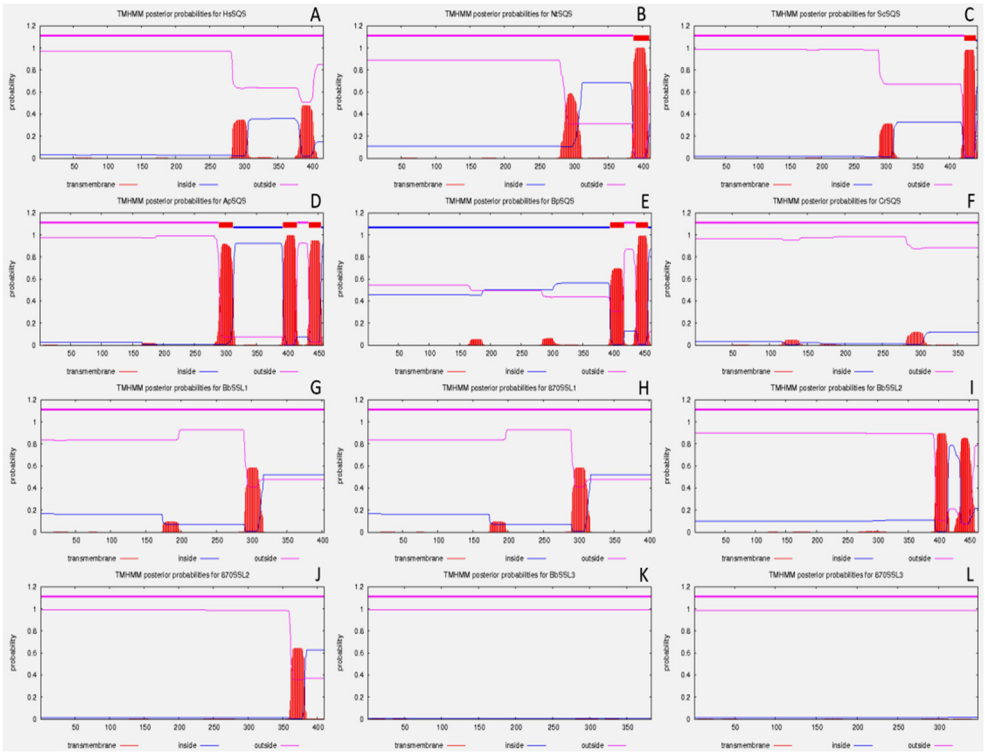

Figure 5. TMHMM plots generated for different SQS and SSL amino acids sequences. (A) *Homo sapiens* – HsSQS, (B) *Nicotiana tabacum* – NtSQS, (C) *Saccharamyces cerevisiae* –ScSQS, (D) *Auxenochlorella protothecoides* – ApSQS, (E) *Bathycoccus prasinos* – BpSQS, (F) *Chlamydomonas reinhardtii* – CrSQS, (G) *Botryococcus braunii* SSL-1 – BbSSL1, (H) AICB 870SSL-1 – 870SSL1, (I) *Botryococcus braunii* SSL-2 – BbSSL2, (J) AICB 870SSL-2 – 870SSL2, (K) *Botryococcus braunii* SSL-3 – BbSSL3, and (L) AICB 870SSL-3 – 870SSL3.

Only one TMM was observed in plants and yeast, in the following taxa: *Arabidopsis thaliana, Nicotiana tabacum* (Fig. 5 b)*, Solanum chacoense* and *Saccharomyces cerevisiae* (Fig. 5 c).

The highest rate of variability was observed in algae, the number of predicted TMM varies between three in *Auxenochlorella protothecoides* (Fig.5 d) and zero in the SQS from *Chlamydomonas reinhardtii* (Fig.5 f) and SSL from *Botryococcus* (Fig.5 g-l); as well, two THMM were observed in *Bathycoccus prasinos* (Fig.5 e).

Different papers about subcellular localization in rat hepatic cells (Stamellos *et al*., 1993) and yeasts (Zhang *et al*., 1993) showed that SQS is embedded in endoplasmic reticulum membrane through α-helix TMM. On the contrary, in this TMM prediction experiment we were unable to identify the membrane-spanning C-terminal motifs. Thus, more extensive investigation is further required.

Conclusions

In the present study we successfully identified and analyzed three squalene synthase-like cDNA fragments in the *Botryococcus terribilis* AICB870 stain.

Light and fluorescence microscopy observations showed that *Botryococcus* sp. AICB 870 presents similar features with a *Botryococcus terribilis* species, as described by Komárek and Marvan (1992), FanésTreviño *et al.* (2010), de Queiroz Mendes *et al.* (2012) and Hegedűs *et al.* (2014).

In order to extract high quantities of good quality RNA from *B. terribilis* AICB 870, the most suited method is that proposed by Ghawana *et al*. 2011. Using this protocol we were able to extract large quantity and high quality of RNA from the studied strain.

Based on the cDNA fragments from *B. braunii*, we were able to design new primers pairs, which successfully amplified the related cDNA fragments from *B. terribilis strain* AICB 870.

Bioinformatic analysis of nucleotide and translated amino acid sequences including G+C content, nucleotide frequencies, amino acids frequencies, computed Mw/pI and transmembrane motif prediction showed a high degree of similarity between the SSL identified as pertaining to *Botryococcus braunii* and those generated in the present work.

Multiple sequence alignments revealed four conserved domains, two aspartate-rich motifs and one NADPH binding domain including those from *B. terribilis* AICB 870, which were identified and analyzed for the first time in the present work.

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Differential responses of components of the antioxidant defense system to high salinity stress in the lesser duckweed (*Lemna minor* L.)

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SUMMARY. Salt stress causes oxidative damage in plants, and it induces protective mechanisms through enzymatic and non-enzymatic components of the antioxidant system. Different components of this system exhibit specific degrees of tolerance toward certain salt concentrations. Their differential responses may contribute not only to a better understanding of the functional interconnections in the antioxidant defense system, but also to a more efficient selection of physiological and biochemical markers of stress reactions of plants, in the effort for an early and precise bioindication of oxidative damage caused by high salinity of the environment. In this context, the molar ratio between the reduced and the oxidized form of ascorbic acid is a more sensitive marker of oxidative stress than the total amount of this vitamin in the biomass of lesser duckweed. Glutathione content exhibits a more moderate variation with increasing salt stress than the concentration of carotenoid pigments in the fronds exposed to constant photon flux density. From among the antioxidant enzymes, ascorbate peroxidase was found to be the most sensitive, and superoxide dismutase was the most resistant to oxidative stress caused by increasing salinity. Catalase and glutathione reductase activities decreased under severe salt stress. Efficiency of the antioxidant system can be monitored by membrane damage through lipid peroxidation. Antioxidants of duckweed are useful tools for indication of increasing salinity of aquatic environments.

Keywords: ascorbate, carotenoids, protective enzymes, salt stress.

Introduction

A large variety of environmental stress factors exerts convergent changes in plant metabolism by inducing oxidative damage, this is why antioxidant defense is a basic manifestation of cross-tolerance for different abiotic and biotic stresses.

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Oxidative stress is caused by over accumulation of harmful reactive oxygen species, generated by disturbances in basic physiological processes, such as the light reactions of photosynthesis, photorespiration, aerobic respiration, and other oxidative processes (Apel and Hirt, 2004; Pogany *et al.*, 2006; Smirnoff, 2005). High amounts of singlet oxygen, superoxide radical, hydrogen peroxide, alkyl-peroxides and hydroxyl radical are produced in plants exposed to drought, heavy metals, air pollutants, certain pesticides, excessive photon flux density and UV-B radiation, extreme temperatures, as well as by increased salinity of terrestrial and aquatic habitats (Fodorpataki *et al.*, 2014; Khanna-Chopra and Selote, 2007; Mittler, 2002; Wang *et al.*, 2009). While low concentrations of reactive oxygen species have a useful role in signaling of developmental and environmental changes, their high amounts are chemically harmful to vital biomolecules, such as unsaturated fatty acids in membrane lipids, chlorophylls, nucleic acids and proteins. As a physiological response to the oxidative damage, concerted changes occur in plant metabolism, in order to develop stress tolerance. The network of antioxidative protection processes, which relies on the pronounced metabolic plasticity of plants during hardening, represents a key mechanism of cross-tolerance, which enables plants to defend themselves against various environmental stress factors (Chattopadhyay, 2014; Laloi *et al.*, 2004; Shah *et al.*, 2001). The knowledge of how oxidative stress modulates plant metabolic processes during the physiological acclimation to adverse growth conditions, enables us to monitor the early effects of environmental changes, and to influence plant production in the direction of inducing accumulation of protective metabolites which not only confer a better survival of plants and a sustained primary production in terrestrial and aquatic ecosystems, but also possess health-promoting qualities for consumers, including humans (Mahmoudi *et al.*, 2010; Oh *et al.*, 2009; Pallag *et al.*, 2009; Rios *et al.*, 2008).

Salt stress is one of the most frequent environmental impacts that impair plant development in both aquatic and terrestrial habitats, in connection with global climate warming that enhances evaporation of water (Djanaguiraman and Prasad, 2013). Beside osmotic dehydration and chemical toxicity of sodium ions, salt stress increases the amount of reactive oxygen species in plants. This is why salinity tolerance is related to an increased amount and activity of antioxidants, while salt sensitivity is associated with down-regulation of protective enzymes involved in detoxification of oxygen radicals and peroxides (Bartha *et al.*, 2011; Bordi, 2010; Zushi *et al.*, 2009). An early detection of changes in the quality of aquatic environments, caused by increased salinity and by different agents of water pollution, is possible by using biochemical markers related to oxidative stress in test organisms such as various algae and duckweed (Fodorpataki and Bartha 2008; Karatas *et al.*, 2009; Radic *et al.*, 2011; Tkalec *et al.*, 2007; Zhang *et al.*, 2011).

The aim of the present work is to reveal differences in the activity of antioxidant enzymes and in the dynamics of protective biomolecules in the lesser duckweed exposed to different degrees of salt stress, in order to identify those biochemical markers that are most suitable for an efficient bioindication of oxidative stress induced by increased salinity of the aquatic environment.

Materials and methods

Plant material and growth conditions. Lesser duckweed (*Lemna minor* L.) individuals were collected from a small lake in Ernei (Mureş county, Romania), rinsed with 10 mM NaOCl for 30 s, and introduced in axenic cultures grown in Steinberg's inorganic nutrient medium (Fodorpataki *et al*., 2014) in an environmental test chamber (MLR-351H, Sanyo), at 22 ºC and a constant illumination with a photon flux density of 330 μM m^{-2} s^{-1} provided by white fluorescent lamps. Experimental variants were set up under the same conditions in Petri dishes, in 5 replicas, the starting cultures containing 500 individuals with one fully developed frond. Duckweed cultures were treated for 7 days with 40 mM, 80 mM, 120 mM or 160 mM of sodium chloride (p.a.), the control being grown in pure Steinberg solution.

Ascorbic acid content and reduced ascorbate to oxidized dehydroascorbate ratio. Ascorbic acid (vitamin C) content was determined according to Kampfenkel *et al.* (1995). 0.5 g of duckweed (fresh weight) was homogenized in a prechilled mortar with 4 mL of 6% trichloroacetic acic (TCA), than centrifuged for 15 min at 4 ºC with 15600 g. 200 μL of supernatant was introduced in sodium phosphate buffer (pH 7.4) containing TCA, dithiothreitol, ethanolic solution of 2,2'-dipyridyl, ortophosphoric acid, and completed with N-ethylmaleimide and iron(III) chloride. After 1 h incubation at 42 ºC with continuous mixing, absorbance of the mixture was measured at 525 nm. Standard curve was obtained with 25-100 nM ascorbic acid dissolved in 6% TCA. The assay is based on reduction of ferric ions to Fe(II) by reduced ascorbate, then Fe(II) forms a coloured complex with 2,2'-dipyridyl. Dehydroascorbate is reduced to ascorbate by dithiothreitol, the excess of the latter isremoved with N-ethylmaleimide, and total ascorbic acid is determined spectrophotometrically by the 2,2'-dipyridyl method. Concentration of dehydroascorbate is calculated from the difference of total ascorbic acid and reduced ascorbate (without pretreatment with dithiothreitol).

Glutathione content. Glutathione concentration in duckweed extract was determined according to Razinger *et al.* (2008). 0.5 g fresh plant material was homogenized in 3 ml of 5% sulfosalicylic acid and centrifuged for 10 min at 4 ºC with 14000 g. 0.1 mL of supernatant was supplemented with 2 μL 3-ethanolamine and 2 μL 2-vinylpyridine and incubated 1 h at room temperature for determining the oxidized glutathione. Glutathione concentration was measured as increase of absorbance at 412 nm as a result of reduction of 5,5-dithio-bis(2-nitrobenzoic acid), in a reaction mixture that also contained potassium phosphate buffer (pH 7.5), EDTA and NADPH. After incubation of the reaction mixture for 10 min at 30 ºC, 10 μL of glutathione reductase (50 units mL^{-1}) were added to 50 μL sample.

Carotenoid pigment determination. 0.25 g fresh weight of duckweed fronds were immersed in 5 ml dimethylformamide and kept for 48 h in darkness until complete extraction. The extract was centrifuged for 10 min at 4000 g, and the carotenoid content of the supernatant was determined spectrophotometrically (with a V-530 UV-Vis Spectrophotometer, Jasco), based on its absorbance at 480 nm (Zhang *et al*., 2013).

Ascorbate peroxidase (APX) activity. Determination of APX activity was performed through the oxidation of ascorbic acid initiated by addition of hydrogen peroxide, and measured by decrease in the absorbance of the reaction mixture at 290 nm. 0.5 g fresh duckweed was ground in a prechilled mortar with 5 mL extraction solution containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM Na_2-EDTA, 1 mM ascorbate and 2% water-soluble polyvinyl-pyrrolidone. The homogenate was centrifuged at 15000 g for 20 min, then 50 µL of supernatant was resuspended in a mixture of 1.75 mL phosphate buffer (pH 7.8) containing 1 mM Na_2-EDTA and 0.1 mL of 10 mM ascorbic acid. The reaction was initiated by the addition of 0.1 mL of 20 mM hydrogen peroxide, and after a period of 40 s the decrease of absorbance at 290 nm was measured for 3 min. Reference mixture contained distilled water instead of hydrogen peroxide, an extinction coefficient of 2.8 mM^{-1} cm^{-1} was used for ascorbic acid, and the APX activity was expressed as scavenged hydrogen peroxide in unit of time per unit of protein quantity.

Protein content of duckweed was determined with Bradford's method, using bovine serum albumine as standard (Bartha *et al.*, 2010).

Superoxide dismutase (SOD) assay. 0.5 g of fresh duckweed was ground in a prechilled mortar with 2.5 mL extraction solution consisting of 50 mM potassium phosphate buffer (pH 7), 1 mM Na_2-EDTA, 1 mM ascorbic acid and 2% water-soluble polyvinyl-pyrrolidone. The homogenate was centrifuged for 20 min at 15000 g and the obtained supernatant was used as the source of enzyme. Determination of SOD activity was based on the fact that in the presence of riboflavine and light, SOD inhibits the formation of formazane from nitro blue tetrazolium (NBT). 0.1 mL of enzyme extract from duckweed was introduced in 3 mL of reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 15 mM methionine, 0.1 mM Na_2-EDTA, then 0.1 mL of 5 mM NBT and 0.1 mL of 0.2 mM riboflavine were added. The mixture was intensely illuminated for 15 min, then absorbance of the generated formazane was measured at 560 nm. References were the mixtures kept in darkness, and blank samples contained no enzyme extract. One enzyme unit is the amount that inhibits by 50% the reduction of NBT to formazane in the presence of light. The specific SOD activity was expressed as enzyme units in 1 mg protein content of the plant extract, and protein content was determined as mentioned above for ascorbate peroxidase (Eraslan *et al*., 2007).

Catalase (CAT) activity. Enzymatic activity of catalase was determined spectrophotometrically, by measuring change of absorbance at 240 nm due to consumption of hydrogen peroxide during 1 min at 22 ºC, in a mixture containing plant extract corresponding to 10 µg protein in 1 mL of 50 mM potassium phosphate buffer (pH 7.5), and 0.1 mL of 200 mM hydrogen peroxide to start the reaction (Sairam *et al*., 2005). Protein content was determined as for ascorbate peoxidase.

Glutathione reductase (GR) assay. Glutathione reductase activity was determined by the increase in absorbance at 412 nm due to formation of 2-nitro-5-thiobenzoic acid by reaction of reduced glutathione with 5,5-dithio-bis(2-nitro-benzoic acid). The

reaction mixture contained homogenized plant extract corresponding to 10 μg protein, 1 mM EDTA, 2 mM NADPH and 15 mM 5,5-dithio-bis(2-nitrobenzoic acid) in 0.7 mL of 0.2 M potassium phosphate buffer (pH 7.5), and was incubated at 22 ºC for 40 min, than the reaction was started by addition of 50 μL of 20 mM oxidized glutathione (GSSG) and change of absorbance was monitored for 15 min (Panda *et al*., 2003).

Lipid peroxidation assay. Lipid peroxidation was measured as the amount of malondialdehyde determined by the reaction with thiobarbituric acid (TBA). 0.5 g of duckweed (fresh weight) was homogenized in 10 mL of 0.1% TCA and centrifuged at 15000 g for 15 min. 1 mL of supernatant was mixed with 4mL of 0.5% TBA dissolved in 20% TCA, the mixture was incubated at 95 ºC for 30 min and cooled instantly in ice bath. After centrifugation at 10000 g for 10 min, the absorbance of the supernatant was measured at 532 nm and corrected for 600 nm. Malondialdehyde content was calculated according to its absorbance coefficient of 155 mM^{-1} cm^{-1} (Panda *et al.*, 2003).

Statistical analysis. Experimental data were statistically analyzed in R environment (version 2.14.1), using one-way ANOVA and the post-hoc Tukey HSD test for the significance of differences between treatments. The results were expressed as the mean ± standard error, and a value of $P < 0.05$ was considered to be statistically significant.

Results and discussion

Oxidative stress is a common side effect of high salinity, along with osmotic stress leading to imbalanced water status and with chemical toxicity of excess sodium ions that accumulate over time in plant cell. Increased generation of reactive oxygen species is also associated with various other environmental stress factors, being a main feature responsible for development of cross tolerance towards different external constraints. Plants defend themselves against oxidative damage with the concerted action of interconnected enzymatic and non-enzymatic antioxidants, induced specifically by overproduction of certain reactive oxygen species (Gill and Tuteja, 2010). Carotenoid pigments (especially some xanthophylls) prevent overproduction of singlet oxygen. Ascorbate, glutathione, ascorbate peroxidase and glutathione reductase interact in scavenging excessive amounts of hydrogen peroxide, superoxide dismutase detoxifies superoxide radicals, while tocopherol deactivates hydroxyl and alkyl-peroxyl radicals. Catalase, peroxidases and peroxiredoxins also contribute to protection against inorganic and organic peroxides. The various components of the antioxidative defense system of plants are induced by different stress signals and exhibit different levels of sensitivity to various concentrations of reactive oxygen species related to the degree of stress. This is why under a given stress condition the amount or the activity of different antioxidants vary according to various patterns, indicating different levels of sensitivity, tolerance or resistance. As a consequence, various antioxidants have different indicative values as

functional markers of stress status, and knowledge of their differential behaviour under a range of stress conditions enables a better understanding of the mechanism of acclimation processes, a more sensitive evaluation of the quality of environment for given organisms, as well as more efficient procedures of phytoremediation using these organisms. In this context, the ubiquitous duckweed is a well-suited indicator of water pollution for freshwater ecosystems (Parra *et al.*, 2012).

Ascorbic acid (vitamin C) is the most abundant antioxidant in the water-soluble phase of different cell compartments, its highest amounts being found in chloroplasts. It participates in the Halliwell-Asada-Foyer redox chain that scavenges hydrogen peroxide. When duckweed plants were exposed for several days to different degrees of salt stress, their total ascorbic acid content registered a moderate, but statistically significant increase in the presence of 80 mM and 120 mM NaCl, with a further increment at 160 mM (Fig. 1).

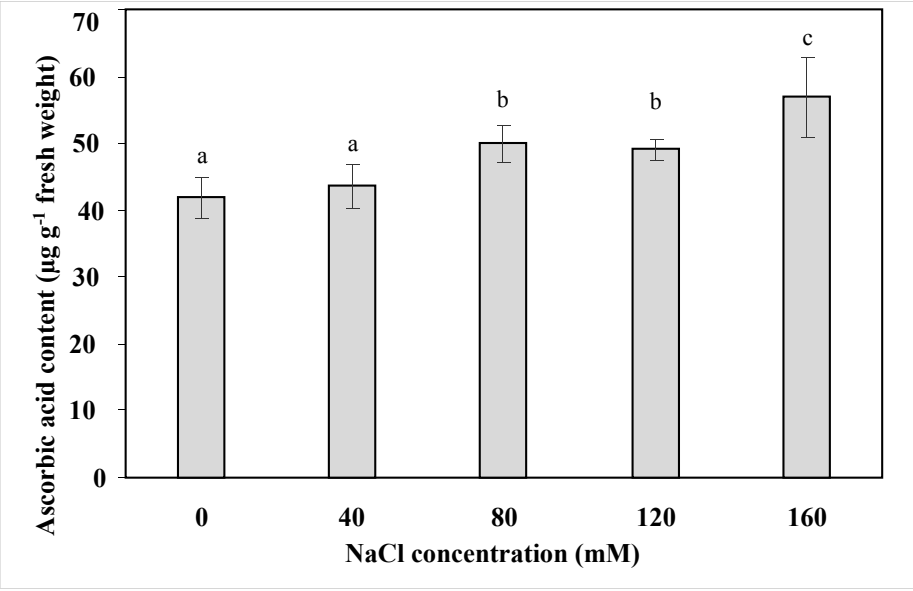

Figure 1. Ascorbic acid (vitamin C) content of duckweed (*Lemna minor* L.) fronds exposed for one week to different degrees of salinity stress. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

This result reflects that duckweed plants enhance the biosynthesis of vitamin C (from mannose and galactose) to cope with the increasing amount of hydrogen peroxide that has to be reduced to water. Increased ascorbic acid content was also reported in plants exposed to other stress conditions, such as high light intensity, UV irradiation, air pollution with sulfur dioxide, low temperature and drought stress (Kampfenkel *et al.*, 1995; Khanna-Chopra and Selote, 2007).

Because under several moderate stress conditions the total amount of ascorbic acid changes in a hardly detectable degree, the molar ratio between the reduced and the oxidized form of vitamin C might be a more sensitive marker of impaired metabolic homeostasis. In the case of duckweeds grown for one week in an aquatic environment containing increased amounts of sodium chloride, the reduced ascorbate to oxidized dehydroascorbate decreased in a higher extent than the variation of total ascorbic acid concentration, as salt stress became more intense (Fig. 2). While in control plants around 90% of vitamin C was in the reduced state, this percentage became reduced to 70-80% in the presence of 80 mM NaCl (moderate salt stress) and to approximately 50% at 160 mM NaCl (severe salt stress). For wheat it was demonstrated that in salinity tolerant genotypes this ratio exhibited a much moderate decrement than in susceptible ones (Sairam *et al*., 2005), while in lettuce the improved antioxidant capacity was correlated with maintainance of a higher ratio between the reduced and the oxidized form of ascorbic acid (Rios *et al*., 2008).

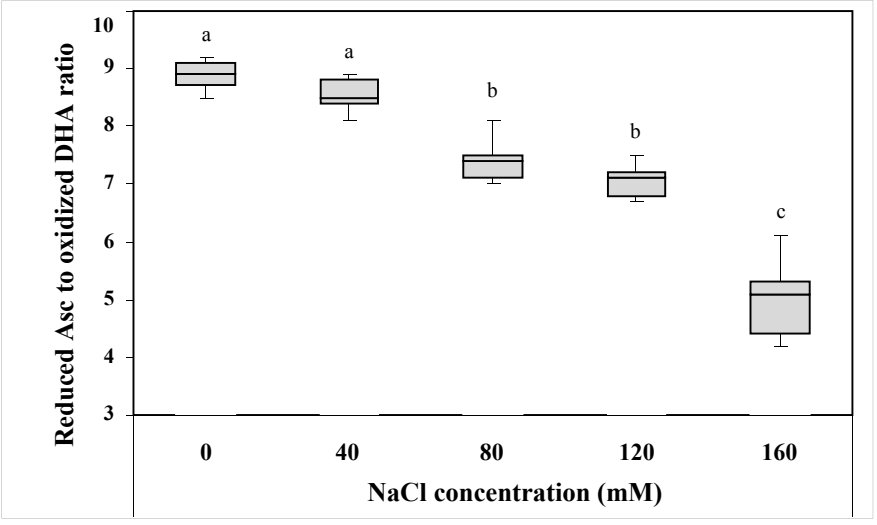

Figure 2. Molar ratio between the reduced ascorbate (Asc) and the oxidized dehydroascorbate (DHA) in fronds of duckweed exposed for one week to different concentrations of sodium chloride. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Along with ascorbic acid, glutathione (a tripeptide consisting of glutamic acid, cysteine and glycine) is another important non-enzymatic component of the antioxidant defense system, and plays a crucial role in the redox homeostasis of plant cells. By ensuring regeneration of the reduced vitamin C during the scavenging of hydrogen

peroxide molecules, glutathione ensures the continuous functioning of the Halliwell-Asada-Foyer redox chain, while it also contributes to protection of several functional proteins against oxidative damage (Chattopadhyay, 2014; Rouhier *et al*., 2008). Our experiments showed that the free glutathione level is kept at a constant level in cells of duckweed exposed to salinity, significantly higher glutathione content being registered only at salt concentrations that reached or exceeded 120 mM (Fig. 3). As a consequence, under the experimental conditions described above, glutathione level in duckweed is a less sensitive biochemical indicator of salt stress as compared to ascorbic acid. More intense changes in glutathione content were reported in lettuce leaves exposed to lower levels of salinity (Mahmoudi *et al*., 2010), and even for duckweed exposed for short time to cadmium toxicity (Razinger *et al*., 2008).

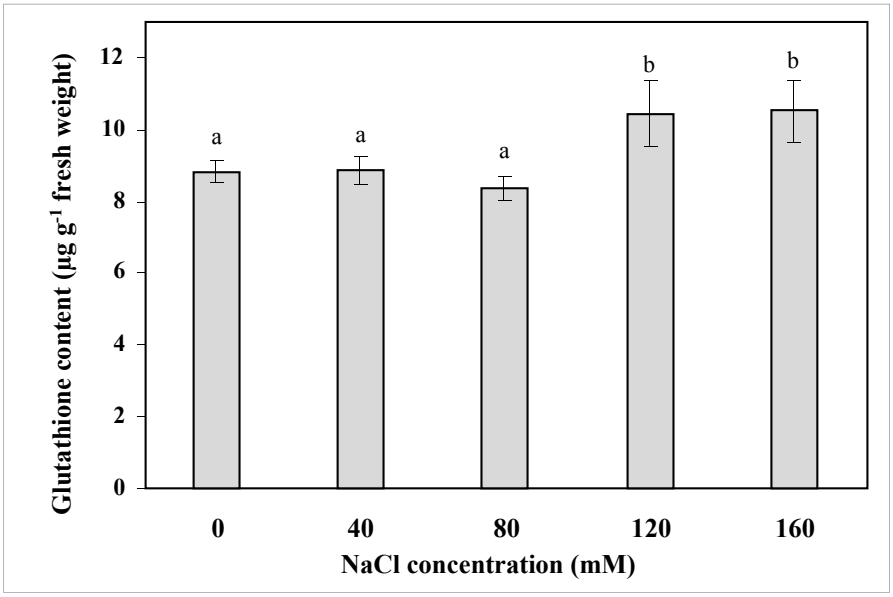

Figure 3. Glutathione content of duckweed (*Lemna minor* L.) fronds exposed for one week to different degrees of salinity stress. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Carotenoid pigments, synthesized by plants as accessory photosynthetic pigments of the thylakoid membranes of chloroplasts, also have an important antioxidative function, especially under excessive photon flux densities, when limitation of energy use in carbon assimilation leads to photooxidative damage. Increasing amounts of certain carotenoids (especially the zeaxanthin and antheraxantin components of the xanthophyll cycle) prevent formation of singlet oxygen by dissipating the excess light energy

absorbed by chlorophylls, and inactivate the newly generated singlet oxygen in a reaction followed by heat dissipation from the excited carotenoid molecule. As hydrophobic, membrane-integrated molecules, carotenoids play a determining role, together with vitamin E, in preventing oxidative damages of membrane lipids, thus in maintaining integrity, selective permeability and normal functions of biomembranes (Mittler, 2002; Zushi *et al*., 2009). Our results show that total carotenoid pigment content of duckweed plants is maintained at a relatively constant level until salt stress becomes severe (120-160 mM NaCl for 7 days), when carotenoid level significantly increases as part of the antioxidant defense mechanisms that tends to protect thylakoid membranes against photooxidative damage. The higher carotenoid content makes salt-stressed duck-weed a more valuable food source for its consumers, because carotenoids are general health-promoting metabolites for all living organisms (Smirnoff, 2005).

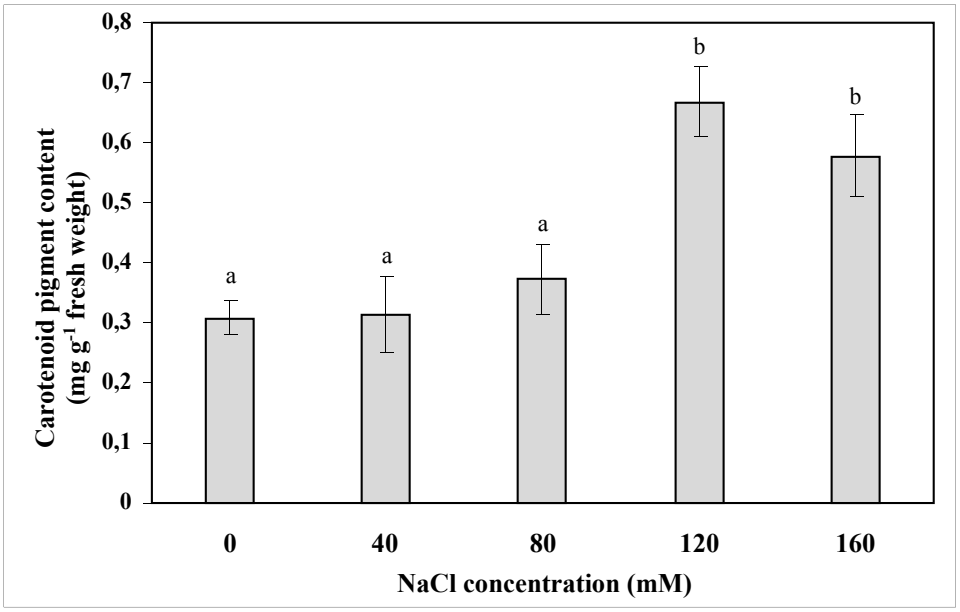

Figure 4. Carotenoid pigment content of duckweed (*Lemna minor* L.) fronds exposed for one week to different concentrations of sodium chloride. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Several non-enzymatic antioxidants act together with enzymes that catalyze the transformation of reactive oxygen species into nontoxic products. In many cases these enzymes are associated in functional complexes (metabolomes) to increase the efficiency of antioxidant protection. E. g. the hydrogen peroxide generated by superoxide dismutase is scavenged by ascorbate peroxidase, while dehydroascorbate reductase and glutathione

reductase regenerate the reduced form of ascorbate and glutathione needed for continuous protection against newly generated peroxide molecules. These enzymes have different levels of sensitivity towards oxidative stress and their catalytic activity is down- or up-regulated according to the redox state of the cell compartments in which their function is induced by several environmental constraints (Pogany *et al*., 2006).

Ascorbate peroxidase (APX) has different isozymes in chloroplasts, mitochondria, peroxisomes, cytosol and cell wall, the cytosolic one being mostly sensitive to oxidative stress. Our experiments revealed that APX activity is very sensitive to different degrees of salt stress, and therefore is a very suitable marker of stress tolerance when duckweeds are exposed for 7 days to various salinity levels. APX activity increased progressively with salinity up to 120 mM NaCl, registering significant differences among the plants exposed to different salt concentrations. Under conditions of severe salt stress caused by 160 mM NaCl, APX activity decreases below the values of control plants, reflecting its pronounced sensitivity to high salt stress (Fig. 5). This down regulation is most probably related to the lowered amount of reduced ascorbate under high salinity conditions, considering that APX activity is dependent on the concentration of reduced vitamin C. Its inhibited activity at 160 mM NaCl may be also related to overaccumulation of hydrogen peroxide, taking into account that APX scavenges hydrogen peroxide in its micromolar concentration range, being inactivated by higher amounts of this reactive oxygen derivative (Shigeoka *et al*., 2012).

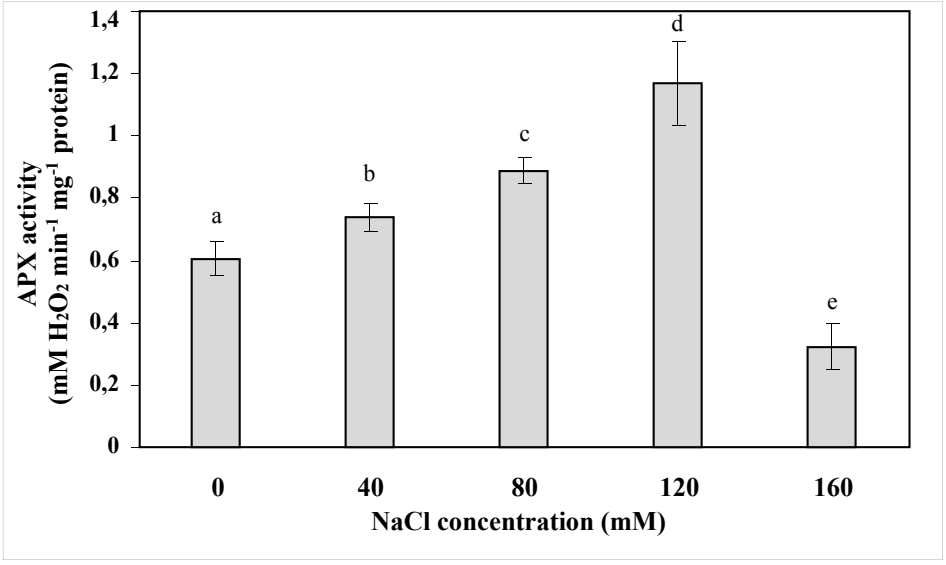

Figure 5. Hydrogen peroxide-scavenging activity of ascorbate peroxidase (APX) in fronds of duckweed exposed for one week to different degrees of salinity stress. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Superoxide dismutase (SOD) also has several isozymes in different plant cell compartments, and in contrast with ascorbate peroxidase, it is a very stable enzyme (because of very few α helices and several β sheets in its molecular structure) and exhibits a very high catalytic rate, being inactivated only by millimolar amounts of hydrogen peroxide that forms during conversion of superoxide radicals (Alscher *et al*., 2012). In this context, it is explainable that under our experimental conditions the SOD activity did not exhibit significant modification upon mild stress exerted by 40 mM NaCl, it increased moderately under exposure of duckweed to 80 mM and 120 mM NaCl, and at the very high salinity level of 160 mM, instead of inhibition (as in case of APX) it registered a significant increase in the catalytic activity (Fig. 6). Under conditions of more moderate salt stress, several authors have found in different terrestrial plants that SOD level does not change significantly (Bartha *et al*., 2011; Mahmoudi *et al*., 2010; Wang *et al*., 2009).

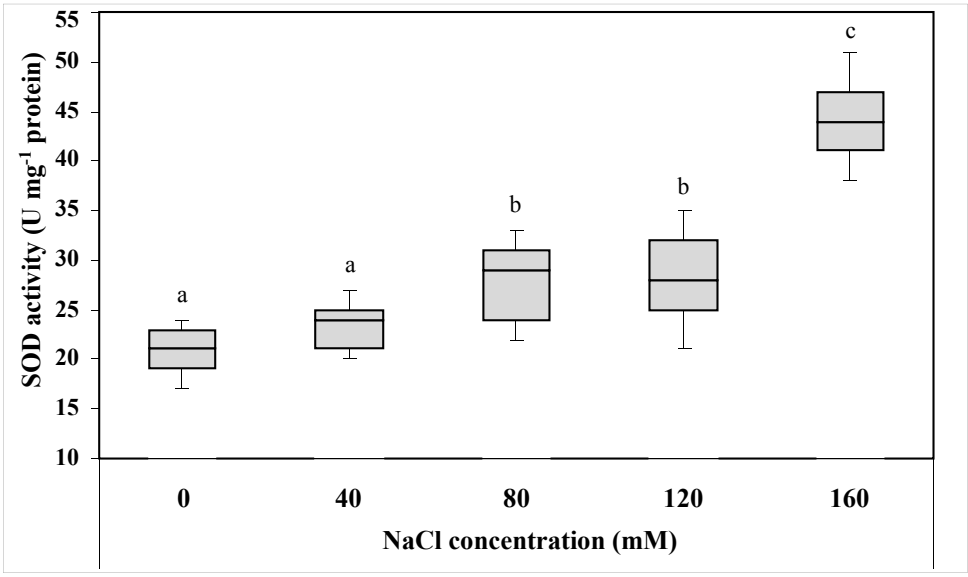

Figure 6. Superoxide dismutase (SOD) activity in duckweed (*Lemna minor* L.) fronds exposed for one week to different concentrations of sodium chloride. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

While ascorbate peroxidase regulates hydrogen peroxide level in the micromolar range, catalase (CAT) has a low affinity to hydrogen peroxide, so it scavenges it only when it accumulates in micromolar concentrations in micro-bodies of plant cells (peroxisomes, glyoxysomes, uricosomes). It is easily photo-inactivated, and as a compensation for this, it has a high turnover rate (Mittler, 2002). Because higher amounts

of hydrogen peroxide accumulate under more severe salt stress, in duckweed exposed for a period as long as 7 days to different salinity levels, one can notice that 40 mM and 80 mM NaCl do not induce significant changes in catalase activity as compared to control conditions, 120 mM NaCl induces an obvious increase of this enzyme's catalytic activity, while severe salt stress caused by exposure to 160 mM results in drastical inhibition of hydrogen peroxide-scavenging activity of CAT (Fig. 7). These results are in agreement with the sensitivity of this enzyme only to higher concentrations of hydrogen peroxide (generated by more severe salt stress), and they also suggest that catalase activity is not a suitable marker for distinction between different levels of moderate salt stress in duckweed, but it can indicate distinct levels of severe salt stress.

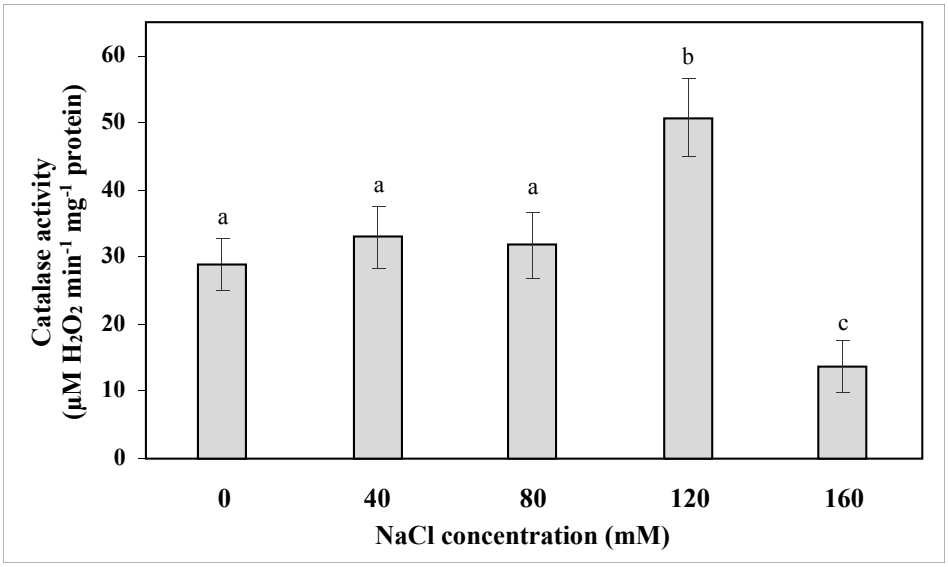

Figure 7. Enzymatic activity of catalase in fronds of duckweed exposed for one week to different degrees of salinity stress. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Glutathione reductase (GR) has an indirect role in the antioxidant defense: it does not anihilate any of the reactive oxygen species, but it regenerates the reduced form of glutathione, which is required for sustained scavenging of excess amounts of hydrogen peroxide in the Halliwell-Asada-Foyer redox chain, along with ascorbate (Chattopadhyay, 2014). In accordance with the results concerning variations of glutathione content of duckweed exposed to different degrees of salt stress, GS level exhibits no significant variation at lower salt concentrations (40 mM and 80 mM), and

it increases at 120 mM NaCl to ensure an efficient regeneration of the elevated amount of glutathione in the effort of plants to cope with oxidative stress associated with higher salinity. At even higher salt con-centration (160 mM), even though glutathione content continues to increase, GR activity declines significantly, probably because of enzyme damage caused by oxidative stress. Like catalase, this enzyme is not suitable for the early detection of milder salt stress, but its activity varies in a wide range when salinity reaches different higher values (Fig. 8). In another set of experiments, conducted also with duckwed, Zhang *et al*. (2013) have found that GR activity increases progressively with intensification of oxidative stress induced by water pollution with an organic xenobiotic substance, over a wide range of its concentrations. This substance probably caused a lower oxidative stress than the salt concentrations used in the present experiments.

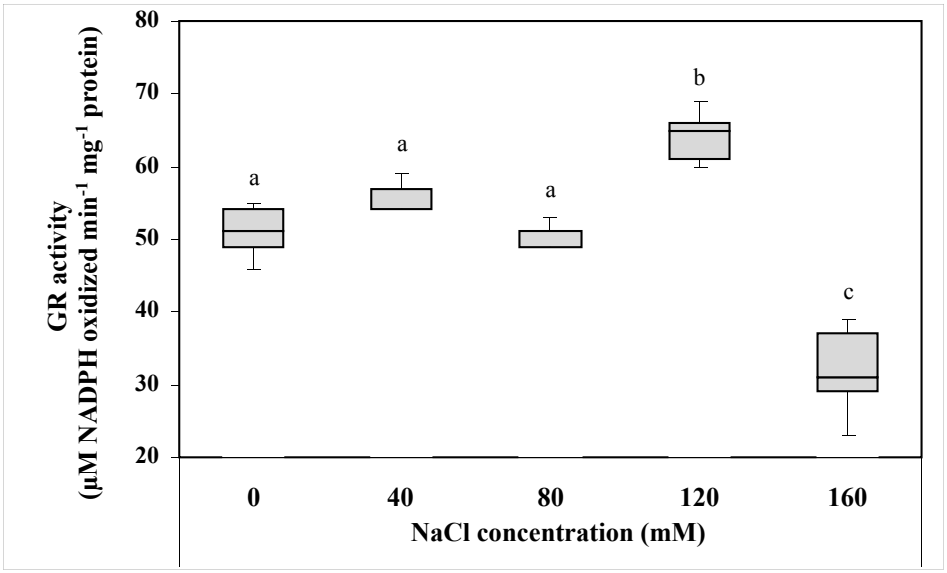

Figure 8. Glutathione reductase (GR) activity in duckweed (*Lemna minor* L.) exposed for one week to different concentration of sodium chloride. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Efficiency of antioxidant defense may be evaluated by the degree of membrane damage due to peroxidation of unsaturated fatty acids in the lipids structure of membranes. When environmental stress factors induce enhanced generation of hydrogen peroxide and alkyl peroxides, the excess amounts of these reactive oxygen species, if they are not quickly detoxified by specific enzymatic and non-enzymatic components, oxidative membrane damage results in formation of lipid peroxide derivatives, the most frequent and most toxic of these being the malondialdehyde (MDA). Our results

reflect that mild and moderate salt stress exerted for one week on duckweed plants does not significantly increase membrane damage by lipid peroxidation, because the concerted action of different antioxidants anihilates deleterious increment of peroxide concentrations. Enhanced membrane lipid peroxidation, manifested in increased generation of malondialdehyde and other related, thiobarbituric acid-reactive substances, occurs only upon exposure of duckweed to heavy stress condition induced by 160 mM sodium chloride (Fig. 9). This is in agreement with the results that indicated that ascorbate peroxidase, catalase and glutathione reductase activities markedly decrease at this salinity level, while superoxide dismutase activity, which generates hydrogen peroxide, increases. Even though ascorbic acid, glutathione and carotenoid pigment concentrations become higher, in lack of a proper antioxidant enzyme activity these protective molecules cannot prevent oxidative membrane damage if salt stress is pronounced. Similar results, when the antioxidant defense system could not prevent peroxidation of membrane lipids and oxidative stress became irreversibe, were reported especially for heavy metal toxicity in crop plants such as wheat, rice and lettuce (Eraslan *et al*., 2007; Panda *et al*., 2003; Shah *et al*., 2001).

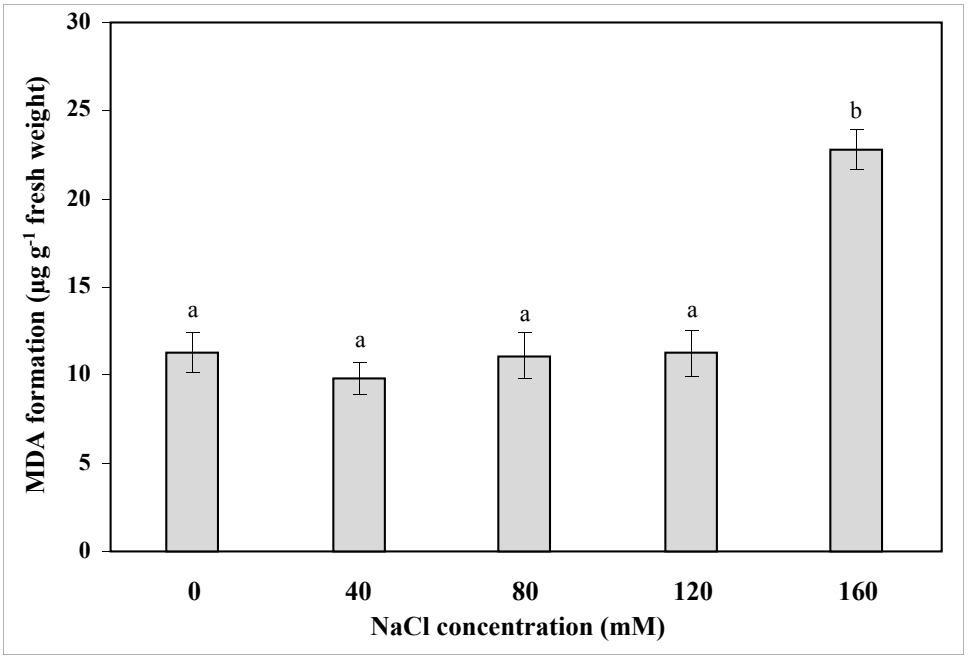

Figure 9. Degree of oxidative membrane damage by lipid peroxidation, revealed by accumulation of the toxic breakdown product malondialdehyde (MDA) in fronds of duckweed (*Lemna minor* L.) exposed for one week to different degrees of salinity stress. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Conclusions

Selected antioxidants of duckweed may be useful biochemical markers of oxidative stress induced by increased salinity of the aquatic environment. While the molar ratio between the reduced and the oxidized form of ascorbic acid (vitamin C) progressively decreases with enhanced salinity, the concentration of carotenoid pigments is increased only by pronounced salt stress caused by 120-160 mM sodium chloride in the nutrient solution. Enzymatic activity of ascorbate peroxidases is stimulated by milder salt stress, but it is decreased by salinity as high as 160 mM. In contrast, superoxide dismutase activity is significantly increased only by higher salt concentrations. Glutathione reductase and catalase activities are inhibited by salt stress induced with 160 mM sodium chloride, but are enhanced by moderately high salinity (120 mM). Oxidative membrane damaged, evaluated by the degree of peroxidation of unsaturated fatty acids, intensifies only at high salt concentrations, due to the effective protection ensured by different antioxidants at lower levels of salt stress. The ratio between reduced and oxidized form of ascorbic acid is a more sensitive stress marker than variation of the total amount of ascorbate. Moderate salt stress is indicated properly by increased ascorbate peroxidase activity, while pronounced salt stress may be monitored by high superoxide dismutase activity, by enhanced membrane lipid peroxidation and by increased carotenoid content of the duckweed biomass. The results demonstrate that the components of the antioxidative defense system behave differently under various degrees of oxidative damage caused by increased salinity, some of them being resistant to mild stress and tolerant to heavy stress, while others are tolerant to mild stress and sensitive to more severe stress conditions. In conclusion, measurement of changes in the dynamics of some selected antioxidants gives only a partial insight in the whole defense system against various degrees and forms of oxidative stress, and integrated stress tolerance of plants may be better evaluated if more enzymatic and non-enzymatic components of the antioxidant system are taken into account.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, 1, 2015 (p. 57-67)

Comparative study of trichomes in three parental *solanum* species and their somatic hybrids, cultivated in greenhouse or phytotron

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SUMMARY. Trichomes represent one of the most important anatomical structure of plants, involved in protecting them against herbivores or other pathogens. The aim of our study was to compare trichome morphology in *Solanum* parental species and sexual or somatic hybrids cultivated in a greenhouse or phytotron. The analysis were performed on the leaf abaxial epidermis by using light and epifluorescent microscopy. In total, six out of the eight types of *Solanum* specific trichomes, firstly described by Luckwill (1943), were found. There were not significant differences between the plants grown in phytothron or greenhouse. However, we observed variation in the trichomes morphology in different hybrid plants as compared to their parents.

Keywords: Colorado potato beetle, *Solanum chacoense*, somatic hybrids, trichomes.

Introduction

It is necessary to use the most valuable cultivars of potato to obtain an extended crop production. Genetic resources which are useful to improve the quality of commercial species were exploited just to a limited degree along the time mainly because sexual incompatibility. The literature and technology available until this moment, allow us to find new possibilities to operate with the existing resources (Bradshaw *et al*., 2006).

Trichomes arise from epidermal cells and they are common in all terrestrial plants. They have different shapes, can be easily observed and also, they serve as an excellent model system to analyse molecular mechanisms corresponding to plant cell differentiation, such as cellular death, supervision of the cellular cycle and morphogenesis (Yang and Ye, 2013).

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In 1943, Luckwill was the first who grouped the *Solanum* trichomes in eight different types, based on their different morphological characteristics. Later, Channarayappa *et al*. (1992) revised the classification made by Luckwill. However, in our study, we used Glas *et al*. (2012) trichome phenotipical traits table, which is a summarised version of the above mentioned researchers results (Table 1). Properly, there can be distinguished: four types of glandular (I, IV, VI, VII) and four non-glandular hairs (II, III, V, VIII) (Glas *et al.,* 2012).

Table 1.

Description of glandular and non-glandular trichomes types, based on Luckwill (1943) and Glas *et al*. (2012).

TRICHOME TYPE	GLANDULAR/ NON-GLANDULAR	DESCRIPTION
I	GLANDULAR	Tiny trichomes, made by six to eight cells with a small, round cell at the tip and a globular, multicellular base.
II	NON-GLANDULAR	Similar to the first type, but shorter, with a globular and multicellular base.
III	NON-GLANDULAR	Fine hair, made by 4-8 cells and a unicellular, horizontal base.
IV	GLANDULAR	Analogous to type I, but shorter, the head is constituted by a glandular cell.
V	NON-GLANDULAR	Similar to type IV, but has no secretory cells.
VI	GLANDULAR	Short and delicate, with four cells head.
VII	GLANDULAR	Undersized trichomes, the heads are composed by 4-8 cells.
VIII	NON-GLANDULAR	Unicellular base and a bending tip, made by one single cell.

Glandular trichomes possess a large variety of glands. Differences between them consist in chemical composition of the secreted, accumulated or absorbed substances and in the way they are produced. The chemical compounds in glandular trichome cells are secreted through their secretive extremity. A typical glandular hair is composed of uni- or multicellular base, uni- or multicellular stalk and uni- or multicellular head. Sometimes, a binding cell between the head and the stalk part can be identified. However, some uncommon, specific shaped trichomes were also described (Werker, 2000).

Non-glandular hairs have different morphology and anatomy. Generally, their classification is made according to their morphology. They can be uni- or multicellular, branched or unbranched. The connections between thrichome cells can be visible or not. There are differences concerning the thrichome cells shape, length, size, vertical symmetry or asymmetry and uniformity of the hairs width. The diameter of trichomes could be variable along the hair, therefore they can have sharped, cut or rounded vertices (Werker, 2000).

It is well known that wild *Solanum* species represent important sources of resistance to Colorado potato beetle (CPB) *(Leptinotarsa decemlineata)* (Jansky *et al.*, 1999) and other diseases and pests.

Somatic hybridization is among the most utilized and efficient methods for a successful transfer of genetic resistance from the wild species into potato cultivars gene pool (Chen *et al*., 2004; Pelletier *et al.,* 2011). That way the development of CPB resistance to insecticides is avoided by using resistant host plants (Austin *et al.*, 1985; Deimling *et al*., 1988; Helgeson *et al.,* 1988; Jansky *et al*., 1999). Colorado potato beetle is one of the most dangerous herbivore of potato. Even a single generation can destroy more than 40% of the annual production, causing large financial losses every year (Noronha *et al.,* 2002; Pelletier and Dutheil, 2006). One of the most effective wild *Solanum* species against CPB attacks is *Solanum chacoense*.

The purpose of this study was to reveal some morphological details about different trichome types found in *S. chacoense*, two *S. tuberosum* cultivars and some of their derived somatic or sexual hybrids. Somatic hybrids (SHs) have been previously obtained by mesophyll protoplast electrofusion. The trichomes were analysed by optical microscopy and compared in the plants grown in a greenhouse or phytotron.

Materials and methods

The plant material consisted in twenty genotypes, grown both in phytotron and a greenhouse, and as such used to compare their trichomes types and morphology.

In the phytotron, day-night alternation was of 16 h light period and 8 h dark. Other environmental factors were represented by 40% humidity, 21ºC temperature and 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity. The growing conditions in the greenhouse were: photoperiod of 16 h, temperature maintained at 24ºC, sunlight intensity and the proper humidity which was reached with daily soil watering.

In our experiment, two accessions of *Solanum chacoense* were used: PI 458310 (the high leptine producer) grown in phytotron, and 138, from Groß Lüsewitz Genebank, grown in a greenhouse. From *S. tuberosum* ssp. *tuberosus* two commercial cultivars Delikat and Desiree were also used. Besides these genotypes, a series of somatic hybrids were studied too.

Table 2.

The somatic hybrids which were assessed for trichome morphology.

HYBRIDS/BCs	PARENTAL GENOTYPES	OBSERVATION
1552/1	*S. tuberosum* cv. Delikat *S. chacoense* 138	
1552/1/1 1552/1/2 1552/1/3 1552/1/4 1552/1/7 1552/1/18	1552/1 *S. tuberosum* cv. Sonate	The hybrid was backcrossed with the cultivar Sonate and the resulted hybrids were named BC1s
1553/1/7	1553/1 *S. tuberosum* cv. Sonate	The hybrid was backcrossed with the cultivar
1552/1/7/1 1552/1/7/2	1552/1/7 *S. tuberosum* cv. Romance	The hybrid was also backcrossed with cultivar and were obtained BC2s
Dk.S10.5 Dk.S10.13 Dk.S10.35 Dk.S10.43 Dk.S10.61	*S. tuberosum* cv. Delikat *S. chacoense* PI 458310	S. chacoense PI 458310 is a DNA mismatch repair (MMR) deficient accession, which contains a mutant *Atmsh2* gene in antisense orientation, from a *Arabidopsis thaliana* gene
De.C7	*S. tuberosum* cv. Desiree *S. chacoense* PI 458310	
De.P5.5 De.P11.5	*S. tuberosum* cv. Desiree *S. chacoense* PI 458310	S. chacoense PI 458310 is MMR deficient, with complementary negative *Atmsh2* gene.

In our study, abaxial epidermis of the third and fourth leaves was removed, placed in distilled water on a microscope slide and investigated by optical microscopy.

We intended to succeed with identification of all type of trichomes which are present on each potato genotypes.

This method allow us to observe a series of morphological features, like contact areas between trichome parts, the segments where the hair is attached to the epidermis, tip form and the number of cells in the glandular cap.

Pictures were taken using Olympus digital camera Camedia C-5060 and then, processed using LabSens software, with the adjustment of the adequate parameters such as light, exposure, image size or contrast.

Results and discussion

More types of trichomes were recognized in the greenhouse grown plants, than in a phytothron (Table 3). The following genotypes: 1552/1, 1552/1/1, 1552/1/2, 1552/1/18, Dk.S10.35, Dk.S10.61, De.C7 and De.P11.5 had the same types of hairs.

Table 3.

Trichome types identified in the plant genotypes cultivated both in greenhouse and in a phytothron. Types I and VIII were not present in any of the genotypes analysed.

GENOTYPE	PLANTS GROWN IN PHYTOTHRON						PLANTS GROWN IN GREENHOUSE					
	TRICHOME TYPE						TRICHOME TYPE					
	II	III	IV	V	VI	VII	II	III	IV	V	VI	VII
1552/1	+	+	+		+	+	+	+	+		+	+
1552/1/1	+	+			+	+	+	+			+	+
1552/1/2	+	+		+	+	+	+	+		+	+	+
1552/1/3	+	+	+	+	+		+	+	+		+	+
1552/1/4	+	+		+	+	+	+	+	+	+	+	+
1552/1/7	+	+	+		+	+	+	+			+	+
1552/1/7/1	+	+	+		+	+	+	+	+	+	+	+
1552/1/7/2	+	+			+	+	+	+	+		+	+
1552/1/18	+	+	+		+	+	+	+	+		+	+
1553/1/7	+	+	+		+	+	+	+		+	+	+
S. chacoense PI 458310	+	+		+	+	+						
S. chacoense 138							+	+	+		+	+
DELIKAT	+	+			+	+	+	+	+	+	+	+
DESIREE	+	+			+	+	+	+	+	+	+	+
Dk.S10.5	+	+	+		+		+	+	+		+	+
Dk.S10.13	+	+			+	+	+	+		+	+	+
Dk.S10.35	+	+	+		+	+	+	+	+		+	+
Dk.S10.43	+	+		+	+		+	+	+		+	+
Dk.S10.61	+	+	+		+	+	+	+	+		+	+
De. C7	+	+		+	+	+	+	+		+	+	+
De.P5.5	+	+	+	+	+		+	+	+		+	+
De.P11.5	+	+	+		+	+	+	+	+		+	+

In some cases, distinguishing between type V and II or III of non-glandular trichomes was difficult if they were incompletely developed *i.e.* in different growth stages. Because of this, we have compared only the trichomes that we have considered completely developed. In all analysed genotypes, trichomes types II, III and VI were present, regardless of the plant developmental stage. Type VII trichomes occurred in all greenhouse grown genotypes, but this type was not observed in some phytotron grown plants (1552/1/3, Dk.S10.5, Dk.S10.43 and De.P5.5) (Table 3). Trichomes type I and

VIII were not identified either in the parents or within their derived somatic hybrids or back-crosses. The two cultivars of potato used in our experiments showed very similar types of trichomes, with additional trichomes IV and V when the plants were grown in a greenhouse. The two accessions of *S. chacoense* could not be compared because each was grown either in phytotron or greenhouse. But, the main trichomes types *i.e.* II, III and VI and VII were present. The hybrids, with or without MMR deficiency, presented also the main trichome types, but there were some variations between them, with some missing or some having additional trichome types (Table 3).

The length of non-glandular trichomes was higher in the greenhouse grown plants than in the phytotron plants. Most of the plants grown in phytotron had 3-5 cells and only those which were developed in greenhouse had six or more cells.

The hair's fluorescence is a feature that could be detected in all the analysed genotypes, in some cases was more intense and less powerful in others. The reflected colours of the trichomes were also extremely interesting characteristics, because in most observation the colour was green, but occasionally the sixth type of hairs revealed red fluorescence (Fig. 1). The green fluorescence of the hairs might be caused by some kind of phenolic compounds, as such chemicals are produced in tomato trichomes or other *Solanum* species. On the other hand, red fluorescence, which was encountered rarely, in some genotypes, can indicate the biosynthesis of anthocyanins, azulenes or related compounds, since it does not look like chlorophyll fluorescence and these compounds were described as fluorescing in red (Roschina, 2008; Roshchina, 2012).

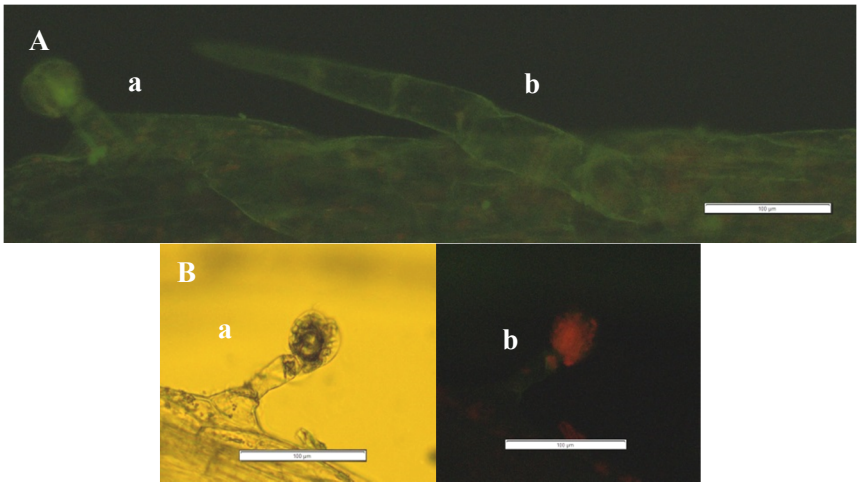

Figure 1. A - Types VI(a) and III(b), analysed in the MMR deficient somatic hybrid DK.S10.20 grown in greenhouse; B – Type VI, observed in direct light (a) and epifluorescence, with red fluorescence (b), in the MMR deficient somatic hybrid DK.S10.40, which was also grown in greenhouse; Bar = 100 µm.

After performing the microscopically examination, we observed that the cuticula which cover both glandular and non-glandular trichomes, contained some small modifications on cell wall like granules. These can provide a harsh (scabrous) surface to the trichomes. At the non-glandular hairs, these granulations were easier to be observed (Fig. 2).

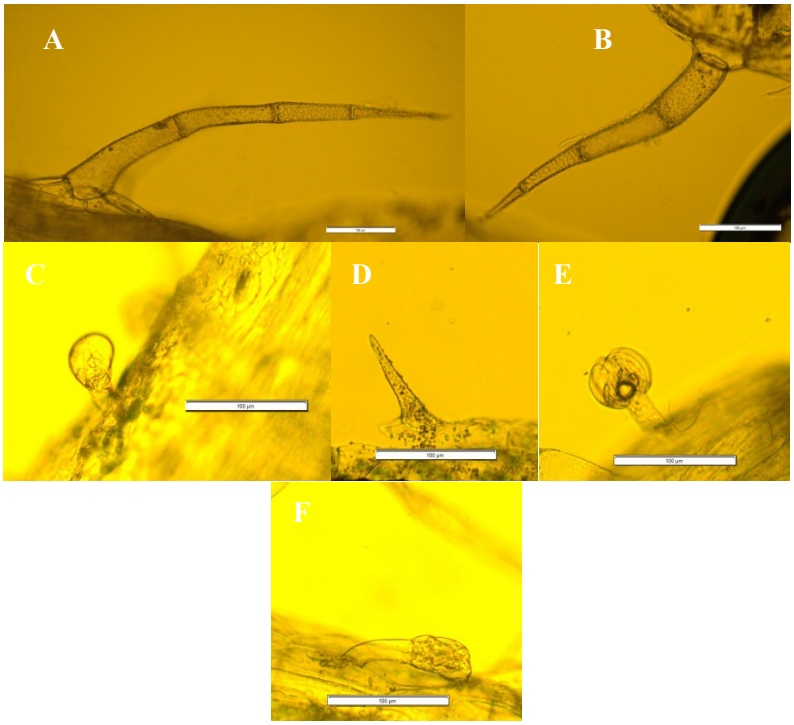

Figure 2. Trichomes types observed in plants cultivated in a greenhouse; A -Type II, in the sexual hybrid, *Solanum tuberosum* cv. Pannonia; B - Type III in *Solanum chacoense* 138, Bar = 10µm; C - Type IV in SH 1552/1; D - Type V in BC1 1552/1/4; E -Type VI in DK.S10.5; F – Type VII, in SH 1552/1/2; Bar = 100µm.

In hair types VI and VII, the secretory area was darker than in type IV, where this part of the cap was more transparent. The secretory part of type IV trichomes had unicellular construction. The types VI and VII contained multicellular secretory head with four cells. Lengths of secretory cells in type VI trichomes was larger than their width. The multicellular head of the type VII was more voluminous than in the one of the other types of glandular trichomes. In this context, our results are in accordance with those of the authors that we have cited in the introduction.

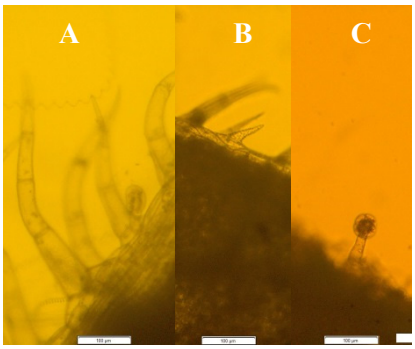

Figure 3. Plants grown in phytotron; A - Type II, *Solanum tuberosum* cv. Desiree; B – Type V, SH 1552-1-4; C - Type VI, somatic hybrid DK.S10.5; Bar = 100 µm.

Within the current study, the glandular hairs had relatively constant dimensions in almost all the genotypes, but this was not the case for non-glandular trichomes.

In the literature, it is certified that glandular and non-glandular hairs are thought to be important assets for plants, because they are supporting their defensive reactions against insects or pathogens. Compared to other biological structures, these epidermal extensions present an impressive diversity of shapes that confer an original appearance.

Nevertheless, *Solanum* trichomes are still classified in eight distinct types and two major classes, depending if their secretory function is available or not. Luckwill (1943) was the first to describe and compile trichomes forms in the genus *Solanum*, wich were especially based on analysing the tomato hairs. Later, this paper was modified and adapted to more species in the genus *Solanum* (Glass *et al.,* 2012). In our studies we tried to compare what is known from the tomato trichomes with the types of morphologies we described in potato cultivars Delikat and Desiree, a wild species representing the best source of resistance to Colorado potato beetle and their somatic or sexual hybrids.

According to Dai *et al.* (2010), Reeves (1977) and Lemke and Mutschler (1984), *Solanum habrochaites*, *S. lycopersicum* and *S. pennellii* are three representative species as concerning trichome morphology in *Solanum* genus. Dai *et al.* (2010) distinguished and shortly described the following types: I, III, IV, V, VI and VII. It can be observed that the non-glandular type II, which is present in all genotypes we analysed, was missing in these species of tomato. By the contrary, we have not found type I. Another major difference is that tomato type V conformation is constituted by one to four cells, unlike that of potato, which has a unique, elongated cell. Comparing with tomato, all the potato leaves possessed type VI with four terminal cells. Trichomes with bi-cellular heads, specific for above mentioned tomato species, were not observed in our genotypes. Tian *et al.* (2012) related in their article that the

presence of trichomes on tomato (*Solanum lycopersicum*) mutants leaves surfaces may prevent Colorado potato beetle's activity, even inhibit their appetite or mobility. In potato it is thought that mainly glandular trichomes represent a defence mechanism by repelling the beetles, but from our assays, glandular trichomes density corellates well with hybrid plant resistance to Colorado potato beetle (Mărgineanu *et al*., 2014).

Due to the trichomes positioning on the leaf, Pelletier *et al.* (2011) also considered them a real way of resistance against herbivore actions. Another opinion speculated in the same paper, is that glandular trichomes could increase potato plants resistance degree (Flanders *et al.,* 1992, Pelletier *et al*., 2011). They have classified glandular hairs of potato in only two types: A with four cells in the tip and B, which is longer than the previous one (Flanders *et al.,* 1992, Pelletier *et al.,* 2011). *Solanum berthaultii* is another *Solanum* wild species studied in relation to CPB resistance, more than *S. chacoense*. Many of the cultivated potato varieties are hybrids of *S. berthaultii,* because it possesses glandular trichomes and also because its way of resistance is well-known (Pelletier *et al*. 2011). Resistance tests made and described by some authors prove that trichomes, especially the glandular ones, symbolize a natural approach instrument to reduce the injuries caused by insects. Plants defensive reactions achieved through glandular trichomes, are performed by secretion of different chemical compounds. These substances could interfere with the feeding process or could induce important damages in the beetle larvae or adults (Pelletier *et al.* 2011).

Conclusions

At all the analyzed genotypes, we could identify types II, III and VI of the trichomes.

Types II and III of trichomes are very similar, the only difference between them was their base. The secretory head of the type VI consisted of four cells. Thus, we can consider that our results are not different from those indicated in the literature.

We can conclude that the cells are very well delimited from each other and we observed a series of morphological details like cell junctions or binding zones where the hairs attach to the epidermis, and a specific fluorescence due to the accumulation of secondary metabolites, most probably involved in plant defencing against herbivores.

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Ecological analyses on benthic diatom and invertebrate communities from the Someşul Mic catchment area (Transylvania, Romania)

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SUMMARY. The present study focused on benthic diatom and invertebrate communities from the Someşul Mic catchment area, between Someşul Rece and Apahida localities. Five sites were chosen, and they were sampled in spring, summer and autumn 2014. The area experienced major human impacts on aquatic communities, all caused by the existence of numerous urban and rural centers: discharges of waste waters into the natural streams, hydro-technical works for the production of energy or for water storage, hydropeaking, pollution coming from point or diffuse sources. The presence and the indicator value of the diatom species identified in the study area, together with the relative abundance of benthic invertebrate taxa were used to characterize the ecological status of the five sampling sites. Both diatoms and invertebrates showed the highest ecological status at the sampling site located on the Someşul Rece River, while the sampling sites located in or downstream Cluj-Napoca displayed the most impacted conditions. However, eutrophic conditions were characteristic to all sampling sites, showing affected biotic communities even in habitats with low human impacts.

Keywords: bio-indicators, human impacts, relative abundance, saprobity, trophic state

Introduction

Diatoms can be found in almost all aquatic habitats, so they can be used for comparison of streams, lakes, wetlands, oceans, estuaries, and even some ephemeral aquatic habitats (Amoros and van Urk, 1989). Diatoms are valuable indicators of environmental conditions in rivers, because they respond directly and sensitively to

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many physical, chemical, and biological changes in lotic ecosystems, such as temperature (Descy and Mouvet, 1984), or nutrient concentrations (Pringle and Browers, 1984; Pan *et al*., 1996).

The species - specific sensitivity of diatom physiology to many habitat conditions is manifested in the great variability in biomass and species composition of diatom assemblages in rivers (Patrick, 1961).

Diatoms have one of the shortest generation times of all biological indicators (Rott, 1991). They reproduce and respond rapidly to environmental change and provide early warning indicators of both pollution increases and habitat restoration success.

Benthic invertebrates represent by far the most studied and diverse group of organisms in rivers (Giller and Malmqvist, 1998). Their communities have the ability to reflect physical, chemical or biological changes of their habitat in time and in space (Cook, 1976). Thus, they integrate the effects produced by a complex of disturbing factors, offering valuable information on water quality, including abusive discharges, hard to identify based on a point chemical analysis (De Pauw and Hawkes, 1993; Metcalf, 1989).

The Someşul Mic River is the most important tributary of the Someş River, having a total length of 178 km, an average slope of 8‰, a 1.68 sinuosity coefficient and a total catchment area of 3773 km^2. Located in north-western Romania (Fig. 1), it is formed at the confluence of two rivers, the Someşul Cald and the Someşul Rece, both coming from the Apuseni Mountains. From this confluence in Gilău, the Someşul Mic flows eastwards and then northwards, through several urban and rural centers: Cluj-Napoca, Apahida and Gherla, up to Dej, where it meets the Someşul Mare River (Ghinea, 2000).

For the present study, a region affected by dissimilar impacts was chosen from the Someşul Mic catchment area: the sector stretching between Someşul Rece and Apahida localities. This sector is affected by three types of human impacts, as follows: i) the presence of numerous human settlements, many without waste water treatment plants; ii) the presence of different hydro-technical works for energy production, water supply etc.; and iii) pollution from point and diffuse sources, including industry and agricultural fields.

According to the Someş-Tisa Catchment Area Management Plan (*http://www.rowater.ro/dasomes/*), only 75 to 96% from the waste waters are connected to the central collection systems in Cluj-Napoca, while in Apahida the percentage does not exceed 40%. Secondly, the ICPDR Danube River Basin District Management Plan (2014) pointed out the existence of several hydropower plants in the Someşul Mic catchment area, upstream Cluj-Napoca, with both large (>10MW) and medium (1-10MW) generation capacities. The dam reservoirs from this area are multipurpose facilities: energy production, water supply, mitigation of floods or droughts etc. Thirdly, the same document showed high Total Nitrogen and Total Phosphorus emissions near Cluj Napoca, with long term averages (2000-2008) of more than 20 kg / ha / year for Nitrogen and around 5 kg / ha / year for Phosphorus.

Algal communities from the area were well studied beginning with 1992: in the Someşul Cald River (Rasiga *et al*.,1995/1996, 1996), in the Someşul Rece (Rasiga *et al.,* 1992, 1994, 1996), in the Someşul Mic (Rasiga *et al.,* 1995/1996, Rasiga, 2001), in the Someşul Mare and the Someş River (Rasiga *et al.,* 1998, 1999). Several studies focused on the assessment of saprobity levels using diatoms (Rasiga *et al.,* 1996, 1997, 1998, 1999), or on the assessment of river ecological status based on biotic communities (Momeu and Péterfi, 2007, Momeu *et al.,* 2007). Aquatic invertebrate communities from the study area were also investigated in previous research: Battes *et al.,* 2000/2001, Petrovici and Tudorancea, 2000/2001; Cîmpean and Tudorancea, 2001, Tudorancea and Tudorancea, 2002, Cîmpean, 2004, 2011, Avram *et al.,* 2005, 2009.

Even though the benthic algal and invertebrate communities were well studied in the Someşul Mic catchment area, the present paper focuses on the use of taxonomical richness, bioindicators and relative abundance for assessing the ecological status at five sampling sites, affected by different human impacts of different intensities. Hydropeaking and its major affects on biotic communities should be taken into consideration for further detailed studies.

Materials and methods

The samples were collected in 2014 from five sampling sites in three seasons: spring, summer, autumn. The following code was used to denominate the sampling sites for the present paper: S1_DD.MM.YY, where S1 is the sampling site; DD is the day, MM the month and YY the year of the sampling. Thus, S1_06.05.2014 represents the site located on the Someşul Rece, sampled on 6th of May, 2014 (Table 1, Fig. 1).

Number codes were assigned to sampling sites from upstream to downstream. Thus, the first site, S1, was located on the Someşul Rece River, at the highest altitude, in a deciduous forest, where trees and shrubs represented the riparian vegetation from both river banks, and the river substratum consisted mainly of large and small boulders, gravel and coarse sand. Human impacts were low in this area.

The second site, S2, was situated on the Someşul Mic River, in Gilău, downstream from the Gilău dam reservoir, where the river experienced a significant human impact, due to hydro-technical works: controlled water outlet from the reservoir, periodic alternation between the surge and low water (hydropeaking) and concrete river banks. Riverbed consisted in coarse and fine sand (Table 1, Fig. 1).

S3 was located on the Someşul Mic River, in Grigorescu district, just upstream Cluj-Napoca, where both river banks were covered in herbaceous vegetation, with concrete tiles on the left bank. Small boulders, coarse and fine sand formed the river substratum at this sampling site. The main human impacts in this area were represented by the dam reservoirs Floreşti I and II, located upstream, and possibly by domestic effluents coming from Gilău and Floreşti localities.

Table 1.

Main characteristics of the five sampling sites located in the Someșul Mic catchment area (Alt. – altitude; Max. – maximum).

Sampling site code	Sampling site name	Sampling site location	GPS coordinates	Alt. (m)	Max. depth (m)	Riverbed width (m)
S1_06.05.2014	Someșul Rece	The Someșul Rece River, upstream Somesul Rece village	N 46°41'23.9" E 23°17'45.5"	521	0.30	4.50
S1_23.08.2014					0.05	3.00
S1_02.11.2014					0.20	3.00
S2_06.05.2014	Gilău	The Someșul Mic River, downstream Gilău dam reservoir	N 46°45'09.7" E 23°23'37.2"	410	0.30	7.00
S2_23.08.2014					0.20	6.00
S2_02.11.2014					0.50	5.00
S3_06.05.2014	Grigorescu	The Someșul Mic River, upstream Cluj-Napoca	N 46°45'50.4" E 23°32'46.3"	357	0.50	20.00
S3_23.08.2014					0.50	20.00
S3_02.11.2014					1.00	20.00
S4_06.05.2014	Valea Popești	The Popești Rivulet, tributary to the Someșul Mic River	N 46°47'08" E 23°32'57"	355	0.20	2.00
S4_23.08.2014					0.04	1.80
S4_02.11.2014					0.20	1.50
S5_06.05.2014	Apahida	The Someșul Mic River, downstream Cluj-Napoca	N 46°48'56.6" E 23°44'54.7"	300	> 1.00	35.00
S5_23.08.2014					> 1.00	35.00
S5_02.11.2014					> 1.00	35.00

Figure 1. Location of the sites from the Someșul Mic catchment area, sampled in 2014 (abbreviaton of the sampling sites as in the Table 1) (source: Google Earth 2013).

S4 was placed on the Popeşti Rivulet, a left tributary of the Someşul Mic River, in Baciu District of Cluj-Napoca. Trees and shrubs were present on both river banks, while small boulders, gravel, coarse and fine sand made the substratum. Domestic effluents coming from Popeşti and Cluj-Napoca localities represented the major human impacts in the area, next to the deposits of organic debris left by the old chicken farms. The last sampling site - S5 was situated on the Someşul Mic River in Apahida, about 10 km downstream from Cluj-Napoca. Riparian vegetation on both banks consisted of trees, shrubs and herbaceous plants, while the river substratum was composed of large and small boulders, together with fine sand and clay. Human impacts concentrated at this site were represented by domestic effluents coming from all rural and urban centers upstream, but also by diffuse pollution coming from agricultural lands near-by (Table 1, Fig. 1).

Several physical and chemical parameters were recorded in the field or were measured subsequently in the laboratory. Thus, water temperature and dissolved oxygen were measured using the portable meter YSI 52, while the pH and the conductivity were measured in the laboratory. The values for water discharge, total phosphorus and total nitrogen were taken with permission from the Romanian Waters National Administration data base, The Someş – Tisa Basin Administration (ABAST). Diatoms were sampled by scraping the hard substratum or collecting the sediment using a pipette, while invertebrates were collected using a 250 μm mesh net for qualitative samplings. All samples were preserved in the field with 4% formaldehyde. Identifications were made to the species level in case of algae (Krammer, 2000, 2002, 2003; Krammer and Lange - Bertalot, 1986, 1988, 1991 a, b), and to different taxonomical levels in case of benthic invertebrates (Sansoni, 2001).

The species packing model was chosen to illustrate the numerical variation of diatom and invertebrate taxa along the water discharge gradient, using PAST version 2.14 (Hammer *et al*., 2001).

The ratio of araphid pennate to centric diatoms, namely the A/C diatom index (Stockner, 1972) was used to characterize the trophic state of the five aquatic habitats considered for the present paper. The values of the index indicate oligotrophy (<1), mesotrophy (1-2) or eutrophy (>2).

Multivariate analyses were used to visualize and interpret the data. Principal Component Analysis (PCA) was used for the physical and chemical parameters, due to its ability to project the data on a two dimensional map and to identify trends. Correspondence Analysis (CA) visualizes complex data, primarily data on categorical measurement scales, facilitating understanding and interpretation. Simple CA analyses the relationships between two variables, while Multiple CA (MCA) analyses several categorical variables. Multivariate analyses were performed using XLSTAT Version 2015.3.01.19199.

Results and discussion

Physical and chemical parameters

The physico-geographical conditions differed at the five sampling sites considered for the present study, even from one season to another (see table 1). The maximum depth of the river, for example, varied in most sampling sites, generally having the lowest values during summer. However, no significant correlations were found with the total number of diatom or invertebrate taxa ($p > 0.05$ in case of Spearman correlation).

Moreover, the water discharge measured at S1, S3 and S5 (ABAST database) was highly irregular, ranging from 0.038 m^3/s to 45.3 m^3/s. In summer, a high water discharge was recorded at S3 and S5, but not at S1 (0.552 m^3/s), showing an artificially increased flow of water coming from the hydropower plants located upstream on the Someșul Mic River.

Water discharge had drastic influences on the taxonomical richness of benthic diatom and invertebrate communities (Fig. 2). Only intermediate water discharge values were favorable to biotic communities, the trend showing the lowest number of taxa (expressed as percentage from the total number of taxa identified in the five sampling sites) connected to the highest discharge values.

In fact, these results are similar to the literature. Human impacts on the river natural flow regimes, by means of rapid changes in water releases below hydropower plants, are usually recognized as one of the most serious threats to aquatic biodiversity (Bunn and Arthington, 2002; Poff and Zimmerman, 2010; Chen *et al*., 2015). These changes has drastic effects on all benthic organisms, including diatoms (Smolar-Žvanut, 2013) and invertebrates (Richards *et al*., 2014).

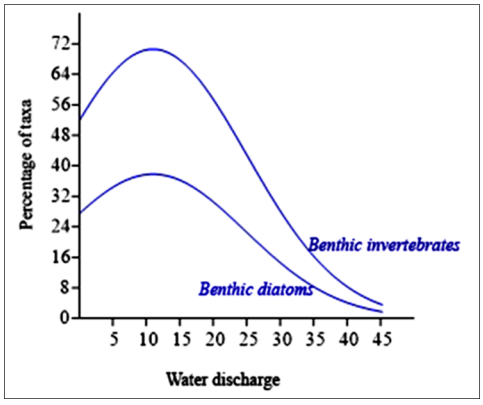

Figure 2. The relationship between the water discharge (m^3/s) and benthic diatom and invertebrate taxa (percentage from the total number present in all sampling sites) (sampling sites S2 and S4 not included).

The PCA biplot showed a tendency of the sampling sites to aggregate according to similar physical and chemical characteristics in different seasons (Fig. 3). Water temperature, pH and water discharge were best explained by F1, while dissolved oxigen by F2.

Conductivity was not discriminated; it was not well linked to F1, nor to F2, thus any interpretation could be hazardous. However, the highest values were recorded at S4 (exceeding 900 µS/cm in all sampled seasons). The conductivity values are linked to the dissolved ions present in the water, including nitrate or phosphate, whose high concentrations are mostly caused by agricultural fertilization.

Water temperature varied with the season, showing similar values in spring and summer 2014 and lower values in autumn. The pH values were circum-neutral, thypical for most surface freshwater systems. Dissolved oxygen ranged between 7 and 11 mg/L, with the lowest value at S4 in autumn 2014. Water discharge had very high values at S3 and S5 during summer 2014, as shown in Fig. 3.

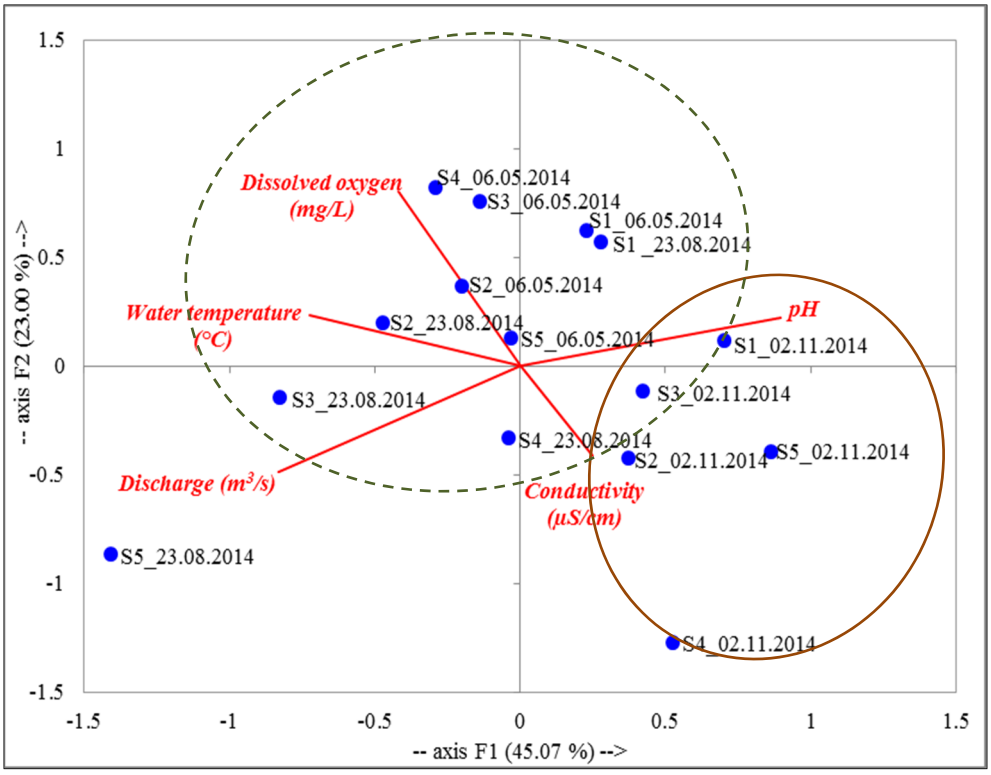

Figure 3. Principal Component Analysis (PCA) biplot (axes F1 and F2: 68.07 %) for the five sampling sites and three seasons considered for the present study, and their aggregation based on physical and chemical parameters (abbreviations as in Table 1).

As for nutrient concentrations, total phosphorus (TP) exceeded the admissible limit in surface waters according to Order no. 161/2006 only at S3 in May 2014. The value exceeded 1.2 mg/L (the Romanian Waters National Administration data base), indicating waters of poor quality as TP was concerned. No limitation was exceeded in case of total nitrogen (TN), however, higher values were recorded at S4 and S5 (over 2 mg/L, according to the Romanian Waters National Administration data base).

Benthic diatom communities

A total number of 116 diatom species, belonging to 27 genera, were identified in the five sampling sites from the Someşul Mic River in 2014 (Table 2). As in previous studies from the area (Rasiga *et al*., 1995/1996), cosmopolitan taxa dominated the qualitative structure of the diatom communities from the Someşul Mic catchment area, while *Navicula* sp. and *Nitzschia* sp. dominated in all samples, in terms of number of species. In fact, numerous species identified in the five sampling sites were tolerant and adapted to survive in habitats with high organic matter loads: *Nitzschia palea, Fragilaria ulna, Fragilaria ulna* var. *acus, Gomphonema parvulum, Navicula accomoda, Navicula goeppertiana, Navicula veneta, Nitzschia umbonata*. Several planktonic algal species were identified in each sampling site, like: *Actinocyclus normanii, Asterionella formosa, Frustulia vulgaris, Melosira varians, Nitzschia fruticosa, Nitzschia intermedia, Nitzschia acicularis*. Their presence in benthic samples was due to several dam reservoirs, located upstream of the sampling sites: Someşul Rece, Gilău or Floreşti.

Eutrophic conditions were found in all five sampling sites, according to the A/C diatom index (Stockner, 1972). In fact, numerous indicator diatom species for eutrophic conditions were identified in the five sampling sites, as follows: *Actinocyclus normanii, Amphora veneta, Asterionella formosa, Cyclotella meneghiniana, Cyclotella radiosa, Cymatopleura solea, Gomphonema parvulum, Gyrosigma acuminatum, Navicula veneta, Navicula viridula, Nitzschia capitellata, Nitzschia fruticosa, Nitzschia intermedia, Nitzschia palea, Nitzschia paleacea, Nitzschia umbonata, Stauroneis phoenicenteron*. These eutrophic conditions can be explained at S4 by the remaining deposits of organic debris coming from the old chicken farms located in the area. However, at S1, where human impacts had low intensities, the bottom discharge coming from a lake located upstream could cause the higher nutrient loads, leading to the development of eutrophic diatom species.

Several halophilic elements were found in the study area: *Gyrosigma scalproides, Navicula cincta, Navicula goeppertiana, Nitzschia trivialis, Nitzschia constricta, Nitzschia hungarica, Nitzschia intermedia, Amphora veneta, Gomphonema augur, Navicula halophila, Navicula salinarum,* or *Nitzschia levidensis*. Rasiga *et al*. (1995/1996) reported similar findings, caused by the presence of salt water habitats in the Someş catchment area (Sălsig). The presence of these halophilic species can be also explained by the influence of diapir folds located on the Zăpodie and Becaş Rivers, as well as in Cojocna.

Table 2.

The qualitative structure of benthic diatom communities from the five sampling sites located in the Someșul Mic catchment area (sampling site codes as in Table 1).

Samples sites (→) **Algal taxa (↓)**	**S1**	**S2**	**S3**	**S4**	**S5**
Achnanthes biasolettiana Grunow in Cleve & Grunow 1880				✓	
Achnanthes bioretii Germain 1957		✓			
Achnanthes flexella (Kützing) Brun 1880			✓		
Achnanthes helvetica (Hustedt) Lange-Bertalot in Lange-Bertalot & Krammer 1989		✓			
Achnanthes lanceolata (Brébisson ex Kützing) Grunow in Van Heurck 1880	✓	✓	✓	✓	✓
Achnanthes minutissima Kützing 1833	✓	✓	✓	✓	✓
Actinocyclus normanii (Gregory) Hustedt 1957			✓		
Amphipleura pellucida (Kützing) Kützing 1844:		✓			
Amphora libyca Ehrenberg 1840			✓	✓	✓
Amphora ovalis (Kützing) Kützing 1844		✓	✓	✓	✓
Amphora pediculus (Kützing) Grunow ex A.Schmidt 1875	✓	✓	✓	✓	✓
Amphora veneta Kützing 1844		✓		✓	
Asterionella formosa Hassall 1850		✓	✓	✓	
Bacillaria paradoxa Gmelin 1788				✓	
Caloneis silicula (Ehrenberg) Cleve 1894				✓	
Cocconeis pediculus Ehrenberg 1838	✓	✓	✓	✓	✓
Cocconeis placentula Ehrenberg 1838	✓	✓	✓	✓	✓
Cyclotella bodanica var. *affinis* (Grunow) A.Cleve 1951		✓			
Cyclotella meneghiniana Kützing 1844			✓	✓	✓
Cyclotella radiosa (Grunow) Lemmermann 1900		✓	✓		
Cymatopleura solea (Brébisson) W.Smith 1851	✓		✓	✓	
Cymbella affinis Kützing 1844	✓	✓	✓		✓
Cymbella cistula (Ehrenberg) O.Kirchner 1878	✓		✓		
Cymbella formosa Hustedt 1955		✓			
Cymbella minuta Hilse in Rabenhorst 1862	✓	✓	✓	✓	✓
Cymbella prostata (Berkeley) Cleve	✓		✓		✓
Cymbella silesiaca Bleisch in Rabenhorst 1864	✓	✓	✓		✓
Cymbella simonsenii Krammer in Krammer & Lange-Bertalot 1985	✓				
Cymbella sinuata W.Gregory 1856	✓	✓	✓	✓	✓
Cymbella tumida (Brébisson) van Heurck 188	✓				✓
Denticula tenuis Kützing 1844		✓	✓		✓
Diatoma ehrembergi (Kütz.) Grunow 1862			✓		
Diatoma hyemalis (Roth) Heiberg 1863	✓				
Diatoma mesodon Kützing 1844		✓	✓		
Diatoma vulgaris Bory de Saint-Vincent 1824	✓	✓	✓	✓	✓
Didymosphenia geminata (Lyngbye) M.Schmidt, A.Schmidt 1899	✓	✓	✓	✓	✓
Diploneis elliptica (Kützing) Cleve 1894		✓	✓	✓	
Fragilaria arcus (Ehrenberg) Cleve 1898	✓				
Fragilaria capucina Desmazières 1830	✓	✓	✓	✓	✓
Fragilaria capucina var. *rumpens* (Kützing) Lange-Bertalot ex Bukhtiyarova 1995			✓		

Table 2 (continued)

Fragilaria capucina var. *vaucheriae* (Kützing) Lange-Bertalot 1980	✓	✓	✓	✓	✓
Fragilaria construens (Ehrenberg) Grunow 1862		✓	✓		
Fragilaria crotonensis Kitton 1869		✓	✓	✓	
Fragilaria exigua (W.Smith) Lemmermann 1908			✓		
Fragilaria parasitica (W.Smith) Grunow in van Heurck 1881			✓		
Fragilaria pinnata Ehrenberg 1843		✓	✓		✓
Fragilaria pulchella (Ralfs ex Kützing) Lange-Bertalot 1980					✓
Fragilaria ulna (Nitzsch) Lange-Bertalot 1980	✓	✓	✓	✓	✓
Fragilaria ulna var. *acus* (Kützing) Lange-Bertalot 1980		✓	✓	✓	
Fragilaria ulna var. *claviceps* Hustedt 1937			✓		
Fragilaria virescens Ralfs 1843		✓	✓		
Frustulia vulgaris (Thwaites) De Toni 1891	✓				
Gomphonema acuminatum Ehrenberg 1832			✓		
Gomphonema angustum C.Agardh 1831	✓				
Gomphonema augur Ehrenberg 1840					✓
Gomphonema olivaceum (Hornemann) Brébisson 1838	✓		✓	✓	✓
Gomphonema parvulum (Kützing) Kützing 1849	✓	✓	✓	✓	✓
Gomphonema pseudoaugur Lange-Bertalot 1979					✓
Gomphonema truncatum Ehrenberg 1832	✓	✓			
Gyrosigma acuminatum (Kützing) Rabenhorst 1853				✓	
Gyrosigma nodiferum (Grunow) Reimer 1966			✓	✓	
Gyrosigma scalproides (Rabenhorst) Cleve 1894			✓	✓	
Melosira granulata (Ehrenberg) Ralfs in Pritchard 1861			✓		
Melosira varians C.Agardh 1827	✓	✓	✓	✓	✓
Navicula accomoda Hustedt 1950				✓	✓
Navicula atomus (Kützing) Grunow 1860					✓
Navicula capitata Ehrenberg 1838			✓	✓	
Navicula capitatoradiata Germain 1981	✓	✓	✓		✓
Navicula cincta (Ehrenberg) Ralfs in Pritchard 1861	✓	✓	✓		✓
Navicula cryptocephala Kützing 1844	✓	✓	✓	✓	✓
Navicula cryptotenella Lange-Bertalot in Krammer & Lange-Bertalot 1985	✓				
Navicula decussis Østrup 1910	✓	✓	✓	✓	
Navicula fonticola Grunow 1880		✓			
Navicula goeppertiana (Bleisch) H.L.Smith 1876					✓
Navicula gregaria Donkin 1861	✓	✓	✓		✓
Navicula halophila (Grunow) Cleve 1894					✓
Navicula lanceolata Ehrenberg 1838	✓		✓	✓	✓
Navicula minuscula Grunow in van Heurck 1880	✓				
Navicula mutica Kützing 1844					✓
Navicula mutica var. *ventricosa* (Kützing) Cleve & Grunow 1880					✓
Navicula radiosa Kützing 1844	✓	✓	✓		✓
Navicula recens (Lange-Bertalot) Lange-Bertalot 1985				✓	✓
Navicula reinhardtii (Grunow) Grunow in Van Heurck 1880			✓		
Navicula salinarum Grunow 1880					✓

Table 2 (continued)

Navicula tenelloides Hustedt 1937			✓		
Navicula tripunctata (O.F.Müller) Bory de Saint-Vincent 1822	✓	✓	✓	✓	✓
Navicula trivialis Lange-Bertalot 1980		✓		✓	
Navicula veneta Kützing 1844	✓				
Navicula viridula (Kützing) Ehrenberg 1836	✓		✓		✓
Nitzschia acicularis (Kützing) W.Smith 1853				✓	
Nitzschia amphibia Grunow 1862		✓	✓	✓	
Nitzschia capitellata Hustedt in Schmidt et al. 1922				✓	✓
Nitzschia constricta (Gregory) Grunow 1880				✓	✓
Nitzschia dissipata (Kützing) Rabenhorst 1860	✓	✓	✓	✓	✓
Nitzschia fonticola (Grunow) Grunow in Van Heurck 1881	✓	✓	✓		✓
Nitzschia frustulum (Kützing) Grunow in Cleve & Grunow 1880		✓	✓		✓
Nitzschia fruticosa Hustedt					✓
Nitzschia hungarica Grunow 1862				✓	
Nitzschia inconspicua Grunow 1862		✓			✓
Nitzschia intermedia Hantzsch ex Cleve & Grunow 1880	✓	✓	✓		
Nitzschia levidensis (W.Smith) Grunow in van Heurck 1881				✓	
Nitzschia linearis W.Smith 1853	✓				
Nitzschia palea (Kützing) W.Smith 1856	✓		✓	✓	✓
Nitzschia paleacea Grunow in Van Heurck 1881					✓
Nitzschia sinuata (Thwaites) Grunow in Cleve & Grunow 1880		✓			✓
Nitzschia sinuata var. *tabellaria* (Grunow) Grunow in van Heurck 1881			✓		
Nitzschia umbonata (Ehrenberg) Lange-Bertalot 1978			✓	✓	
Nitzschia vermicularis (Kützing) Hantzsch in Rabenhorst 1860					✓
Pinnularia borealis Ehrenberg 1843		✓			
Pleurosigma elongatum W.Smith, 1852					✓
Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot 1980	✓	✓	✓	✓	✓
Stauroneis phoenicenteron (Nitzsch) Ehrenberg 1843	✓				
Surirella angusta Kützing 1844	✓	✓	✓	✓	
Surirella brebissonii Krammer and Lange-Bertalot 1987	✓		✓	✓	✓
Tabellaria fenestrata (Lyngbye) Kützing 1844		✓			
Tabellaria flocculosa (Roth) Kützing 1844		✓			
TOTAL TAXA	**48**	**55**	**67**	**50**	**57**

Benthic invertebrate communities

A total number of 17 taxa of benthic invertebrates was found in the five sampling sites (Table 3). Only oligochaetes and dipterans (chironomids and other groups) were present in all five sampling sites. S1 was the only site where stoneflies (Plecoptera) and alderflies (Megaloptera) were present, even if the highest number of taxa was recorded in S3. On the other hand, the lowest number of taxa was found at S2 (Table 3): for example, in spring 2014 only chironomids were present. This decreased

taxonomical richness could be due to high variations of water discharge and to high substratum instability. From the total number of taxa identified at S4 and S5, 7 were common to both sampling sites, and all were characterized as tolerant to habitat degradation.

Integrative analyses

The number of diatom species identified in the study area ranged between 14 and 45, depending on the sampling season, while the number of benthic invertebrates ranged between 1 and 12.

The highest diatom species richness was recorded at S3 in November 2014, with a total number of 45 taxa (Fig. 4). In fact, higher number of diatom taxa were identified in autumn (94) compared to spring (51) and summer (69). These results are partially in agreement with those from the work of Patrick (1977), according to which diatoms have two development peaks, one in the spring and one in the autumn.

This trend of higher number of taxa in autumn was displayed by the benthic invertebrates, too (Fig. 4). In fact, the high water discharge values from August 2014 led to high variations of the water level (hydropeaking), thus negativelly influencing the number of taxa. Similar findings were reported in the literature (Vannote *et al*., 1980, Voelz and McArthur, 2000).

Table 3.

List of benthic invertebrate taxa identified in the five sampling sites from the Someşul Mic catchment area (sampling site codes as in Table 1).

Taxa	**S1**	**S2**	**S3**	**S4**	**S5**
Platyhelminthes			✓		
Nematoda		✓	✓	✓	✓
Annelida, Oligochaeta	✓	✓	✓	✓	✓
Annelida, Hirudinea			✓	✓	✓
Mollusca, Gastropoda	✓		✓		✓
Mollusca, Bivalvia		✓	✓		
Arthropoda, Chelicerata, Acari, Hydrachnidia	✓		✓	✓	✓
Arthropoda, Crustacea, Isopoda		✓			✓
Arthropoda, Crustacea, Amphipoda			✓	✓	
Athropoda, Hexapoda, Insecta, Ephemeroptera	✓		✓	✓	✓
Athropoda, Hexapoda, Insecta, Plecoptera	✓				
Athropoda, Hexapoda, Insecta, Trichoptera	✓		✓		✓
Athropoda, Hexapoda, Insecta, Odonata	✓		✓		
Athropoda, Hexapoda, Insecta, Megaloptera	✓				
Athropoda, Hexapoda, Insecta, Chironomidae	✓	✓	✓	✓	✓
Athropoda, Hexapoda, Insecta, Diptera - others	✓	✓	✓	✓	✓
Athropoda, Hexapoda, Insecta, Coleoptera	✓			✓	
TOTAL TAXA	**11**	**6**	**13**	**9**	**10**

There are numerous systems of assessing the quality of aquatic habitats based on indicator algal species; a thorough review was made by Dokulil, 2003. The saprobic system represents one of the oldest (Kolkwitz and Marsson, 1902), still in use today. Saprobity represents a direct measure of water quality, since xeno- or oligosaprobic waters contain low quantities of decomposing organic matter, indicating clean conditions, while polysaprobic habitats are heavily polluted with organic loads.

From the total number of diatom species, 66 indicated different values of saprobity: from xenosaprobic, to oligosaprobic, β-mesosaprobic, α-mesosaprobic and polysaprobic waters. The most numerous group was the one indicating β-mesosaprobic waters, with more than 12 species in each sampling site. The intermediate group including β-α-mesosaprobic taxa followed, with more than 6 species / sampling site (Fig. 5).

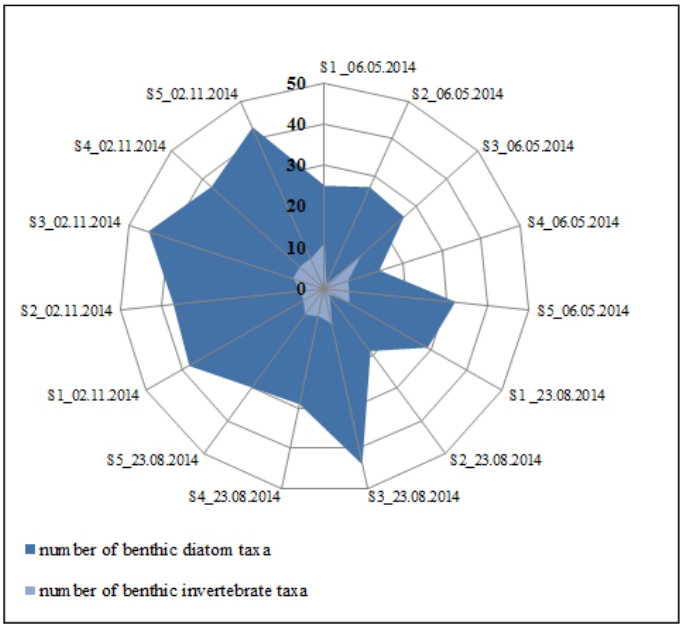

Figure 4. Total number of diatom and invertebrate taxa identified in the five sampling sites from the Someșul Mic catchment area in 2014.

The highest number of diatom taxa indicating polysaprobic conditions was recorded in S4, thus showing the worst conditions, in terms of high organic load, low or no dissolved oxygen content, high content of ammonia or hydrogen sulphide. Only slightly better conditions were indicated by diatom taxa at S5: α-mesosaprobic and α-mesosaprobic - polysaprobic taxa. The remaining sampling sites however were characterized by lower organic loads, as showed in Fig. 5. These results are in agreement with previous works: Rasiga *et al*. (1995/1996) found critical levels of saprobity in the lower Someș River.

The relative abundance was calculated for the 17 taxa categories of benthic invertebrates. As depicted in Figure 6, chironomids and oligochaetes - tolerant taxa - were common to all sampling sites, recording higher abundances in S4 and S5. Stoneflies and alderflies (Plecoptera and Megaloptera), known to survive only in habitats with clean water, were identified only at S1, while flatworms were present only at S3 (Fig. 6).

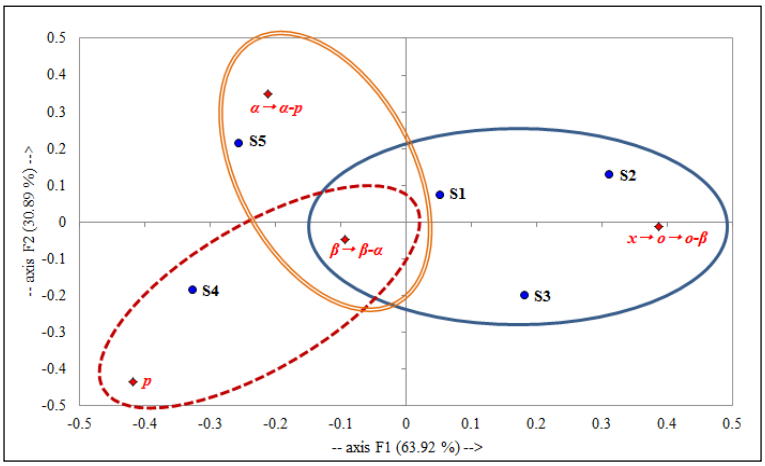

Figure 5. Correspondence Analysis (CA) symmetric plot (axes F1 and F2: 94.80 %) showing the aggregation of sampling sites with the number of indicator diatom species (S1 – S5 as in Table 1; ***x → o → o-β***: xenosaprobic, oligosaprobic and oligosaprobic-β-mesosaprobic taxa; ***β → β-α***: β-mesosaprobic and β-α-mesosaprobic taxa; ***α → α-p***: α-mesosaprobic and α-mesosaprobic-polysaprobic taxa; ***p***: polysaprobic taxa).

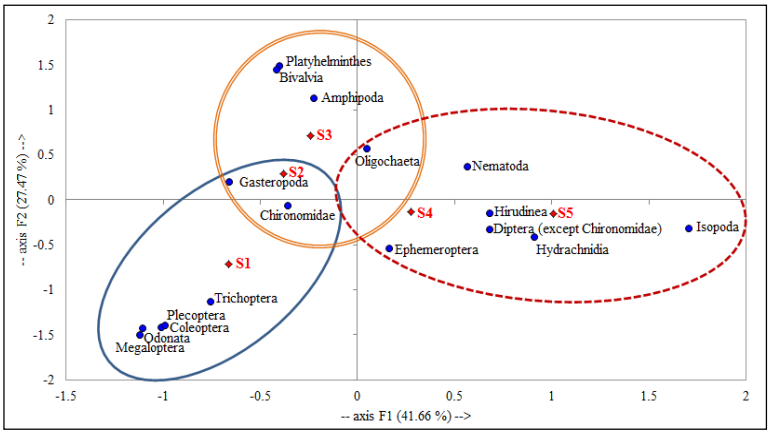

Figure 6. Correspondence Analysis (CA) symmetric plot showing the aggregation of the sampling sites with the 17 taxa categories of benthic invertebrates, in terms of their relative abundances (axes F1 and F2: 69.13 %) (S1 – S5 as in Table 1).

To summarize, benthic diatom and invertebrate communities sampled in 2014 from the Someşul Mic catchment area reflected the major human impacts present in the region stretching from Someşul Rece to Apahida, as shown in Figure 7. The sampling sites S2 and S3 were strongly affected by hydro-technical works: the presence of dam reservoirs, hydropeaking, river regularization etc. At the other sampling sites, other impacts prevaled: the negative effects of waste waters coming from the numerous human settlements and pollution from point or diffuse sources, as agricultural fields treated with fertilizers.

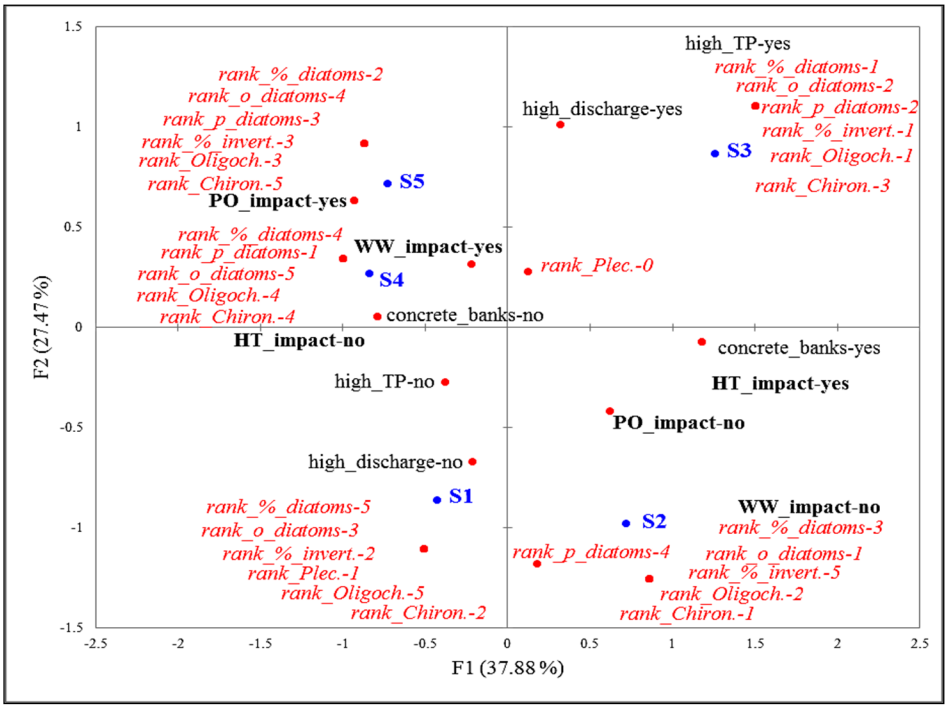

Figure 7. Multiple Correlation Analysis (MCA) symmetric plot (axes F1 and F2: 65.35 %) of the five sampling sites considered for the present study and: i) the main human impacts (**WW_impact**: impact caused by waste waters; **HT_impact**: impact caused by hydro-technical works; **PO_impact**: impact caused by pollution); ii) different biological elements, ranked descending between the five sampling sites (*rank_%_diatoms*: the percentage of diatom taxa present, from the total number; *rank_o_diatoms*: the number of diatom taxa indicating oligosaprobic waters; *rank_p_diatoms*: the number of diatom taxa indicating polisaprobic waters; *rank_%_invert.*: the percentage of invertebrate taxa present, from the total number; *rank_Plec.*: the abundance of Plecoptera taxa present; *rank_Chiron.*: the abundance of Chironomidae taxa present; *rank_Oligoch.*: the abundance of Oligochaeta taxa present; iii) other elements (concrete_banks: the presence of concrete banks along the river; high_TP: high concentration of Total Phosphorus; high_discharge: the presence of high discharge).

At S3, both diatoms and invertebrates recorded the highest percentage, in terms of number of taxa. However, diatoms recorded the highest number of taxa indicating polisaprobic waters, while oligochaetes, a very tolerant group to organic pollution, were ranked with the highest relative abundance. Thus, the highest taxonomic richness was not linked to a good ecological status of the environment.

On the other hand, at S1, both biotic communities recorded lower percentages of taxa: for example, diatoms were ranked 5 (Fig. 7). However, diatoms recorded a higher percentage of oligosaprobic indicator taxa, and the presence of Plecoptera, a group of invertebrates characteristic to clean waters, showed a better ecological status. These conflicting findings depict a less impacted, but still affected community.

S4 recorded the worst ecological status shown by both biotic communities, with the highest percentage of polisaprobic indicator taxa for diatoms, probably due to the nutrient-rich waters coming from the remaining deposits of organic debris left by the old chicken farms. The characteristics of S5 were not very different, also showing negative impacts.

An unclear situation depicted S2, where high hydro-technical impacts led to high abundances of oligochaetes and chironomids. Diatoms however exhibited the highest percentage of oligosaprobic taxa, probably due to the constant flushing of the substratum by water releases from the hydropower plants upstream.

Conclusions

Both benthic diatom and invertebrate communities reflected the ecological status characteristic to five sampling sites from the Someşul Mic catchment area, in the area stretching from Someşul Rece to Apahida localities.

The number of taxa in case of diatoms, and relative abundances of major benthic invertebrate groups showed more balanced conditions at S1, on the Someşul Rece River, where human impacts had lower intensities.

At S3, upstream of Cluj-Napoca, inspite of the highest taxonomical richness, the ecological status was clearly affected, as shown by the high percentage of tolerant or polisaprobic taxa. The influence of the urban center of Cluj-Napoca and its near-by industrial and agricultural facilities were reflected in the characteristics of biotic communites from S4, located on the Popeşti Rivulet, a tributary of the Someşul Mic River, and S5 – Apahida. The conflicting findings about S2, located downstream Gilău dam reservoir, led to the conclusion that biotic communities must be drastically affected by sudden fluctuations of the water discharge.

The present study revealed more or less affected biotic communities at all sampling sites, showing the drastic effects of human influences in the area.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, 1, 2015 (p. 89-94)

Terrestrial isopods (Crustacea, Izopoda) of peat bogs in Romania

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SUMMARY. Nine terrestrial isopod species were identified in five peat bogs in Romania. Out of these six were marshland species (*Ligidium germanicim*, *Ligidium hypnorum*, *Hyloniscus riparius*, *Hyloniscus transsylvanicus*, *Hyloniscus mariae*, *Hyloniscus siculus*) and three woodland species (*Protracheoniscus politus*, *Porcellium conspersum* and *Trachelipus difficilis*). Most species (seven species) were identified in the „Răbufnitoarea" peat bog, which was the smallest habitat, its area being only 1 ha. In the habitat of the Băgău peat bog (3 ha) four species were identified, out of which two were present with a sigle individual only. In the other habitats with a relative large area between 38 and 120 ha, only one or two species were identified. *Protracheoniscus politus* was present in all studied habitats, *Ligidium germanicum* in 3 habitats, all the other species in only one or two habitats. All studied habitats are surrounded with spruce (*Picea abies*) and decidous forests and pastures. The populations of the peat bogs are proceeded from the surrounding habitats. In Romania research considering terrestrial isopods of these habitat types has never been done.

Keywords: peat bogs, marshland, terrestrial isopods, woodland.

Introduction

Ecological and faunistic studies concerning the terrestrial isopods of Romania were made in: woodlands, river banks, hayfields, pastures and scrublands (Radu, 1939, 1950, Radu and Tomescu, 1972, 1976, 1981, Tomescu, 1974 – PhD thesis, Tomescu, 1992, 2010, Tomescu *et al*., 1979a, b, 1995, 2000, 2001, 2002, 2005, 2008, 2011a, b). The study of terrestrial isopods in peat bogs is a first for Romania. The lack of similar studies might be a result of the high level of difficulty in sampling peat bogs. Peat bogs are habitats with a highly acidic soil, which is generally less tolerated by isopods.

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Description of the studied habitats

Our research has been done in five Romanian peat bogs.

Mohoş peat bog with an area of 80 ha, located in the Ciomatu Mare Massive, at 1050 m altitude, surrounded with beech forest. The pitfall traps were set in two distinct habitats of the peat bog: **a**) habitat with birch, where the dominant plant association is the *Vaccinio-Betuletum pubescentis* Libbert 1933, and **b**) habitat with Scots pine, where the dominant plant association is *Vaccinio-Pinetum sylvestris* Kleist 1929, with a layer of *Sphagnum* moss. In both habitats the pitfall traps were set approximately 200 m from the beech forest edge.

Luci peat bog with an area of 120 ha, situated in the Harghita mountains, at 1080 m altitude surrounded by beech forest. On the area of the peat bog there are no open water surfaces, in the moss layer the *Sphagnum* species dominate, blueberry bushes and trees are present in low densities. Pitfall traps were set in three habitats:

- **a**) the open bog area with some sporadic dwarfed pine and birch trees, the dominating association being *Eriophoro vaginati-Sphagnetum recurvi* Hueck 1925.
- **b**) wooded bog with Scots pine and dense blueberry bushes, mostly described as *Vaccinio-Pinetum sylvestris* (*betuletosum nanae*) Kleist 1929.
- **c**) spruce forest, with a dense moss layer, where the dominant association is *Piceetum sphagnoso-Polytrichetosum* Soó 1944 (Tanţău *et al.,* 2003). In all habitats, Barber pitfall traps were set at distance of 200-300 m from the neighbouring beech forest.

Răbufnitoarea peat bog, a protected natural area (1 ha) – Ciomad-Balvanyos, situated in the Bodoc Mountains, at 925 m altitude, sorrounded by a beech forest. Within the peat bog patches of open water are present. The vegetation mainly consists of Sphagnum and scattered birch trees, the dominant plant association being *Eriophoro vaginati-Sphagnetum recurvi* Hueck 1925 (Pop, 1960). Pitfall traps were set approximately 50-100 m from the forest edge.

Fântâna Brazilor peat bog, a protected natural area with a surface of 38 ha, in the Harghita Mountains, at a 950 m altitude, sorrounded by a spruce forest an a pasture. On the area of the peat bog Scots pine and dense blueberry and raspberry bushes are present, the dominant association is *Vaccinio-Pinetum sylvestris* Kleist 1929 (Pop, 1960). Pitfall traps were applied approximately 50-100m from the spruce forest edge.

Băgău peat bog, with a surface area of 3 ha is a protected natural area, at an altitude of 450m, situated near Băgău village at 9 km from Aiud city, Alba County. The bog is surrounded by beech forest. The pitfall traps were placed in the open part of the bog, where open water patches are present, the dominant plant association was *Sphagnetum magellanici* Kastner & Flosner 1933 (syn. *Eriophoro vaginati-Sphagnetum* Pop *et al*., 1987).

Materials and methods

In all studied habitats 15 pitfall traps were set on the 1st of May 2012. The isopods were collected on the 30th of June 2012 and studied in laboratory. Species were identified and the individuals were counted. Due to the low number of individuals collected in the peat bogs (with the exception of the Răbufnitoarea peat bog) cantitative ecological analysis were not possible.

Results and discussion

In the five studied peat bogs nine species of terrestrial isopods were identified, of which six were marshland species (*Ligidium germanicim*, *Ligidium hypnorum*, *Hyloniscus riparius*, *Hyloniscus transsylvanicus*, *Hyloniscus mariae*, *Hyloniscus siculus*) and three woodland species (*Protracheoniscus politus*, *Porcellium conspersum* and *Trachelipus difficilis*) (Table 1).

Table 1.

Terrestrial isopod species identified in peat bogs: number of collected individuals

Species name	Peat bog name								Total number of individuals/species
	Mohoş		Luci			Răbufnitoarea	Fântâna Brazilor	Băgău	
	80 ha		120 ha			1 ha	38 ha	3 ha	
	a	b	a	b	c				
Ligidium germanicum					11	7		6	24
Ligidium hypnorum						44		5	49
Hyloniscus riparius						1			1
Hyloniscus transsylvanicus					2			1	3
Hyloniscus mariae					10				10
Hyloniscus siculus						4			4
Protracheoniscus politus	4	7	3	409		42	37	1	503
Porcellium conspersum	5					14			19
Trachelipus difficilis						3			3
Total captured individuals/peat bog	9	7	3	409	23	115	37	13	

The terrestrial isopod fauna of the peat bogs are originated from the surrounding forests. Some species, which were found in a relative large number of individuals, *Protracheoniscus politus*, *Ligidium hypnorum*, *Ligidium germanicim*,

Porcellium conspersum, *Hyloniscus mariae,* formed permanent populations in the epigaion of the peat bogs. These species tolerate the low pH of peat bogs. Other species, which were collected in a low number of individuals, *Hyloniscus riparius*, *H. transsylvanicus*, *H. siculus*, *Trachelipus difficilis*, are accidentally present species in these peat bogs. This can be caused by the high acidity of these habitats. It is also possible, that other factors as the chemical composition of the detritus on which isopods feed, the desnsity of vegetation, etc. might influence the presence or absence of isopod species in these habitats.

The number of isopod species identified in each habitat is different in concordance with the specific community structure of the nearby forests, and also the area of the peat bogs. The small number of isopod species in large peat bogs can be explain by the number of the isopod species which exist in the sorrounding forests. In the large area peat bogs (Mohoş – 80 ha, Luci – 120 ha, Fântâna Brazilor – 38 ha) a number of 1-3 species were collected (Table 1). In these peat bogs *Protracheoniscus politus* is the dominating species, present in large populations. *Ligidium germanicim* and *Hyloniscus mariae* have permanent populations in the surfaces with spruce and the thick *Sphagnum* layer of the Luci peat bog. In the peat bogs with a small area, individuals of 4-7 species were collected. In the Răbufnitoarea peat bog (1 ha) 3 species were identified in permanent populations: *Ligidium hypnorum*, *Protracheoniscus politus* and *Porcellium conspersum* and 4 accidentally present species: *Ligidium germanicum*, *Hyloniscus riparius*, *Hyloniscus siculus* and *Trachelipus difficilis*. In the Băgău peat bog, all 4 species of which only a small number of individuals were collected, these individuals migrated accidentally from the neighbouring forests. The large number (15) of pitfall traps placed in every peat bog, for a sampling period of two months makes possible the identification of terrestrial isopod populations as permanent or accidental ones in these types of habitats. *Protracheoniscus politus* is the species with the largest distribution, being present in all peat bogs. It's a species with a large spread of ecological needs, tolerant to different levels of environmental factors, this conclusion being also drawn from our previous studies (Tomescu *et al.,* 1971, 2002, 2005, 2008, 104, Mureşan *et al.,* 2003). This species is a woodland species, present in all types of frests in Romania and also inhabiting montane pastures (Tomescu *et al*., 2001, 2002).

Conclusions

- In the peat bogs, wet habitats with *Sphagnum,* the following terrestrial isopod species are present with permanent populations, in a relatively large number of individuals: *Ligidium germanicim*, *L. hypnorum*, *Hyloniscus mariae*, *Protracheoniscus politus*, *Porcellium conspersum*.

- The following species occur accidentally in some of the peat bogs, in very low number of individuals: *Hyloniscus riparius*, *H. transsylvanicus*, *H. siculus* and *Trachelipus difficilis*.

- In the large peat bogs (over 30 ha) a small number of terrestrial isopod species are present (1-3 species), while in the peat bogs of small surface (1-3 ha) the number of species is higher. The small number of isopod species in large peat bogs can be explain by the number of the isopod species which exist in the sorrounding forests.

- The terrestiral isopod populations of the peat bogs are proceeded from the populations of the neighbouring forests, which migrated to these wet habitats. Species which tolerate the ecological conditions of the peat bogs, and form permanent populations.

- The specific structure of the isopod species community in the peat bogs is related to the specific structure of the communities of the neighbouring forests and the specific feature of the ecological factors of each peat bog.

- *Protracheoniscus politus* has permanent populations in all studied peat bogs. *Ligidium hypnorum*, *L. germanicim*, *Hyloniscus mariae* and *Porcellium conspersum* have permanent populations in only one of the peat bogs.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, 1, 2015 (p. 95-105)

The effect of traditional land use of diurnal lepidoptera from Nature 2000 site "Dealurile Clujului Est"

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Cristina Craioveanu[1] and László Rákosy[1]

SUMMARY. The Natura 2000 site "Dealurile Clujului Est" is a vast area (approx. 18.000 ha), with valuable steppe-like and forest habitats still very well preserved. The importance of this site comes from the syntopic presence of 4 *Maculinea* taxons, an extremely rare situation in Europe, and from the fact that the mesophilic medows from this area have the highest plant diversity in the world. In this study we emphasize the effect of mowing and grazing on the butterfly diversity from the site. We used the transect method, walking 6 transects in mowed areas and 6 transects in grazed areas. We used similarity measures and unpaired t-test compare the two different land use types, regarding species number, number of individuals, species diversity and evenness.

Keywords: butterflies, grazing, mowing, similarity, traditional land use.

Introduction

The Natura 2000 site "Dealurile Clujului Est" is located at about 30 km from Cluj-Napoca in the geographical unit "Dealurile Clujului şi Dejului". Semi-natural grasslands are key habitats for maintaining biodiversity in European agricultural areas (Stoate *et al*., 2009), sheltering numerous species whose habitats have been destroyed on vast areas (Baur *et al*., 2006). The importance of the ROSCI0295 – Dealurile Clujului Est site comes from the syntopical presence of 4 *Maculinea* (*M. arion, M. alcon, M. teleius, M. nausithous*) taxons, which is extremely rare in Europe, the presence of some endemic Lepidoptera taxons, like *Pseudophilotes bavius hungarica*, *Cucullia mixta lorica* etc. Moreover, in 2012, Wilson *et al*. registered the global plant richness record for the semi-dry basiphilus grasslands in this area. There are numerous rare species present on the site area, like *Nepeta ucranica, Ranunculus*

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illyricus, Astragalus asper etc. (Bădărău *et al*., 2000). Also in the site we can find one of the largest populations of *Geniolimon tataricum* from Romania and Europe, and the largest populations of *Centaurrea trinervia* from our country. The proximity of Cluj-Napoca metropolitan area is a real menace for the future of the site, its habitats and rare populations within it, due to all the real estate, industrial and agricultural projects that are quickly developing and expanding. One of the reasons these specific structures of the mosaic grasslands from Dealurile Clujului area are still well preserved is that the most part of this site was used as mown meadows until War World Two, while other grasslands from Transylvania were transformed in agricultural crops or became overgrazed. But this doesn't mean that these mosaic grasslands are not affected by the changes in the land use, like intensive grazing, abandonment, drainages, industrial plans, etc. In this study we compare the effect of two traditional land uses, mowing and grazing, on the diurnal Lepidoptera communities from this Natura 2000 Site.

Materials and methods

The Natura 2000 Site "Dealurile Clujului Est" (Fig. 1) is locatet at about 30 km from Cluj Napoca city, in the geographic unit "Dealurile Clujului și Dejului", which is a part of the Someșan Plateau. It covers about 18.000 ha, with valuable steppe-like and forest habitats, still very well preserved. The xeric grasslands are used as pastures and the mesophile ones as meadows.

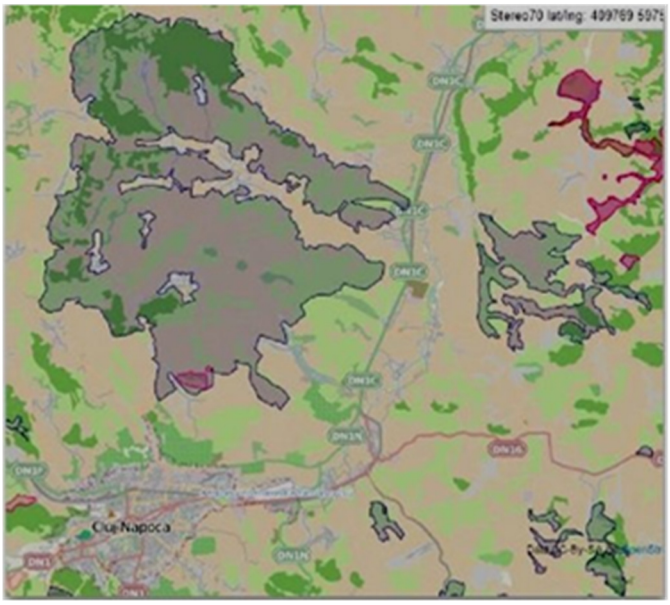

Figure 1. The Natura 2000 Site "Dealurile Clujului Est" map.

The study locations were located at Fânaţul Sătesc, Fânaţul Domnesc and Sechelişte, at about 300 m above sea level (Fig. 2). We used the transect method, taking the samples with the entomological net. The butterfly species and individuals were recorded in an imaginary space of 2.5 m on each side and 5 m ahead. The data was colected from 12 transects (100 m long each), 6 located in mowed areas (4 in Fânaţul Domnesc and 2 in Sechelişte) and 6 located in grazed areas (2 in Fânaţul Sătesc, 2 in Fânaţul Domnesc and 2 in Sechelişte). The surveys where made in good, sunny weather conditions, with temperatures above 18°C, wind-speed less than 15 km/h, between 10:00 and 16:00 -hours, from 21.04.2014 to 15.09.2014, every two weeks. All Rhopalocera and Hesperiidae species from were recorded. The identification of diurnal Lepidoptera was made after Rakosy (2012).

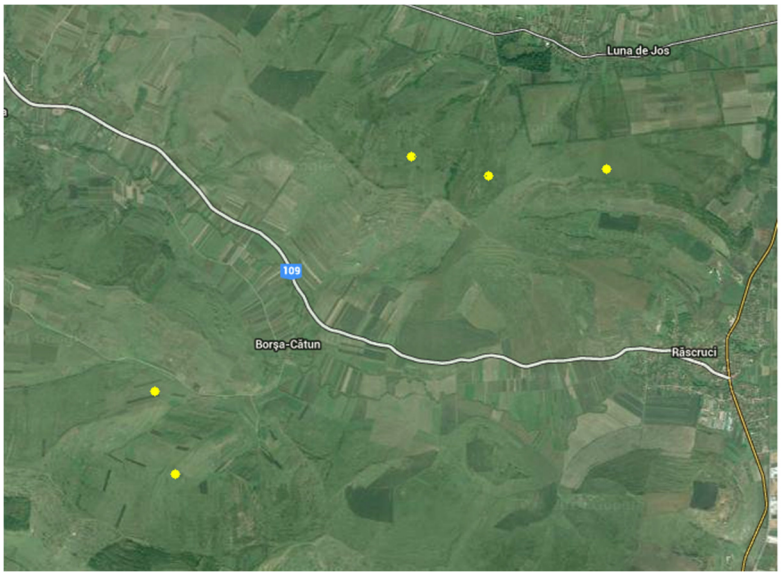

Figure 2. The localization of the study areas, whithin the Site.

In order to check whether we sampled a representative fraction of the local communities we calculated individual-based rarefaction curves (Krebs, 1998). For each transect species richness, individual abundance, Shannon-Wiener diversity index and Pielou evenness index were calculated.

In order to compare the two types of traditional land use, we used an unpaired t-test for individual abundances and diversity indices and Mann-Whitney U-test for species richness, because the data of the latterwas not following a normal distribution. To assess the similarity of the butterfly communities from grazed and mown sites we used single-linked clustering method with Morisita's index of similarity.

Indices, tests and clustering, as well as plots were computed with the program Past 3.0 Statistics (Hammer and Harper, 2001).

Results and discussion

A total number of 53 species of diurnal Lepidoptera (Table 1) were found in the 6 sampling months (from the total number of 213 found in Romania), belonging to 5 families: Nymphalidae (20), Lycaenidae (19), Pieridae (8), Hesperiidae (4), Papilionidae (2). The total number of individuals found in the 12 transects over the sampling period was 3135.

Table 1.

The list of diurnal Lepidoptera from the studied areas.

Taxon	T1 C	T2 C	T3 C	T4 C	T5 C	T6 C	T1 P	T2 P	T3 P	T4 P	T5 P	T6 P	Red List
Hesperiidae													
Erynnistages	2	0	3	1	0	2	0	2	0	0	0	0	LC
Ochlodess ylvanus	2	0	1	23	0	0	0	0	0	0	0	0	LC
Pyrgus malvae	2	1	0	0	1	0	1	0	0	0	0	0	LC
Thymelicus lineola	9	6	0	6	0	0	0	0	2	0	0	0	LC
Lycaenidae													
Celastrina argiolus	2	0	2	0	1	2	0	0	0	0	0	0	LC
Cupido argiades	2	2	0	0	0	0	0	0	0	0	0	0	LC
Cupido minimus	3	1	1	2	1	3	0	0	0	0	0	0	NT
Cyaniris semiargus	4	0	0	2	2	3	0	0	0	0	0	0	LC
Glaucopsyche alexis	1	2	1	3	0	3	0	0	0	0	0	0	LC
Lycaena dispar	0	0	0	0	0	0	0	0	0	0	1	0	VU
Lycaena thersamon	0	2	0	0	1	0	0	0	0	0	0	0	VU
Lysandra coridon	0	1	0	0	0	0	0	0	0	0	0	0	LC
Maculine a alcon	0	10	34	19	19	3	0	0	0	0	0	0	VU
Maculinea alcon xerophila	3	1	1	0	0	0	0	0	0	0	0	0	VU
Maculinea arion	1	0	0	0	0	1	0	0	0	0	0	0	NT
Maculinea nausithous	0	1	22	16	24	18	0	0	0	0	0	0	CR

Table 1 (continued)

Maculinea teleius	0	1	34	24	27	3	0	0	0	0	0	0	EN
Plebejus argus	23	25	24	23	20	25	18	16	0	35	17	13	LC
Plebejus argyrognomon	1	0	1	1	1	0	0	0	0	0	0	0	NT
Polyommatus amandus	0	0	1	0	0	1	0	0	0	0	0	0	LC
Polyommatus daphnis	3	1	0	0	0	1	0	0	0	0	0	0	LC
Polyommatus icarus	22	9	7	11	8	4	9	4	2	2	6	4	LC
Satyrium spini	0	2	0	0	0	0	0	0	0	0	0	0	NT
Nymphalidae													
Apatura ilia	1	0	0	0	1	0	0	0	0	0	0	0	VU
Aphanthopus hyperanthus	3	2	2	2	3	1	1	0	0	0	0	2	LC
Argynnis aglaja	3	1	3	2	4	12	0	0	0	0	1	0	LC
Argynnis paphia	0	0	0	0	1	0	0	0	0	0	0	0	LC
Boloria dia	3	2	4	4	8	10	0	1	0	0	5	3	LC
Boloria selene	0	1	1	0	1	0	0	0	0	0	0	0	LC
Brenthis hecate	14	11	7	8	10	12	0	0	2	1	0	0	VU
Coenonympha arcania	2	0	0	0	0	0	0	0	0	0	0	0	LC
Coenonympha glycerion	9	11	10	7	8	4	2	1	0	1	0	2	LC
Coenonympha pamphilus	35	44	31	31	33	43	42	42	28	33	34	27	LC
Issoria lathonia	1	0	0	0	0	0	0	0	0	0	0	0	LC
Maniola jurtina	134	126	135	114	124	120	36	45	42	32	76	56	LC
Melanargia galathea	47	48	40	37	25	20	14	12	14	21	23	17	LC
Melitaea athalia	2	1	4	1	3	4	0	0	0	0	0	0	LC
Melitaea aurelia	0	0	0	0	0	0	0	0	0	0	1	0	LC
Melitaea cinxia	1	0	1	0	0	0	1	0	2	0	0	0	LC
Melitaea didyma	3	0	0	3	0	0	0	0	0	0	0	0	LC
Melitaea phoebe	2	4	6	4	4	1		0	0	0	0	0	LC
Minois dryas	14	11	1	7	9	8	0	1	3	5	0	0	LC
Vanessa atalanta	1	0	0	1	0	0	0	0	0	0	0	0	LC

Table 1 (continued)

Papilionidae													
Iphiclides podalirius	1	0	0	0	1	0	1	1	19	11	6	2	VU
Papilio machaon	0	0	0	0	0	0	0	0	4	2	0	0	NT
Pieridae													
Anthocaris cardamines	2	1	2	0	3	0	0	0	0	0	0	0	LC
Aporia crataegi	10	6	1	3	3	3	0	1	3	4	0	0	NT
Colias crocea	0	1	0	1	0	1	0	0	0	0	0	1	LC
Colias hyale /alfacariensis	10	16	15	18	16	22	7	4	3	0	14	6	LC
Leptidea sinapis	20	15	12	12	11	13	1	0	4	3	0	0	LC
Pieri snapi	0	0	0	0	0	1	0	0	0	0	0	0	LC
Pieris rapae	0	0	0	0	0	0	0	0	1	0	1	0	LC
Pontia edusa	0	0	0	0	1	0	0	0	0	0	0	0	LC

Abbreviations:

T1C-Transect 1 mowed, T2C-transect two mowed, T3C-transect 3 mowed, T4C-transect 4 mowed, T5C-transect 5 mowed, T6C-transect 6 mowed, T1P- transect 1 grazed, T2P-transect 2 grazed, T3P-transect 3 Grazed, T4P-transect 4 grazed, T5P-transect 5 grazed, T6P-transect 6 grazed, LC-least concern, NT-near threatened, EN-endangered, VU-vulnerable, CR-critically endangered.

Of all the identified species, 15 (28%) are red-listed (Rakosy *et al*. 2002): 6 near threatened (*Cupido minimus*, *Maculinea arion*, *Plebejus argyrognomon*, *Satyrium spini*, *Papilio machaon*, *Aporia crataegi* etc.), 7 vulnerable (*Lycaena dispar*, *Lycaena thersamon*, *Maculinea alcon*, *Maculinea alcon xerophila*, *Apatura ilia*, *Brenthis hecate*, *Iphiclides podalirius* etc.), 1 endangered (*Maculinea teleius*) and 1 critically endangered (*Maculinea nausithous*). Three of the species we found, are on the annex II of The Habitats Directive (*Lycaena dispar*, *Maculinea nausithous* and *Maculinea teleius*).

The rarefaction curve (Fig. 3) shows that for the grazed areas a resonable number of samples have been taken, so a more intensive sampling would help discover only very few more species. But for the mown areas we can see that a higher number of transescts would have yielded an additional number of species to the study.

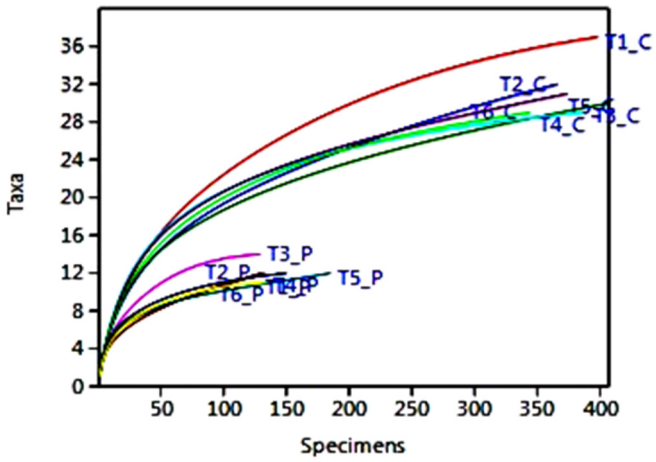

Figure 3. Rarefaction curve for the transects performed in 2014 in two traditional land use types (C-mowed, P-grazed) of the Natura 2000 Site "Dealurile Clujului Est".

Species richness of the mown transects was significantly higher than that of the gazed transects (Mann-Whitney U-test: NC=6, NP=6, MedianC=30.5, U=0, p=0.004) (Table 2, Fig. 4a). Individual abundance in mown transects was also significantly higher than in grazed transects (unpaired t-test: NC=6, NP=6, MeanC=375.2, MeanP=146.5, t=17.8 p<0.001) (Table 2, Fig. 4b).

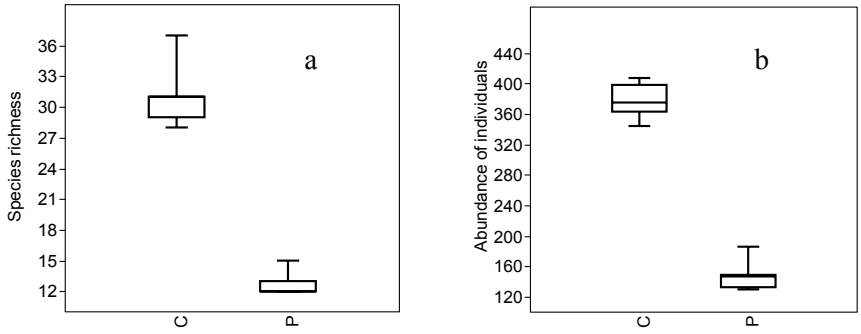

Figure 4. Butterfly species richness (a) and individual abundance (b) in the year 2014, in mown (C) and grazed (P) grasslands from the Natura 2000 Site "Dealurile Clujului Est". Box plots represent 25-75 percent quartiles (boxes), median (line inside the box) and standard deviation (whiskers).

Butterfly diversity (Shannon-Wiener index) of the mown transects was significantly higher than that of the gazed transects (unpaired t-test: NC=6, NP=6, MeanC=2.5, MeanP=1.9, t=9.0, p<0.001) (Table 2, Fig. 5a). Evenness index in mown transects was significantly lower than in grazed transects (unpaired t-test: NC=6, NP=6, MeanC=0.4, MeanP=0.5, t=-4.5, p=0.001) (Table 2, Fig. 5b).

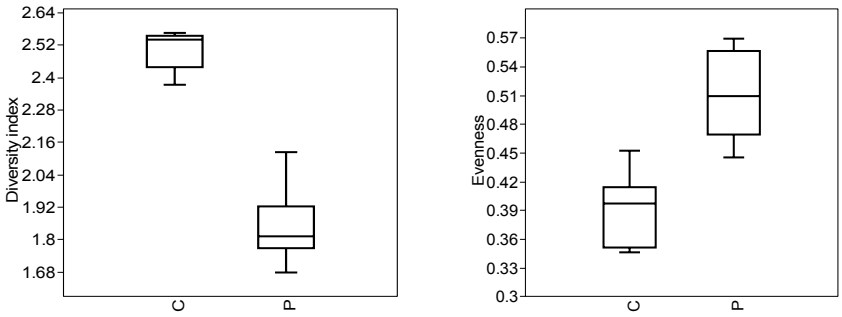

Figure 5. Butterfly diversity (a) and evenness index (b) in the year 2014, in mown (C) and grazed (P) grasslands from the Natura 2000 Site "Dealurile Clujului Est". Box plots represent 25-75 percent quartiles (boxes), median (line inside the box) and standard deviation (whiskers).

Table 2.

Butterfly species richness, abundance, Shannon-Wiener diversity index and Pielou evenness index for each transect performed in the year 2014, in the Natura 2000 Site "Dealurile Clujului Est".

	Species richness		**Abundance**		**Diversity**		**Evenness**	
	C	P	C	P	C	P	C	P
T1	37	12	398	133	2.564	1.810	0.351	0.509
T2	31	12	364	130	2.372	1.676	0.346	0.445
T3	30	15	407	147	2.437	2.122	0.381	0.556
T4	28	12	363	149	2.539	1.921	0.452	0.569
T5	31	13	375	186	2.553	1.807	0.414	0.469
T6	29	12	344	134	2.444	1.766	0.397	0.487

Regarding the species composition, the sampled butterfly communities are grouped by the type of land use (Fig. 6). The comunities from the grazed areas are very similar, as well as the ones from the mown areas.

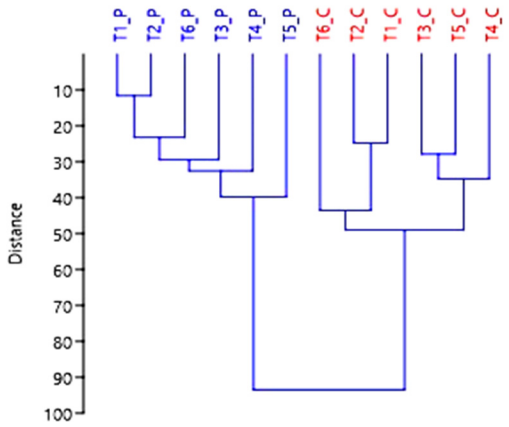

Figure 6. Similarity (Morisita index) of butterfly communities(single-linkage method) of mown and grazed transects, in 2014, in the Natura 2000 Site "Dealurile Clujului Est" (P-grazed, C-mowed).

Overall, we sampled 53 diurnal Lepidoptera species in 12 transects covering two types of land use: mown and grazed grasslands in the area of the Natura 2000 Site "Dealurile Clujului Est". Comparing our results to those of Rakosy and Laszloffy (1997) there are up to 27 further species present in this Natura 2000 Site, however these might be species connected to other habitat types. Furthermore our rarefaction plot showed that we might have discovered several other species in the mown meadows if sampling would have been more intensive. However, when comparing the two land use types, we found significant differences in the butterfly species richness, abundance, diversity and evenness index, showing a higher complexity and higher nature-value of the communities present in mown meadows. If the mown communities would have been sampled even more intensively, the difference between the two land use types would have been even more dramatic.

Even though mown meadows had a higher diversity in butterflies than grazed sites, their evenness was significantly lower, meaning that the number of species is not as evenly distributed over the total number of individuals as in grazed sites. This doesn't mean that they are less valuable communities, but rather that they have several species with higher abundance and many species with very little abundance (1-3 individuals) in the mown meadows, compared to the grazed sites, where there are only few species with a more even distribution of individuals per species. Indeed, if we look at table 1 we can identify several species with low abundaces in mown meadows. These are mostly species with high mobility, low habitat specificity and find

nectar sources or shelter in the mown meadows. Traditionally mown meadows tend to also have a higher structural diversity harbouring also small shrubs. Comparatively grazed areas tend to have a lower structural diversity: vegetation is kept short by constant grazing and shrubs have no opportunity to develop or are intentionally removed. On the other hand if we look at the typical grassland species (e.g. *Erinnis tages*, *Plebejus argus*, *Polyommatus icarus*, *Maniola jurtina* etc.) we will observe a higher abundance in mown meadows. Furthermore, especially sensitive species from the *Maculinea* genus are completely missing in the grazed sites. All these indicate that grazed sites tend to be impoverishd in butterfly communities, and that land use through traditional mowing is a more effective land use to promote butterfly diversity.

Conclusions

To maintain the high biodiversity of the Natura 2000 site "Dealurile Clujului Est", in general, and the diurnal Lepidoptera diversity in particular, we must maintain the grassland ecosystems with traditional mowing and preserve the mosaic landscape by alternating shrub areas, grassland areas and extensive grazing (Page *et al*. 2012). In order to point out even more subtle differences between types of land use (e.g. intensively vs. extensively grazed, hand-mown vs. machine mown) we will continue our study in the following two seasons with a more complex study of the butterfly communities in the same areas.

In addition to this, public awareness and information actions are very important for maintaining the biodiversity of these cultural landscapes.

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Leaf-beetles (Coleoptera, Chrysomelidae) from the Eastern Cluj Hills „Natura 2000" Site

Alexandru Crişan[1,✉] and Mihaela Crişan[2]

SUMMARY. An investigation made in June 2014 on leaf-beetles in "Eastern Cluj Hills, Natura 2000 Site" revealed the presence of 49 species, from 6 subfamilies. Results prove an improvement of general ecological conditions of the area, the xero-mesophilous character of the vegetation and the importance of the group as indicator for human impact. Dominant species of the area, as well as rare and endangered ones, are also mentioned and discussed.

Keywords: leaf-beetles, habitat influence, human impact, rare species.

Introduction

Leaf-beetles were scarcely treated in Romanian scientific literature until the last decade of the 20th century (Seidlitz, 1891; Petri, 1912; Flack, 1905; Marcu, 1927, 1928, 1936, 1957; (old literature); Konnerth-Ionescu, 1963; Negru, 1967, 1968; Ieniştea, 1968, 1974, 1975; Roşca, 1973, 1974, 1976; Bobârnac, 1974 (more recent literature). In the last decade of the 20th century and the beginning of the 21st century some researchers developed more focused studies on faunal and ecological aspects of this group (Crişan, 1993a, b, 1994, 1995, 1997, 2004, 2006a, b, 2007, 2009, 2010, 2011, 2012, 2014; Crişan and Bonea, 1995; Crişan and Teodor, 1996, 1998, 2002, 2005; Crişan and Druguş, 2001; Crişan and Balint, 2007, 2010; Crişan *et al*., 1998, 1999, 2000, 2003; Ilie, 2001; Ilie and Chimişliu, 2000; Maican, 2005; Maican and Serafim, 2001; Gruev *et al.,* 1993). Between these, one of our papers, published in 2000, deals with leaf-beetles in the North-Western part of Transylvania and include also some areas from the Eastern Cluj Hills, Natura 2000 Site (the areas Dăbâca and Vultureni), so that we could discuss and compare these data, this being the first purpose of the present paper. The second purpose was to establish the degree of influence induced by the method of meadow mowing in leaf-beetles, and if the group could be considered a good indicator for the human influence on ecosystems.

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Materials and methods

Leaf- beetle material was collected in June 2014, with the occasion of a large project for monitoring protected species in the Eastern Cluj Hills „Nature 2000"site, developed by a group of researchers. The paper refers only to the leaf-beetles active as adults in that period of the year. Insects were captured with an insect net by striking the vegetation 50 times, approximate 25 m^2, in three repetitions, in each area. We collected material from the following areas:

-Bădeşti village – a natural grassland, about 12 ha, used as hay meadow, not mown at the collection dates;

-Dăbâca village 1. – a large glade, about 3 ha, in a deciduous forest, not mown or grazed at the collection dates;

-Dăbâca village 2. – a pasture in an abandoned meadow, about 5 ha, moderately grazed at the collection dates;

-Dăbâca village 3. – an intensively grazed pasture, about 20 ha, neighboring a deciduous forest;

-Deuş village – a moderately grazed pasture, about 7 ha, not far from a deciduous forest;

-Pâglişa village – a hay meadow extended into an oak forest edge, about 14 ha, partially mown and moderately grazed at the collection dates;

-Vultureni village – an extended meadow, about 25 ha, neighboring a large oak forest, partially mown and grazed at the collection dates.

We remark that in each sampling places wooden vegetation was also present, represented by very sparse bushes or young trees, species as: *Rosa canina, Cornus sanguinea, Crataegus monogyna, Prunus spinosa, Lygustrum vulgare, Viburnum opulus, Corylus avellana, Euonymus europaeus, young Quercus species, Acer campestre, Acer pseudoplatanus, Carpinus betulus, Tilia cordata, Salix caprea, Betula verrucosa* etc., which were also sampled if they were met in the predetermined sampling perimeter.

Leaf-beetle material was collected in 70% alcohol and then was kept dry. Species were analysed in the laboratory, using a stereo-microscope and the appropriate literature (Mohr, 1966; Kaszab 1962-1971; Panin, 1951; Warchalowski, 1993, 2003; Kippenberg and Doberl, 1994; Rozner, 1996; Maican, 2005) for identification and classification.

Results and discussion

1. In the investigated areas we identified a number of 49 species of leaf-beetles from 6 subfamilies, which are listed in Table 1, following the taxonomical order of subfamilies and genera, indicating also the capture date, the number of individuals in each species, and the habitat and place of capture. This represent a

great diversity of leaf-beetles, considering also the fact that it is represented by a single-month capture period. The comparison with the same capture period of the year 2000 (Crişan *et al*., 2000), when only 36 species of leaf-beetles were registered, indicate a general improvement of the habitat conditions in the present, mostly by an enlargement and consolidation of the mown habitats to the prejudice of the agricultural lands.

Table 1.

Leaf-beetles recorded in June 2014 in the „Eastern Cluj Hills" Nature 2000 site.

Crt. No.	Subfamily/ Species	Capture date	No. ind.	Ecol. cat.	Habitat/ place
	I. Clytrinae, Kirby, 1837 **(32)***				
1	*Labidostomis longimana* (Linnaeus, 1761)	05.06	16	p., pr.	Ba.
		05.06	5		Da.1.
		19.06	4		Pa.
		19.06	1		De.
2	*Clytra quadripunctata* (Linnaeus, 1758)	05.06	3	p., sy.	Vu.
		05.06	3		Da.1.
3	*Clytra laeviscula* (Ratzenburg, 1837)	19.06	6	p., sy.	Da.2.
4	*Smaragdina (Monrosia) aurita* (Linnaeus, 1767)	05.06	12	p., sy.	Ba.
		05.06	1		Vu.
		05.06	2		Da.1.
		19.06	1		De.
		19.06	1		Da.3.
		19.06	3		Pa.
5	*Coptocephala unifasciata* (Scopoli, 1763)	19.06	8	p., pr.	Da.3.
	II. Cryptocephalinae, Gyllenhal, 1813 **(78)***				
6	*Cryptocephalus (Cryptocephalus) sericeus* (Linnaeus, 17589	05.06	4	p., eu.	Ba.
		19.06	3		Pa.
		19.06	1		Da.2.
7	*Cryptocephalus (Cryptocephalus) hypochoeridis* (Linnaeus, 1758)	05.06	19	p., eu.	Ba.
		05.06	7		Vu.
		05.06	2		Da.1.
		19.06	1		Da.2.
		19.06	1		De.
8	*Cryptocephalus (Cryptocephalus) octacosmus* Bedel, 1891	05.06	5	p., sy.	Ba.
		05.06	1		Vu.
		05.06	1		Da.1.
9	*Cryptocephalus (Cryptocephalus) biguttatus* (Scopoli, 1763)	05.06	7	p., sy.	Ba.
		05.06	1		Da.1.
10	*Cryptocephalus (Cryptocephalus) violaceus* Laicharting, 1781	05.06	2	m., sy.	Ba.
		05.06	1		Vu.

Table 1 (continued)

11	*Cryptocephalus (Cryptocephalus) bipunctatus* (Linnaeus, 1758)	05.06 05.06 05.06 19.06	1 5 1 2	p., sy.	Ba. Vu. Da.1. Pa.
12	*Cryptocephalus (Cryptocephalus) turcicus* Suffrian, 1847	05.06	5	p., eu.	Ba.
13	*Cryptocephalus (Cryptocephalus) moraei* (Linnaeus, 1758)	05.06 05.06 05.06	1 1 2	o., pr.	Ba. Vu. Da.1.
14	*Cryptocephalus (Cryptocephalus) flavipes* Fabricius, 1781	05.06	12	p., sy.	Vu.
15	*Cryptocephalus (Cryptocephalus) aureolus* Suffrian, 1847	05.06	5	p., eu.	Da.1.
16	*Cryptocephalus (Cryptocephalus) vittatus* Fabricius, 1775	05.06 19.06	1 1	o., pr.	Da.1. De.
17	*Cryptocephalus (Cryptocephalus) virens* Suffrian, 1847	19.06	2	o., sy.	Da.2.
18	*Cryptocephalus (Burlinius) bilineatus* (Linnaeus, 1767)	05.06 19.06	5 1	m., pr.	Ba. Da.3.
19	*Cryptocephalus (Burlinius) exiguus* Schneider, 1792	05.06	1	o., sy.	Da.1.
20	*Cryptocephalus (Burlinius) carpathicus* J. Frivaldsyky, 1883	05.06	4	m., sy.	Da.1.
21	*Cryptocephalus (Burlinius) connexus* Olivier, 1808	19,06	2	o., sy.	Da.3.
	III. Chrysomelinae, Latreille, 1802 **(104)***				
22	*Chrysolina (Chalcoidea) marginata* (Linnaeus, 1758)	05.06 05.06 19.06	6 2 1	o., pr..	Ba. Vu. Pa.
23	*Plagiodera versicolora* (Laicharting, 1781)	19.06	1	o., sy.	Da.2.
24	*Chrysomela (Chrysomela) tremulae* Fabricius, 1787	05.06	1	o., sy.	Da,1.
	IV. Galerucinae, Latreille, 1802 **(33)***				
25	*Galeruca (Galeruca) tanaceti* (Linnaeus, 1758)	05.06 05.06 05.06	7 4 2	p., eu.	Ba. Vu. Da.1.
26	*Galeruca (Galeruca) pomonae* Scopoli, 1763	05.06 19.06	1 1	p., pr.	Ba. De.
27	*Luperus xanthopoda* Schrank, 1781	19.06	1	p., eu.	Pa.
	V. Halticinae, Newman, 1834 **(240)***				

Table 1 (continued)

28	*Phyllotreta armoraciae* (Koch, 1803)	05.06	1	o., pr.	Ba.
		05.06	1		Vu.
29	*Aphthona lacertosa* Rosenhauer, 1847	05.06	6	o., pr.	Ba.
		05.06	7		Vu.
30	*Aphthona caerulea* (Geoffroy, 1785)	05.06	1	m., pr.	Ba.
31	*Aphthona ovata* Foudras, 1861	05.06	2	o., pr.	Ba.
32	*Longitarsus (Longitarsus) lycopi* (Foudras, 1860)	05.06	1	o., pr.	Da.1.
33	*Longitarsus (Longitarsus) pellucidus* (Foudras, 1860)	19.06	1	m., pr.	Da.2.
34	*Asiorestia ferruginea* (Scopoli, 1763)	05.06	1	p., pr.	Ba.
		05.06	3		Da.1.
		19.06	1		Da.2.
		19.06	1		Da.3.
		19.06	1		De.
35	*Asiorestia transversa* (Marsham, 1802)	19.06	2	p., pr.	Pa.
		19.06	1		Da.3.
36	*Asiorestia cyanescens* (Duftschmid, 1825)	19.06	1	p., pr.	Da.3.
37	*Crepidodera aurata* (Marsham, 1802)	05.06	13	o., sy.	Ba.
		05.06	14		Da.1.
		19.06	4		Da.2.
38	*Crepidodera aurea* Geoffroy, 1875	05.06	13	o., sy.	Da.1.
39	*Chaetocnema (Tlanoma) clorophana* (Duftschmid, 1825)	05.06	5	o., pr.	Da.1.
		19.06	1		Da.2.
40	*Chaetocnema (Tlanoma) heikertingeri* Ljubiskev, 1963- Gruev, Tomov, Merkl (1987)	19.06	1	p., pr.	Da.3.
41	*Dibolia (Dibolia) cyanoglosyi* (Koch, 1802)	05.06	1	o., pr.	Da.1.
	VI: Cassidinae, Gyllenhal, 1813 **(30)***				
42	*Hypocassida subferruginea* (Schrank, 1776)	19.06	1	o., pr.	Da.3.
43	*Cassida (Cassida) pannonica*, Suffrian, 1844	05.06	1	m., pr.	Ba.
		05.06	1		Vu.
44	*Cassida (Cassida) rubiginosa* O.F. Muler, 1776	05.06	1	o., pr.	Ba.
45	*Cassida (Cassida) prasina* Illiger, 1798	05.06	1	o., pr.	Ba.
46	*Cassida (Cassida) lineola* Creutzer, 1799	05.06	1	m., pr.	Da.1.
47	*Cassida (Cassida) vibex* Linnaeus, 1767	19.06	1	p., pr.	De.

Table 1 (continued)

48	*Cassida (Mionycha) subreticulata* Suffrian, 1844	19.06	1	o., pr.	Pa.
49	*Cassida (Mionycha) margaritacea* Schaller, 1783	19.06	1	m., pr.	De.

Abreviations: **Crt. No**. = current number; **No. ind**. = number of individuals; **Ba.** = a mown moeadow in Bădeşti village; **Da.1.** = a large glade in a decidous forest, not mowed or grazed at the collection dates, in Dăbâca village; **Da.2**. = a pasture in an abandoned meadow, moderately grazed at the collection dates, in Dăbâca village; **Da.3**. = an intensively grazed pasture neighbouring a deciduous forest, in Dăbâca village; **De**. = a moderately grazed pasture, not far from a deciduous forest, in Deuş village; **Pa.** = a hay meadow extended to an oak forest edge, partially mown and moderately grazed at the collection dates, in Pâglişa village; **Vu.** = an extended meadow, neighbouring a large oak forest, partially mown and grazed at the collection dates, in Vultureni village area.
*- represent the number of species mentioned in Romanian fauna (Maican, 2005).
o= oligophagous species; p= polyphagous species; m= monophagous species;
pr= praticol; sy.= sylvicol; eu.= euritope.

2. The distribution of the number of species according to the subfamilies (Fig. 1.) indicates the domination of Cryptocephalinae and Halticinae, subfamilies which have many xero-mesophilous species, these indicating also the predominant character of the grassland habitats in the investigated area.

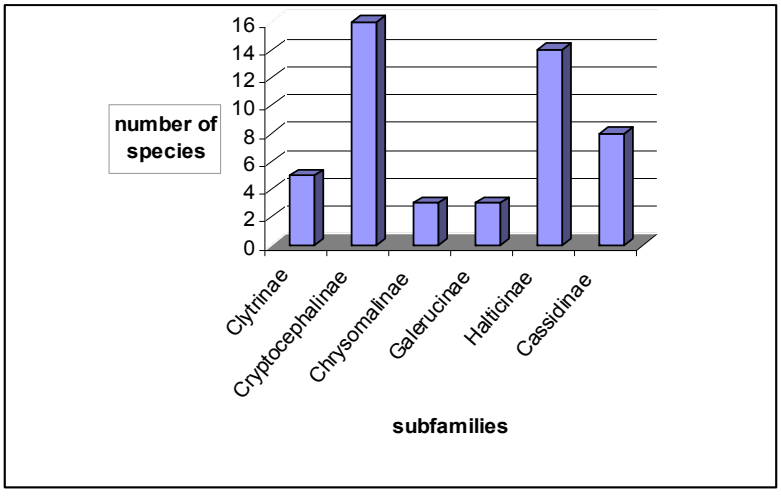

Figure 1. The distribution of the number of leaf-beetle species caught in June 2014 in Eastern Cluj Hills Nature 2000 Site, according to the subfamilies.

In 2014 we found a higher number of species belonging to the Cryptocephalinae subfamily, compared to the number of species belonging to the Halticinae subfamily. Cryptocephalinae species prefere high grasses and compact vegetation, whereas Halticinae are rather found in low and discontinuous vegetation. Compared to the year 2000 (Crisan *et al*. 2000), when Halticinae were dominant, the predominance of Cryptocephalinae in 2014, indicates the evolution of the physical structure of grassy vegetation from a low and discontinuous one, resulted from intensive grazing, to a high and continuous, resulting from a moderate grazing.

3. The distribution of the number of species according to the sampling areas and habitats (Fig. 2.) indicate that in the areas Bădeşti and Dăbâca 1, more than a double number of species were present, compared with Dăbâca 2, Dăbâca 3, Deuş, and Pâglişa, while at Vultureni a mean situation was registered. Considering the fact that the investigated habitats do not differ significantly in pedo-climate conditions (all are meadows on tilted areas in the proximity of a forest), we explain this result as a consequence of the different land use method of these habitats (hay meadow versus grazed pastures). This result indicates also that the leaf-beetles could be used as indicator group of the degree of human impact in the grassland habitats. Because it is a phytophagous group it is sensitive to the changes in the composition and physical structure of the vegetation.

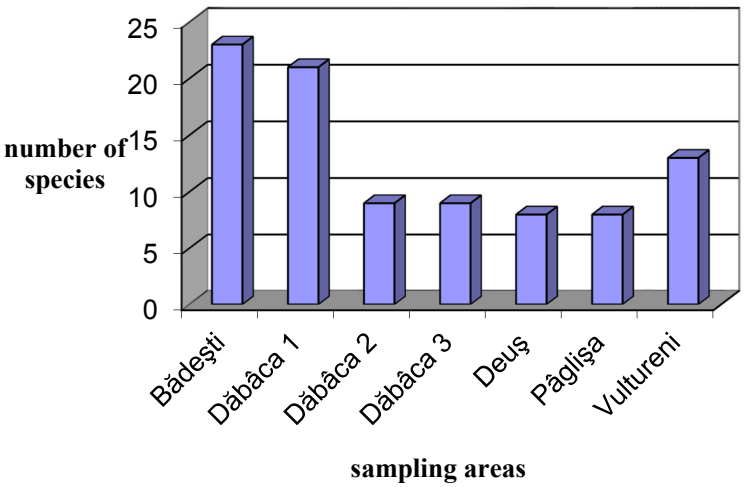

Figure 2. The distribution of the number of leaf-beetles species recorded in June 2014 in Eastern Cluj Hills Nature 2000 Site, according to the sampling areas.

4. Many of the recorded species, like: *Labidostomis longimana* (26 individuals), *Smaragdina aurita* (20 individuals) (Clytrinae); *Cryptocephalus hyppochoeridis* (30 individuals) (Cryptocephalinae); *Galeruca tanaceti* (13 individuals) (Galerucinae); *Aphthona lacertosa* (13 individuals) and *Crepidodera aurata* (31 individuals) (Halticinae) were dominant in the investigated areas. These species were dominant also in the investigations from the year 2000 (Crisan et al., 2000), being species well adapted to the ecological conditions of the Eastern Cluj Hills Site. Other species like: *Cryptocephalus turcicus, C. virens, C. carpathicus* (Cryptocephalinae); *Chrysomela tremulae* (Chrysomelinae); *Asiorestia cyanescens, Dibolia cyanoglosi* (Halticinae) and *Cassida subreticulata* (Cassidinae) are rare and endangered species for the investigated zone.

Otherwise, the situation of rare and endangered species is different, comparing with the investigations from the year 2000: The species *Cryptocephalus aureolus* and *C. schneideri*, found rarely in Vultureni and Dăbâca areas in 2000, were not present in the investigations made in 2014 in Eastern Cluj Hills Site, whereas other three species of *Cryptocephalus* (mentioned above) have penetrated the zone. The species *Phyllotreta armoraciae*, mentioned as rare (1 individual at Bobâlna) in the investigations from the year 2000, have spread to Bădeşti and Vultureni in 2014, and could not be considered as rare any more. A similar situation was illustrated by *Chaetocnema clorophana* (1 individual at Bobâlna in 2000), that extended its area to Dăbâca in 2014, and it is not very rare any more in this area.

Conclusions

In the area "Eastern Cluj Hills, Natura 2000 Site" we identified 49 leaf-beetle species from 6 subfamilies.

The species registered indicate both the xero-mesophilous character of the investigated area and the evolution of the physical structure of the grassy vegetation from low and discontinuous to high and continuous, as a result of a change in the intensity of grazing.

Different methods of land use were reflected in the distribution of leaf-beetles, these being an important indicator of human impact in grassland habitats.

Certain leaf-beetle species, dominant in the area, are better adapted to the area's conditions, whereas others, registered as rare and endangered, are scarce adapted to these conditions.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, 1, 2015 (p. 119-135)

Assessing small hydropower plants impact on Eurasian otter. Case study: the Buzău River, Romania

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SUMMARY. In recent years, in Romania has been registered a substantial increase in the number of small hydropower plants (SHPs) as an alternative of renewable energy source. The construction of small hydropower plants on the rivers of Romania's NATURA 2000 areas is often controversial, being violated national and European legislation. This study aims to assess the otter distribution in the area of a SHP construction project and to find out which is the potential impact of such projects on otter. The standard otter survey methodology proposed by the IUCN Otter Specialist Group was applied for otter evaluation in the area of the SHPs construction and a literature review was used for assesing the impact of the SHPs before construction. The Eurasian otter presence was found in all the survey areas even if the species was absent from the data form of the Natura 2000 Site ROSCI0103 Buzau Everglade, where the study is located. The potential impact of the SHP construction on otter, was not assessed by the assessor in the environmental assessment impact study, for this reason. That's why we review the litterature and use the GIS technology, for understanding and evaluate the potential impact on otter. We found out that this protected top predator of the aquatic ecosystem, the Eurasian otter is potentially affected by the decrease of food resources, destruction of otter holts due to reduced river flow from 21.6 m³/s to less than 5 m³/s and the heavy machinery and workers who work in the riverbed.

Keywords: Conservation, Eurasian Otter, Impact, Small Hydropower Plant.

Introduction

The mountainous area of the Carpathians is the location for the construction of a large number of Small Hydropower Plants (SHPs). In Romania, over 411 small hydropower are in different stages of planning/authorization and construction, and

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more than a quarter of them are proposed to be located within or at the limit of protected areas. Nearly 300 projects have been approved for construction nationwide (Kraljevic *et al.,* 2013).

The Buzău river is found in the SE of Romania, in the Bending Carpathians, the Bending Subcarpathians and Buzău Plain, and is classed as Natura 2000 Site ROSCI0103 Buzau Everglade. The upstream of Buzău River was 'invaded' by small hydropower projects (SHPs) – 5 small hydropower plants with 1 catchment were proposed for this Natura 2000 site in 2012 (Zaharia, 2012).

These SHPs are supported by European Union funds and green certificates allocated on the basis of a national scheme with no ecological criteria attached.

Romania has pledged to increase the proportion of electricity production from renewable resources to 35 % by 2015, and to 38 % by 2020 (Ministry of Economy 2007).

In most of the projects, where small hydropower plants were being developed, despite the area's Natura 2000 status, a lot of problems arose right from the beginning. The most of the SHPs have: no spatial planning process, no proper public consultation, no Environmental Impact Assessment (EIA), nor cumulative impact assessment with other small hydropower projects from the area where is undertaken (Kraljevic *et al*., 2013). In most cases, neither the required Natura 2000 assessment or project permit issued by environmental protection authorities was obtained, also the connectivity of the Natura 2000 sites it was not taken into account. On the other side hydropower installations are considered under Annex II of the EU's EIA Directive, in most of the SHPs building projects, authorities decided that the SHPs should not be subject to an EIA procedure and pulled out from the EIA study at the screening stage.

The European Commission established environmental legislation criteria when proposing a new hydropower scheme. The developer must determine if it is likely to have a 'significant' effect on the integrity of a Natura site. If a significant effect is likely, the 'competent authorities' are required to carry out an 'appropriate assessment' to determine if these effects will be (a) significant; and (b) adverse. Key impacts on listed species and habitats may include anything potentially affecting (among others): number and distribution within the site; breeding success; survival and mortality rates (European Commission, 2000).

The European otter, top predator of the aquatic ecosystem (Clavero *et al.*, 2003), is directly affected by the decrease of food resources, destruction of otter holts due to reduced river flow from 21.6 m^3/s to less than 5 m^3/s and the workers and heavy machinery that work in the riverbed.

Some of the major short term impacts of the SHPs on otters are: (1) increase in accessibility and human presence; (2) movement of heavy machinery and workers; (3) deforestation with habitat loss and fragmentation; (4) change from lotic to lentic ecosystem; (5) lower prey availability and harsher capture; (6) changes in land use adjacent to the reservoir; (7) changing the course and the flow of the river; (8) hydrotechnical development and sewage of the river (Santos *et al.,* 2008).

It is expected, that otters will use "refuge" areas to respond to these impacts. "Refuge" areas are those where species would find refuge to avoid direct conflict with human interventions due to the implementation of the hydropower project.

The goal of this study is to understand the pre-impact and the expected post-impacts, through monitoring the threatened populations of the European otter (*Lutra lutra*) in the area of the Buzău river located in south-eastern Romania, by answering to the following questions:

(1) What is the conservation status of the otter, before project implementation?

(2) How does the otter respond to the project construction impacts and which phase is most critical?

(3) How does deforestation/digging in the riverbed/early flooding/reduced river flow affects otter population distribution?

(4) Which will be the otter distribution after the small hydropower plants building project in the area?

(5) Which are the "refuge" areas?

Materials and methods

The hydropower project is located on the upper Buzău river, in the Bending Subcarpathians, south - eastern Romania, Buzău County. This area is characterized by a highly heterogeneous species, characteristic of different ecosystem components, such as: the azonal meadow, forest fragments, shrub layer, herbaceous stratum, grasslands and agricultural fields.

The existing habitats on the site are: sand beaches - 36%; rivers, lakes - 9%; swamps, bogs - 8%; natural grasslands, steppes - 2%; arable land (crops) - 4%; grassland - 20%; other arable land - 15%; deciduous forest - 12%; other artificial land (settlements, mines, etc) - 2%.

Two types of habitats of community importance, which occupy 40% of the Natura 2000 site Meadow Buzău, are representative for the hydropower construction area: 3240 Alpine rivers and their ligneous vegetation with *Salix elaeagnos* (20%) and 92A0 *Salix alba* and *Populus alba* galleries (20%).

The climate is continental, with hot, dry summers (with precipitation mostly in the form of showers) and cold winters occasionally marked by strong blizzards, and heating intervals causing snow melting. Average temperature is of 12,7 ˚C, maximum temperature is 40,3 ˚C and minimum is – 18,4 ˚C. Annual precipitation levels vary between 500–700 mm (Ielenicz, 2007). The flow rate of the Buzău river in the project area is of 21.6 m^3/s (National Administration "Romanian Waters" 2010). Human settlements are concentrated in cities and in villages located along the Buzău river course.

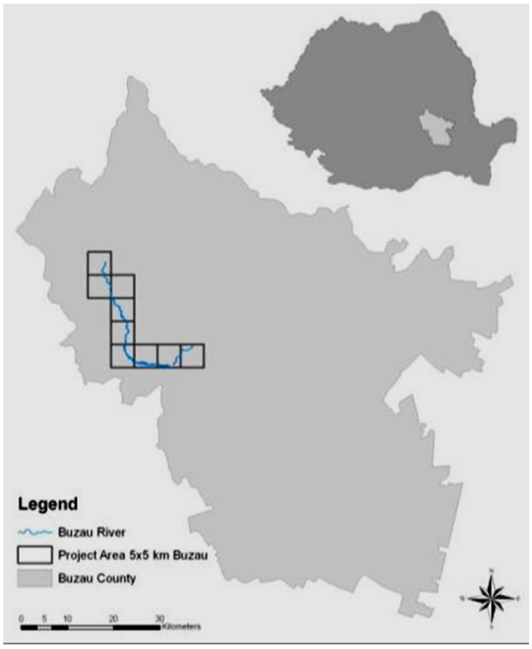

Figure 1. Study area

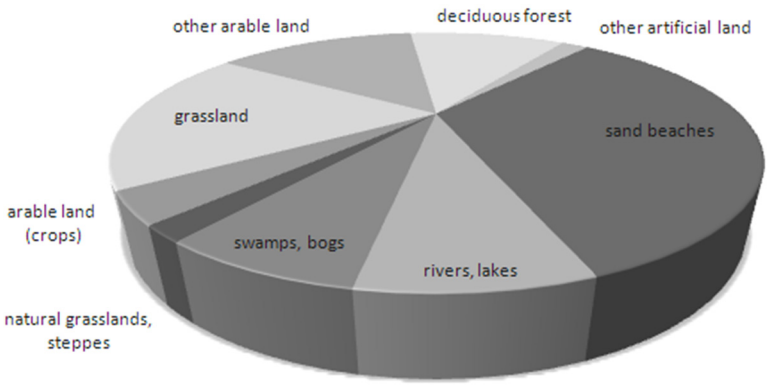

Figure 2. Type of habitats from the hydropower project area

In the initial phase of the study, it was monitored the status of the otter population impact before the construction of the hydropower project starts on the Buzău river and based on GIS data analysis we make assumptions on the distribution of otter after construction. Currently we present the preliminary results obtained in the monitoring before project's start, because the construction has not yet started due to lack of environmental permits.

Prior to field work it was designed a monitoring program based on available information regarding european otter from literature review of other european countries, since there was no available information for the study area. In addition otter was not even mentioned as being present in the area, judging by the standard data form of the Natura 2000 Site of Community Interest Meadow of Buzău.

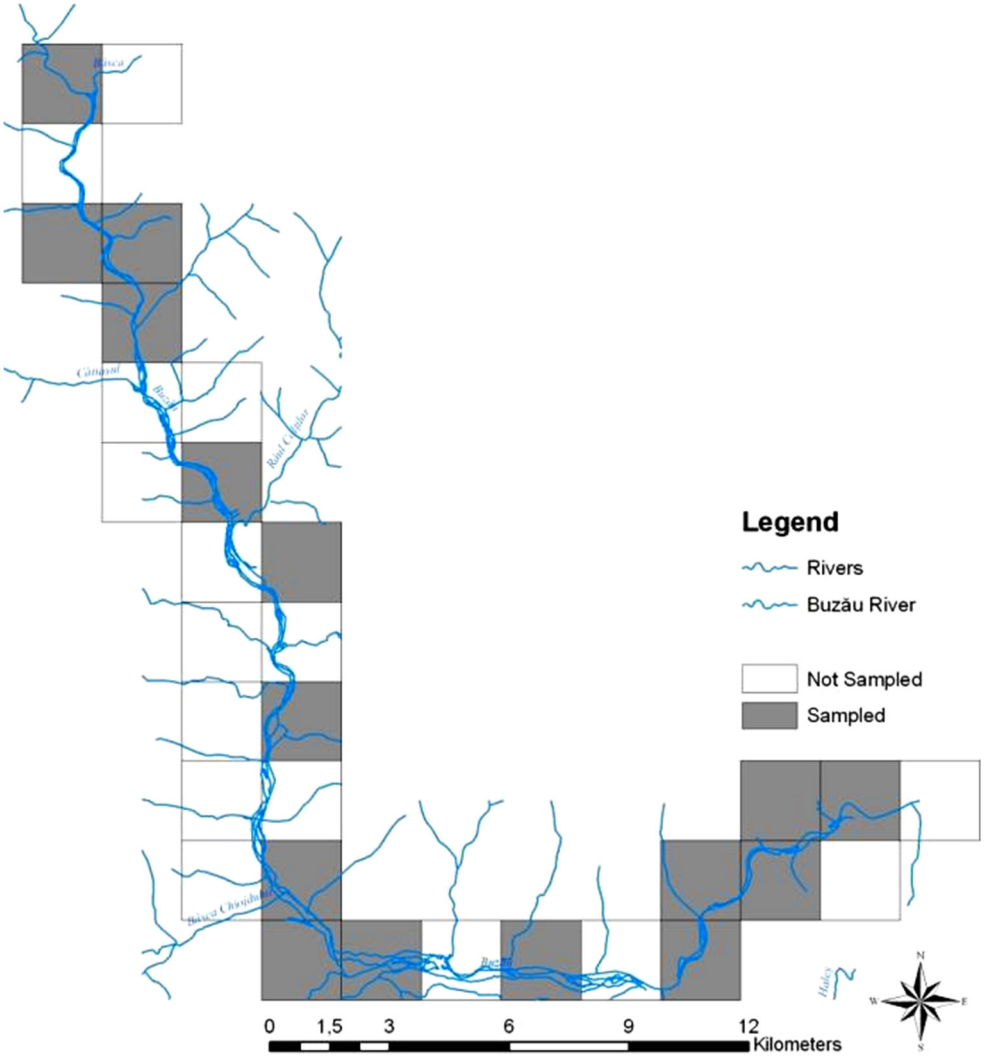

Figure 3. Sampling 2x2 km Grid and Selected Cells: Otter Presence Sampling

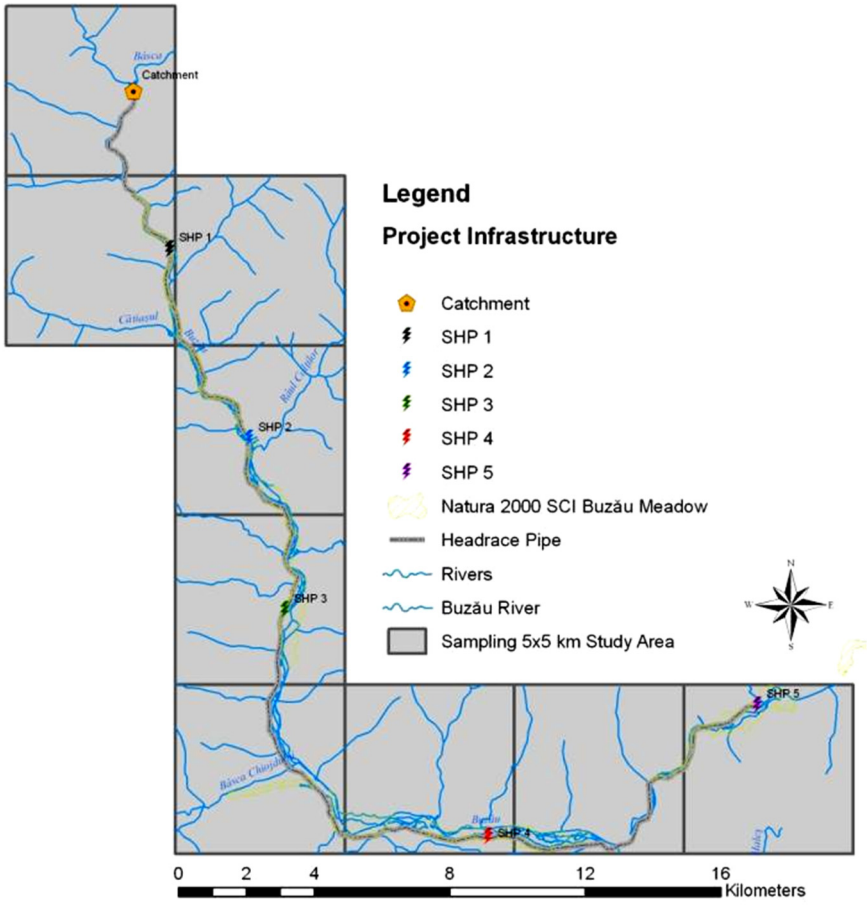

Figure 4. The Buzău River – SHPs Project Area, 5x5 Sampling Grid

The study area was subdivided into a grid of 5 km² cells of the Stereo 70 coordinate system for assessing community structure in the first phase of sampling (Fig. 4) and 2 km² cells randomly selected with different riverine and riparian habitats sampled for Europeean otter presence (Fig. 3). Also another criteria of choice of the cells was - areas where the hydropower project has a high impact: areas that will be built the 5 SHPs, area where will be construct the catchment, and sections where headrace pipeline undercrosses the riverbed.

Linear transects were walked by two observers. These cells were surveyed for otter presence in linear pedestrian transects along water components (rivers, streams and ponds) found within the cell, of at least 600 m (Reuther *et al.*, 2000).

Otter signs of presence (footprints, scats, latrines, burrows, scent marks) were identified and the geographical location was recorded with a GPS device, Garmin Etrex H for further mapping.

Table 1.

Digital data layers in GIS of the Buzău river, SHPs project assessment

Type of data	**Source**	**Format**	**Classes**
Landcover	Europeean Environment Agency	Polygon/Shapefile	CLC 2006
Project infrastructure	Contractor	Line/Shapefile	Catchment, SHPs, Headrace pipe
Water sources	geo-spatial.org	Line/Shapefile	Rivers and streams
Altitude	NASA/ Shuttle Radar Topography Mission	Raster/GeoTIFF	Level Curves
Otter presence data	Present Study	Point/Shapefile	Presence/absence
Otter impact data	Present Study	Point/Shapefile	Presence/absence
Prey availability	Present Study/ Patriche *et al*., 2012	Point/Shapefile	Presence/absence

To analyze the data it was created a Geographic Information System (GIS) database for area impacted by the construction of hydropower project. The Geospatial data used for this GIS database were from diverse sources; some of them were updated, and were used to evaluate species-habitats relationships.

Data on otter presence/absence was recorded as geographical locations. The Europeean otter presence data was plotted in maps of distribution.

Otter population nucleus area and boundaries were determined using a fixed kernel method applied to 100% of the geographic locations of otters presence.

This method was used to assess species distribution range expansion before the construction.

In order to understand the problem it was overlaped all the layers from the Table 1, to determine the most suitable areas for otters before the project implementation and evaluate which factors are contributing for otter's distribution after disturbance and which will be the "refuge" areas. It was examined the effect of land cover, project infrastructure, prey availability, water sources as potential explanatory variables driving range variation and/or habitat selection patterns. We used the landcover units from GIS data (Table 1) and extracted layers of suitable habitats for otter. Suitable habitats were selected accordingly to otter species requirements described in the literature and the results from our monitoring program, so for the otter we selected riparian vegetation, river courses and streams.

Results and discussion

The conservation status of the otter, before project implementation

The otter showed a great incidence in the project area, covering 75 % of the sampled area. A high density of signs of presence (majorly tracks and spraints), left by otters could be identified in the area where it should be built the hydropower project.

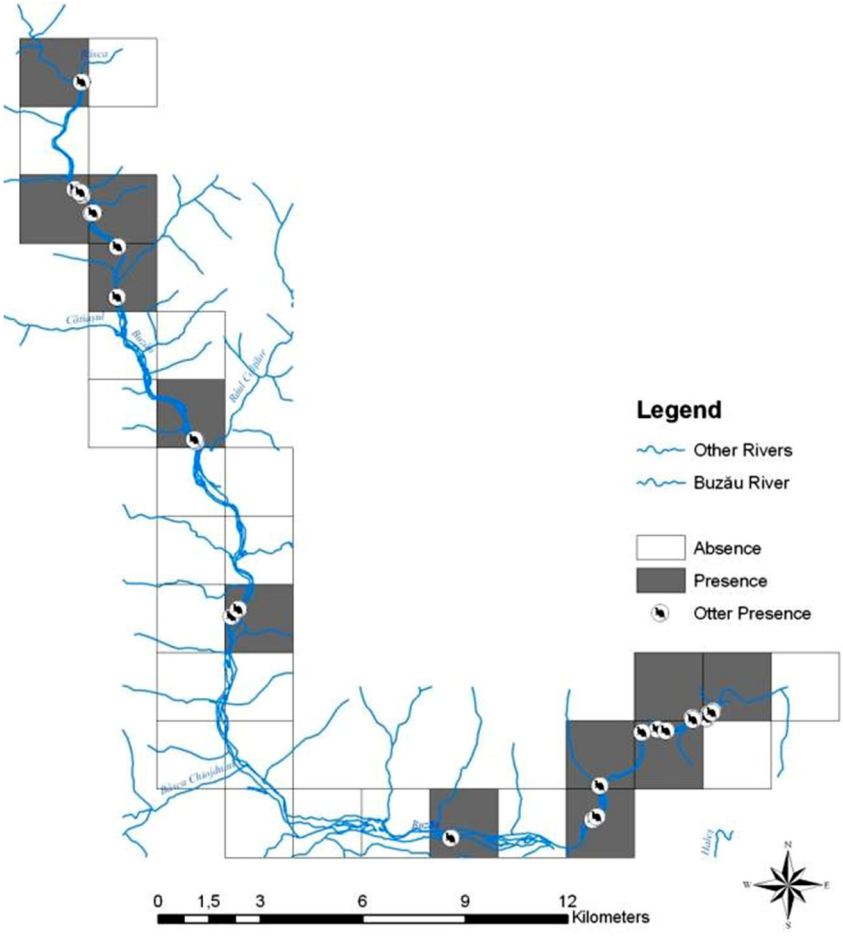

Figure 5. Otter distribution patterns before implementation of the SHPs project

During the survey, based on tracks, it were identified 3 female otters with cubs, thing that suggest a good reproduction rate and a strong and healthy population.

The study revealed two important hotspots, for the otters (Fig. 6), in the project area. One is situated in the north of the project area between the Catchment and the Small Hydropower Plant 1 and another one is situated in the south-east part of the project around Small Hydropower Plant 5. The presence of the otter it was not continous, the distribution is fragmented by the areas with a high level of human activities.

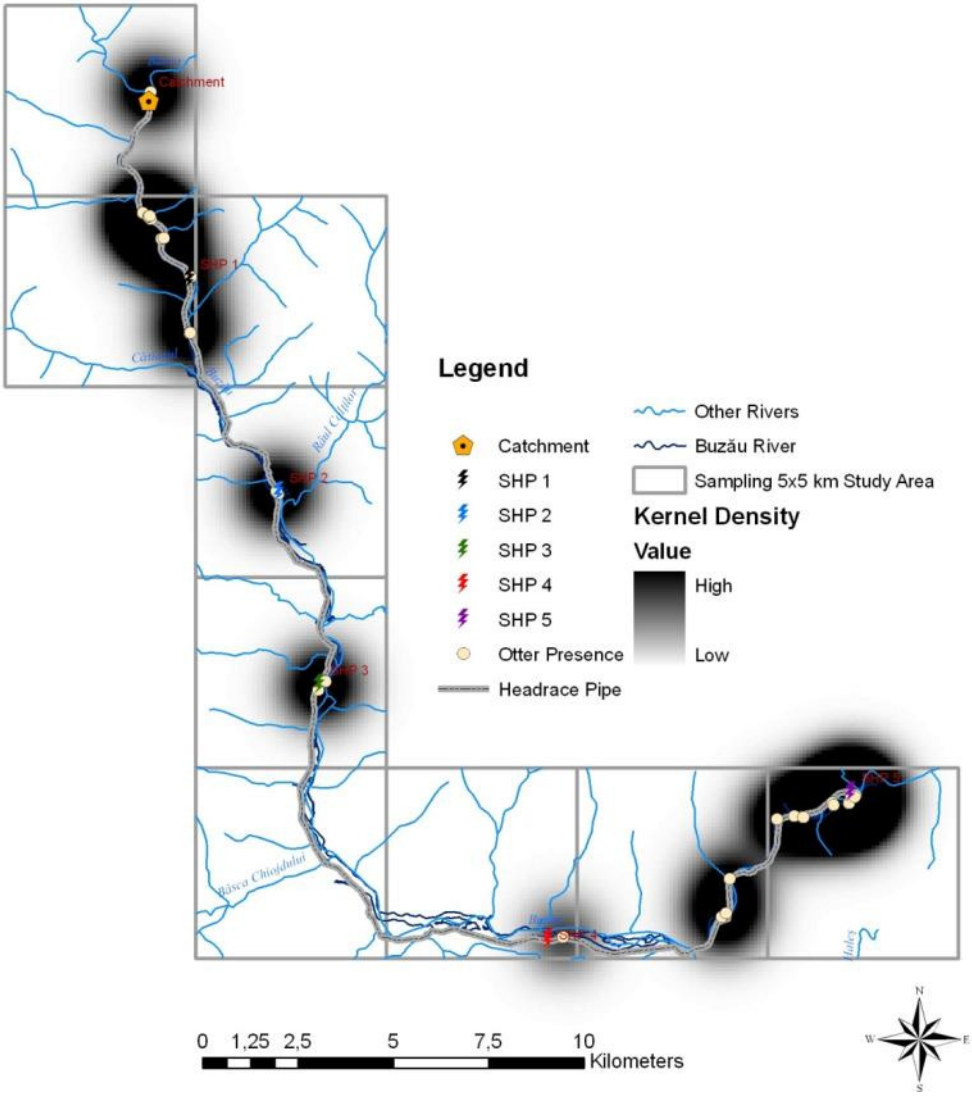

Figure 6. Otter Hotspots before the implementation of the SHPs project

In the study area numerous anthropogenic activities with a negative impact in the quality of the habitat occupied by the otter, were observed. The major threats on otter are: extraction of sand and gravel from the riverbed, incorrect storage of household waste, water pollution and modification of the river functions. (Macdonald *et al*., 1983, Tüzün *et al.,* 2004).

Figure 7. Otter latrine on a stone in the Buzău riverbed

Signs of otter presence, were identified, also in areas with an intense human activity like: fishing, grazing, transport system (railway or major road) even if this activities had a daily presence.

The otters prefer this area with human activities due to rich trophic potential of fish and amphibian fauna, identified in the area. The high density and diversity of fishes and amphibians reveal a good ecological and chemical status, which was confirmed by a study made in 2010 by the National Administration "Romanian Waters".

In order to examine the role of prey availability in otter distribution, fishes and amphibians were considered the main food resource. Many captivity experiments studies have demonstrated otter preference for fish and, among fish, for larger (intermediate) sizes. (Erlinge, 1968, Topping and Kruuk, 1996). In 2012 for the project area, was made an ichthyologic survey, using as method the electro-fishing it were found 11 species of fishes and 10 species of amphibians in 14 monitoring stations located in key areas of the project: catchment, the 5 SHPs area and in the area where the headrace pipe undercross the river (Patriche *et al.,* 2012).

Before the construction has started, in this river sector that may be adversely affected by hydropower project, it was discovered a healthy and vigorous otter populations with a strong growth trend.

Project construction impacts on otter

In order to understand the impact of the project during the implementation, it is required to make a short brief description of the infrastructure, of its characteristics and operating mode.

The Catchment will have a capacity of 9,000 m³, it will be build in the riverbed and as building material will be used reinforced concrete, the river diverting will be also required. Catchment infrastructure located to the right bank will permanently occupy in the riverbed an area of 57.81 m².

Figure 8. The catchment and the surrounding area

The headrace pipe has a diameter of 3200 mm and a total length of 40,501 m. It will be buried in the ground and completely embedded in concrete in areas where is laid in the riverbed of the Buzău river. During the construction of the headrace pipe, in the riverbed and on riverbanks will be dug a trench with a width of 6 meters to allow handling of headrace pipe, this way will be used 20,112 m². For the temporary storage of the excavated material, along the route of the pipeline, will be used an extra 33,520 m² (a corridor of a width of approximately 10 m).

Digging for the headrace pipe will cause the destruction of riparian habitat that can not be mitigated or compensated through habitat creation because such ecosystems cannot be recreated. It can only return through natural succession which is believed to take few decades, and may not occur at all if too extensive an area is damaged. The riparian vegetation from the banks represent the perfect place for otter holts and for the otter resting places.

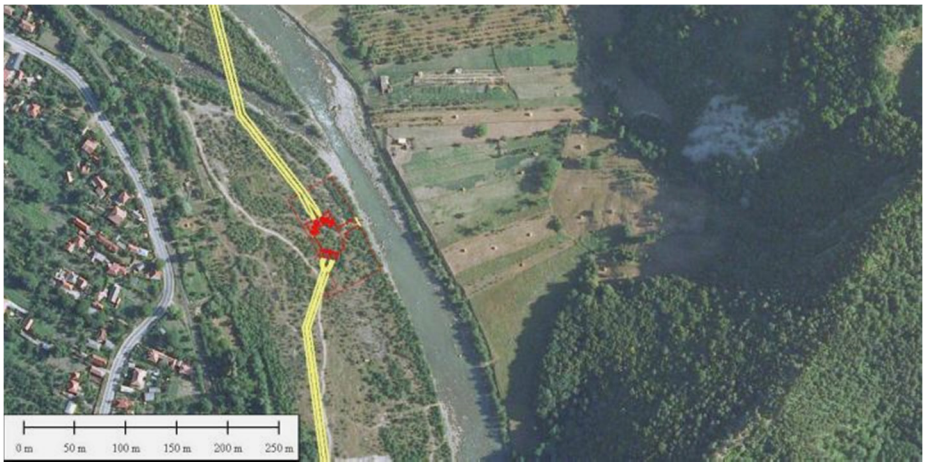

Figure 9. Small Hydropower Plant no. 1 and the surrounding area

For the Small Hydropower Plants placement will be used about 100 m² for each, so for the 5 SHPs, will be permanently occupied a total area of 500 m² and 1000 m² temporarily, during construction activities (200 m² for each building).

The temporary and the permanent infrastructure should occupy an area in the site, as small as possible. As less space is affected, the project is less harmful to otters, considering the fragile wetland habitat from the site.

In this project it is necessary the undercrossing of the Buzau riverbed by the headrace pipe, for making this work, the river will be diverted from a bank to another. The headrace pipe will undercross the Buzău river in 7 points.

Figure 10. Example of work in the riverbed – the Capra River, the Făgăraş Mountains (Source: *www.romaniapozitiva.ro*)

Heavy machinery such as bulldozers, cranes, excavators, pick hammers, trucks, concrete mixers will work in the riverbed: dig into the riverbed, divert the river, reinforce the banks with concrete, create access way and platforms for storing construction materials and will remove the riparian vegetation.Estimated construction time is 60 months.

In the construction phase a part of the otter holts will be destroyed by the heavy machineries or by the changing of the river course and by the flow decrease.

During this time almost all the otter activities in the area of the project will disapear because of the intense human activity and the synergy of pollution sources. Prior to project implementation start, the otter presence was 75%, but during the construction phase, the presence of the otter would be less than 10% of the area.

The otter impact will be also indirect, caused by the lack of trophic potential, food resources decrease due to accentuated water turbidity caused by the work in the river bed, and the long construction time (60 months), such the otter population concentrated in the area will focus on the Buzău river tributaries, having a high flow and a trophic potential that can meet the habitat requirements of the otter.

When the hydropower project is under construction, the disturbance produced by machinery, light, presence of workers and other activities affect the otters, which will then try to escape to adjacent habitats. In addition, the the lack of food resources displaces resident animals to nearby areas. A phenomenon known as the reservoir's extended effect affects mammalian species, which move from their original home range areas to adjacent areas already occupied by the same species (Alho, 2011).

Impact on otter during the operating phase

During operating phase there will be no further changes to the riverbed and terraces of the Buzău river. But the biggest change, which has also a major impact, will decrease by more than 3 times the flow of the Buzău river. From a multiannual flow of 21.6 m^3/s to be reduced to a minimum rate of 5 m^3/s.

In general, the closer the flow is to natural levels and patterns, the fewer species will be affected. Furthermore, flows adequate for water needs of the otter, may well be insufficient for maintaining healthy aquatic communities which provide food for otters. It is probably true to say that a minimum flow perceived as acceptable to maintain fish species assemblage and abundance would be enough to reduce or eliminate effects on otter.

The heavily altered ecosystem provides poor fish resources for otters (Chanin, 2003, Kloskowski *et al*., 2013). It might be argued that at high densities, small fish may form a rich food supply (Topping and Kruuk 1996), but with the very small prey size, the energetic costs of hunting and daily calorific requirements of otters may be difficult to balance (Mason and Macdonald, 1986).

Figure 11. The Buzău river flow in October

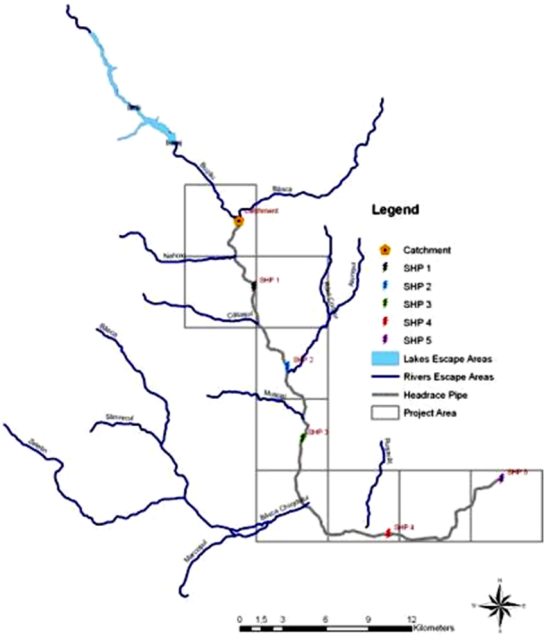

Figure 12. "Escape" areas for otters after hydropower project implementation

A minimum downstream compensation flow of 5 cubic meters per second is under discussion. This is unlikely to be sufficient to prevent problems for piscivorous vertebrates, like the otter, even the maximum release under discussion of 10 cubic meters per second is unlikely to mitigate all of the negative effects of a reduced flow. The compensation flow should be maintained as high as possible preferably 15 cubic meters per second (or more) also during the summer (The Wildlife Conservation Society, 1995).

At low flow levels of the river, the area affected by the hydropower project, could no longer sustain the present otter population, it will be forced to turn to other areas, called "refuge" areas.

Even during construction phase the otter population will be focused to quiet areas that could fulfill their habitat requirements. As can be seen in the „escape areas" map (Fig. 12) the most important rivers and lakes that could be a habitat for otters are: Bâsca (12.6 m^3/s), Bâsca Chiojdului and its tributaries, the Buzău River, upstream the Catchment and Siriu Lake (155 millions m^3 hydropower reservoir). All the areas of refuge are located upstream the project area, due to poor ecological quality of the river Buzău, high population density and intense human activities from downstream.

Forced to occupy new territories, the otters need to fulfill its daily needs and activities. Social contact, familiarity with the area, and social organization are factors that may influence the individuals daily activity, in addition to food gathering.

Otters are not moved into an empty space, but must fit into an existing biological context. This context implies occupied territories, already used home ranges, intra and inter competition for food, space and mate, behavioral interactions, carrying capacity based on offer of ecological resources, and so on (Alho, 2011).

During the initial phase of higher densities of animals in areas adjacent to the Buzău river Small Hydropower Plants project, the competition for food, otter holts, resting places and other ecological resources is tighter. Additionally, the phenomenon known in behavioral ecology as the principle of xenophobia makes free ranging individuals, without fixed home ranges, more vulnerable to be submissive in disputes with resident species for ecological resources, like available food, space and other vital requisites. The result is that soon the otters displaced by the effects of the hydropower project will die or move to another area, the previous ecological densities will return, and the final result is the unsuccessful attempt of the displaced otters to establish themselves in another area (Alho, 2011).

Conclusions

Otters surveys are known to be crucial because they provide important information on species distribution, abundance, habitat and may serve as potential indicators of the impacts of human activities on the aquatic environment. The hydropower projects often result in irreversible loss of habitat, which is particularly crucial for threatened carnivores as the otter who we addressed herein.

This study presents preliminary data; the results before the project's implementation and make assumptions on what will be the situation during construction and operation phases. The study will be continued if the projects similar with this will receive all approvals, analyzing then the real situation.

Monitoring the impact of Small Hydropower Plants project from Buzau river on otter population has shown that this project could have a major negative impacts in the population's distribution, abundance and habitat during and after the implementation.

The negative impact starts with the riparian habitat loss since the construction of the SHP project and finish with the decrease by more than 3 times the flow of the Buzău river, during the operating phase. At low flow levels of the river, the area affected by the hydropower project, could no longer sustain the present otter population, it will be forced to turn to other areas, called "refuge" areas.

Most impacts can be avoided or reasonably mitigated if the projects are correctly, planned, designed and controlled.

And also, more attention must be paid on especially cumulative impacts and monitoring studies on every process of planning and building of the Small Hydropower Plants.

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Baulks, cultural heritage elements as ecological corridors in some traditional Romanian landscapes

Cosmin Ivaşcu[1,✉] and László Rákosy[1]

SUMMARY. The key objective of the current study is to make a general analysis about baulks, one of the structural elements that define a natural – cultural landscape, and that can be considered in a way "elusive". Baulk can be defined as a narrow stripe of land acting as a border between two agricultural fields that are used in a rather traditional manner. They have a important role in the traditional rural communities, marking private properties, and there are special rules for the management of these structures. Within the landscape, baulks work as ecological corridors, linking the various natural and anthropic habitats. The interconnectivity of the different habitats and the maintenance of a high degree of biodiversity is practically assured, in the landscapes where these structures have been conserved. We especially focused on the baulks found in Banat, Maramureş and for other regions we used data from references.

Keywords: baulk biodiversity culture ecological corridors

Introduction

The contemporary social ecological system is mainly characterized by two dynamic aspects, namely: *growth* (Bargatzky,1986)) and *change,* as an effect of growth, changes that transcend the social system and the contemporary human communities, and that have an effect also on the enviroment that human communities inhabit, effects which range from positive effects to negative ones, and which are contributing to the ongoing process of the erosion of biodiveristy.

Enviroment is a part of nature, and nature is understood and conceived in varying ways, according to the different viewpoints, ideologies, scientific branches or even the cultural contexts one looks upon it. Mostly nature is viewed as an external

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reality different from man or human society, which in it's pristine shape is considered to be untouched by man and his activity (Bargatzky,1986). This romantic concept is considered nowadays to be obsolete, because man has had an impact on every corner of nature, in a way or another (Bargatzky,1986).

On the other hand, man as integral part of nature, in his pursuit for survival, is using his intelligence and culture as more effective way of coping with enviromental constraints, perpuating his species and succesfully adapting to the enviroment (Schutkowski 2006, Sutton and Anderson, 2010).

In the view of these, we will use the concept of enviroment to outline the type of nature that is shaped and transformed by human society and it's subsistence activities. As long as human activities were maintaining the subsistence of local and regional communities, modelation as an efect of human activity on biodiveristy has had an overall positive effect in Europe. An exception could be considered the extinction of several big mammals in Europe, during the XVI and at the end of the XVIII centuries, when *Bos primigenius*, *Equus ferus gmelini* gone extinct (Filipaşcu, 1969). A radical shift in balance, occurs when people start moving from extensive subsistence agriculture, which sometimes is controlled by ethical percepts with regional or ethnic nuances (Cristea and Rákosy, 2011; Rákosy 2011), to intensive agriculture, driven only by yield and profit.

Actual landscapes are fragments of nature, that belong both to the cultural and the natural enviroment, a particular type of landscape is considered to be the *cultural landscape*, which is a result of the interaction between humans and nature, and which owes its existence and persistence in time due to human presence and it's activity. The concept of cultural landscape has a long history, bearing the imprints of the main paradigms that influenced scientific research at various times (Calcatinge, 2013).

Even within the concept of cultural landscape there are some major differences and distinctions to be made (Calcatinge, 2013). Urban cultural landscapes or the different theories that label and classify the various cultural landscapes (Calcatinge, 2013), are not within the field of our research or interest. We will insist instead on what is commonly considered as being a natural – cultural landscape, sometimes also reffered as natural – cultural *landschaft* (Rákosy, 2011), which is usually understood and used to outline the part of nature that is shaped by traditional activities, as part of subsistence agriculture, and due to that, assures a high degree of biodiveristy (Rákosy, 2011). Some classical theories speak about a natural landscape, that has a series of dynamic elements and some stable ones, within the dynamic elements, human activity is considered to be most important driving factor that shapes the structure and dynamics of a given landscape (Teaci, 1983). Thus it seems very hard or almost impossible to make a clear demarcation betwen human activity and the notion of landscape or *landschaft.*

Another very interesting concept which has been coined and used within the biology school of Cluj Napoca, by some researchers like Nicolaie Boşcaiu (Cristea and Rákosy, 2011), or Alexandru Filipaşcu (Filipaşcu, 1977) is that of the *ethnoecosistem*. The *ethnoecosistem* concept was utilized, but sadly it was not defined until very recently by Cristea and Rákosy (2011, manuscript). This considers an *ethnoecosistem* to be: "A partycular type of secondary ecosystems, with an important note of originality and a susbtantial state of native life, a type which is adjusted, and maintained in time by specific human communities by "ethical instructions" (specific cultural practices), in a determined geographical space".

A different viewpoint on the natural – cultural landscapes, is one linked to the ethnic factor, which is considered to be responsible for shaping, the perception, representation and the differentiation of certain landscapes (Gnädinger *et al.*, 2011), as a result the concept of *ethnic landscape* was coined and it refers to: "areas, that are perceived in a certain, diverse, often characteristically way by one or more ethnic groups. The perception depends mostly on the properties of the area, which are the result of actions and interactions between human and natural factors (Gnädinger *et al*., 2011, p.4).

Beside the more obvious aesthetical values, some elements have some very special functional and structural values, given by the human community, which allowed their development. But despite these values and roles which the human community is giving to a certain element, it can also have a function that is unknown, or acknowledged on a small scale. In this case both functions, the social one and the one which is imprecisely aknowledged, define the multifunctionality and the importance of the certain elements. From this point of view we can acknowledge and understand the variety of definitions which are given to a landscape (Gnädinger *et al.,* 2011) and why the different specialists have so many different viewpoints on the issue (Cristea and Rákosy, 2011), as the landscape is viewed separately by geographers, tourists, consevationists, landscape architects or ecologists (Godart and Deconick, 2003), we see how different elements of the landscape can have different significance and functions, but taken alltogether they offer us a more complex view upon the landscape as whole and it's valor for biodiveristy and human culture.

Baulks as structural element of the natural – cultural landscape

Certain structural elements of the landscape, features such as green fences, woodsides, orchards, terraced slopes etc., are typically taken into consideration when it comes to analysing the characteristics of a landscape (Gnädinger *et al*., 2011).

However within the natural – cultural landscape there are some other structural elements, that can be considered a bit more "elusive" but which have an outstanding cultural and ecological importance, and are also structuring the landscape. "The baulk" is certainlty such an element, which has both cultural and ecologcal importance.

A baulk is a strip of land that separates two agricultural fields, whether they are cultivated or not, practically it can be considered as a border which delineates the different private properties within a given community, especially the agricultural fields. In some cases and in some regions of Romania, baulks are separating orchards, backgardens, hay meadows, vineyards etc. Baulks are usually managed differently by the two neighbours and special property rules apply.

Materials and methods

For this study we investigated two villages from two distinct historical regions of Romania, Maramureș in Northern Transylvania and Banat in the South – West of Romania. The linear stripes of land that separate the different terrain types and properties, have different names, but have the same role althought a slight different management can be observed. The different shapes is due to geographic diferences in both regions, although the social role is exactly the same. In Banat we investigated the village of Forotic which is situated in the ethnographic region of the Caraș Valley, county Caraș – Severin. In Maramureș county, we conducted our research in Ieud, a village situated on the Iza Valley. We used semi – directed interviews, but the results will be published in another paper. We relate our findings with literature data from other regions of Romania.

The origin of baulks in Romania

The general Romanian term for a baulk is "răzor" (according to Dicționar Tehnic englez – roman, 1997), but this landscape structural element has a variety of names, according to region, shape and even village. The most widespread name is "răzor', in the historical regions of Banat it is known as a "slog", in Maramureș it is named "hat" or "mejdă", in other regions of the country it is known under different names such as dâlmă, mejdină, mejdrină, mezuină, răstav (Scriban, 2013). The significance and function of this structure is the same all over Romania, including the hilly – mountainous Maramureș where *hat* or *mejdă* is considered to be a strip of land that separates two cultivated terrains, more certainly two agro - terraces, but also in regions with lower altitudes, where as simple furrows (Stănică, 1937), strips of uncultivated land or a border of trees (Scriban, 2013), they separate and mark private properties. What we name under the generic Romanian term "răzor" or English baulk had, the initial role of delineating different parcels which were utilized in various ways, being a symbol of private property and of neighbourhood, thus being implicitly a symbol of traditional extensive agriculture, with terrains that were differently utilized.

In Romania the genesis of baulks can be definitely linked to certain changes that have arisen within an community, being a result of a shift from absolute collective ownership ("devălmășia absolută", Stănică, 1937) of land, to the collective proportional ownership and then finally to the private property (Stănică, 1937). It is not our task, within this article, to analyse the different customs and practices of land division in the traditional Romanian culture, for it is known that is the appanage of the advent of the group and individual property (Togan, 2005). Some authors have suggested that division was done in accordance with the topography of the place, the forms resulting being thus similar to geometrical figures, having as fix marks barrows**,** hummocks, springs, rivers and only in rare cases isolated ancient or old trees (Stănică, 1937), although in medieval documents trees weren't at all rare in demarcations, some were specially marked with symbols or the coat of arms of the country (Giurescu, 1975). These demarcation practices may had some magical or mythical implications according to other authors (Togan, 2005).

Due to geographical factors, two types of baulks have been originated, which depend on the relief and nature of the landscape. One that is common at low altitudes like small hills and plains, and another one that separates two agricultural terraces, which is a horizontal line on the hill, and ranges from the hilly to the mountainous regions, up to 1200 - 1400 meters (Someșan, 2011, Idu, 1999).

The exact origin of baulks is hard to determine, but some researchers suggest the hypothesis that the various structures have their origin in different historical. The baulks we encounter in the hilly areas of the Transylvanian Plain, but also in the Para - Carpathian and Intra - Carpathian depressions, are of possible Pre-Roman age, while those which are in the mountainous areas on the ridge of the mountains, could have more recent origins, connected with the Migration Period and the early Medieval Age (Someșan, 2011).

In Romania baulks that have been made on the crest of hills and mountains, are the result of terracing and agricultural use of these relief units (Fig. 1, 2), their direction is transversal on the cliff, their forms is horizontal and they have some very sharp dividing stripes. These baulks have a more pronounced aspect because of the sharper cliffs and due to a long time agricultural use. The horizontal form of the terraces towards the versant, is the result of ploughing starting from the top and the gradual down throw of the furrow. In time the result of this ploughing method was that some terraces are divided by baulks of a relative height of 7-8 m, sometimes the breadth of those being even bigger than of the actual cultivated terrace (Someșan, 2011).

The form and shape of the agro- terraces has been developed in such a manner that it prevents or at least minimizes the dangers of soil erosion but also the washing of the natural fertilizers by rainfall (Someșan, 2011).

Figure 1. Agro – terraces and baulks ("haturi, mejde") in Botiza village, Maramureș.

Figure 2. Terraced hills in the villages Bogdan Vodă, Maramureș

Another type of baulk is the one found in regions with low altitudes and by default agro - terraces are not be found here. However their function is the same, they are a boundary between cultivated terrains, their orientation is parallel with the direction of those. In some regions of Banat, such a baulk is named *slog*, in some regions it has a standard admeasurement of 40 cm, while other informants told us that the normal admeasurement of this structure is that of a furrow (more or less). The *slog* was made after plotting a terrain in the following way, a strip of land was left unploughed between the two neighbours which had a width of a furrow or was meticulously measured to be 40 cm wide. This newly created strip of unploughed land was also marking the border between the two newly separated parcels, but on the other hand the baulk itself was subject to a division and particular type of property, so half of it was the property of one neighbour and the other half was the property of the other

one. In the case of 40 cm baulk, it was divided giving 20 cm to each one of the neighbours. In the traditional custom and rules of land division, in this area, the *slog* was a mandatory structure which normally was covered by grasses (Fig. 3 a, b), very rarely bushes were left growing on them for an additional mark, even less trees were planted or left to grow, for shade.

Figure 3. Baulks ("slog") in Banat, village Forotic, covered by spontaneous vegetation, dividing tillage grounds (a) and crop fields (b).

In another historical region of Romania, in Oltenia or Lesser Wallachia, in the village of Orodel, these structures are named *răzoare* (the standard Romanian name), and they have originated around 1858 when the joint proprietors were divided into sole proprietorships (Stănică, 1937). Their main role was to divide the different cultivated areas, together with some bushes that were left on them: " The baulk is a simple furrow made by the plough, which is renewed each time the places are ploughed, and by this, it's replacement is very easily done. As a consequence of this, quarrel, trials, beatings and sometimes even murders were not very uncommon." (Stănică, 1937, p. 29). Baulks with grasses were called in local speech "*pârciuri"*, being fewer at that time than the ones represented by simple furrows. "Between some fields, the baulks had bushes, blackthorn and hawthorn left to grow on them as marks, other baulks were planted in this sense with quince, the bushes and the planted fruit trees were the marks which maintained the border, the baulk was being drawn as a straight line" (Stănică, 1937, p.29).

Another traditional structure of the cultivated areas, found in many parts of the country, but with different meaning and role, is the "obraț". In some regions the term refers to a measurement unit used in land division, or to the unplanted margins of a vineyard (Scriban, 2013). In Banat (Fig. 4), this structure has a special logistical role, as it represents the common road situated at the end of the cultivated terrains which assures the access of all land owners to their properties. For this reason it is the common property of all neighbours and therefore it is especially designated by all neighbours when land division is done. The "obraț" is separated by the actual cultivated terrains by an indicator furrow. It is fallow ground, ploughing and planting of trees is prohibited on this stripe of land, and, since it is a common property, any damage brought to it is sanctioned. Its width is of approximately two meters, just enough to let a carriage pas on it.

Figure 4. Obraț – a communally managed field road with spontaneous vegetation specific to the region of Banat, village Forotic.

The traditional and the current management of baulks.

In past times these structures have been subject to an intensive management, which also had some deep social implications. For example in Banat when ploughing was done near a baulk, it was done in such manner which avoided bringing any damages to it. It was accepted to cover the baulk with a furrow thus transforming it into a hilly linear structure next to the neighbouring ploughed terrains. But the next year, when the new ploughing was being executed, it was bared of the soil bed so that it would again become visible. So this band was left to fallow, and people were not allowed to touch it even with the harrow, so that the slightest damage would not occur to it.

Every neighbour was supposed to clean his part of the baulk, bushes and shrubs springing up, were considered to be a shame, and which had some repercussions upon the one who did not respect this ethical exigence. Sometimes, very rarely, as an additional sign to help mark it's presence or when ploughing was done, to avoid trespassing, a tree was planted on it (for example acacia or fruit trees). Regarding the traditional management we can observe that this structure, was left to fallow, but bushes and shrubs were removed, people being bound to this by the local management system (unpubl. results).

Thus, after the baulk was created, beside these "technical" instructions regarding the management of the structure, some other aspects of this issue can be considered under "ethical instructions", a necessary result of the common vicinity. By this we consider the ethical exigence of not damaging the baulk at any costs, some locals relate that is better to let pieces of your land into the baulk then to bite from the baulk (unpubl. results). To avoid bringing any damage to the baulk, was an ethical exigence with highly deep implications upon the life of the neighbours, but in a broader sense also upon of the whole local community, it thus pictured the mutual respect, and assured a sort of social stability within the community and the avoidance of conflictual states.

Almost the same type of management is found in the mountainous area of Maramureș, here the baulks are called "mejdă" or "hat" and they are also divided in two halves, between the two neighbours. A significant difference is that here, more than in other regions, shrubs and bushes, or even trees were left to grow on the baulks, for better marking this border (Fig. 5), but also for utilitarian purposes (for example: firewood or for tools). As the shrubs and bushes broaden on the structure, they were cleared by each individual neighbour, which also managed his part of the baulk, in the way he wanted, some were mowing it, some grazed it with their cows that were not sent in the mountains etc.

Figure 5. Shrubs and bushes left on baulks as marks, in Ieud, Maramureș.

An interesting interdiction is regarding the planting of trees or fruit trees on these structures, so that it wouldn't shade the neighbour's terrain. Also if there were some fruit trees, and the fruits fell on the side of the baulk, which belonged to the neighbour, automatically these fruits became his property as well, and not of the person who owned the tree. Despite the fact that various shrubs and trees were left on the baulks as an additional mark (for example: *Prunus spinosa, Alnus glutinosa, Salix Caprea, Quercus robur, Populus alba, Fraxinus excelsior* etc.), most commonly these structures were mowed, the resulting hay was carried home in a blanket ("ciumău" is the local term for designating such a transport and also its quantity which is limited by the person's strength and his tool – the blanket). In Maramureș, there wasn't any social commitment in clearing the baulk, mowing it was a necessary labour, which in turn brought an surplus of food for the domestic animals. Also the trees that were left to grow on the baulk, had been the subject of an active management, their branches were chopped (Fig. 6) for two reasons: to avoid shading the neighbour's terrain and to stimulate the growth in height of the tree. Other trees that were planted by locals on these structures are mainly spruces (*Picea abies*), because of their importance in the local economy.

Figure 6. Maramureș, Ieud. A pollarded tree left to grow on a baulk.

A slightly different management was that of the baulks found in the region of Oltenia, not all of them were left for fallow, the ones that had grassland vegetation on them had an distinctive local name "pârciuri". Here also some spontaneous shrubs (like *Prunus spinosa* and *Crataegus sp.*) or planted fruit trees like quince (*Cydonia oblonga)* served as additional mark for the baulk. The importance of these baulks for the stability of the social system was highly remarkable, because trespassing or damaging these limits has had negative conflictual effects within the community like beatings, lawsuits or even sometimes murder (Stănică, 1937).

Nowadays due to some very important social and historical factors, the management of baulks has had a major shift, but it is still different in the individual regions of the country. The beginning of the communist regime in Romania marked also the start of the "socialization of agriculture", and baulks along with other structures of traditional agriculture have been seen as symbols of and outdated farming system, connected with private property, an barrier to the modern scientific socialist development of society, as they saw it. As a result a process of destruction was waged upon these traditional structures of extensive agriculture like baulks, wood-pastures (Sutcliffe *et al.,* 2014), wetlands (Teaci, 1983) etc. The process of agricultural collectivization has started in 1948 and ended in 1962, it had resulted in a fundamental shift within the structure of the rural communities, thus also affecting the traditional Romanian landscape. Some of its effects were the massive emigration to the city, "agricultural mechanization" and the "aging" and "feminisation" of rural work-force in agriculture (Surd, 2003).

After 1990, and the fall of the communist regime in Romania, most of the former owners had reclaimed their land, as result, baulks have naturally reappeared in the natural-cultural landscape of most regions, while in some places they have never disappeared completely (in more remote regions which were not so affected by collectivization). Even nowadays in the village of Ieud in Maramureș, although there was a partial collectivization, there is a traditional pre-collectivization type of management of these baulks. Meanwhile, in other regions, like Banat, the management had undergone some major mutations. In some regions the mechanization of the agriculture meant that bushes and shrubs are no longer removed from the baulks, and some of them have been completely covered by this type of vegetation (*Prunus spinosa*, *Crataegus monogyna*, *Rosa* sp.) (Fig. 7 a, b).

a.  b. 

Figure 7. Bushes of *Salix sp*. (a) and *Prunus spinosa* (b) covering some baulks, after active local management ceased, in Banat, village Forotic, Romania.

In the ethnographical region of "Ținutul Pădurenilor" in Hunedoara county, Transylvania, the agro-terraces are several centuries old (some authors suggest that they have a pre-roman age), and they haven't been cultivated for forty or fifty years. The abandonment of these agro- terraces is due to the migration of work force to the highly industrialized urban centers of the county, but also because of the abandonment of wheat cultivation, since this is supplied from other regions. Nowadays those ancient agro-terraces are covered by grassland, bushes or even secondary forests (Coste and Arsene, 2003).

Baulks also differ in their length and width by region. We have measured a standard width of 40–45 cm, but some baulks had a width of almost two meters, in Banat. Those found in Maramureş had an even more considerable size, this is mainly because of the local geographical factors: these structures follow the backfall of the hills and mountains on which they are found. For this reason, the average width starts at 70 cm and height at 40 cm, but there are baulks which are 3.6 m wide and 1.6 m height.

The ecological functions of baulks

Baulks, regardless of their various forms and local denominations, are a linear landscape elements, in which fragments of local flora and fauna can survive, enhancing their ecological, agro-ecological and aesthetical functions. They constitute a sanctuary for the flora and fauna that has been less affected by pesticides, they are literally and functionally binding corridors between the various types of ecosystems. In many cases these baulks work as reservoirs and seed banks supplying the local biodiversity.

Biodiversity is definitely higher on baulks, than on the neighbouring agricultural terrains, we can assume that species distribution is more balanced and that the presence of species considered rare is also higher.

Baulks give shelter to a higher number of predatory coleoptera (Carabidae) than any other form of crop fields (Raskin *et al*., 1992), from where these are largely affected by pesticides. For many bird species these structures represent a niche where they can survive even after the traditional use of the terrains has been abandoned (*Perdix perdix, Coturnix coturnix, Crex crex, Lullula arborea, Lanius collurio, L. minor, Miliaria calandra, Oenanthe oenanthe*). Many mammalian species use them as shelter (hedgehog, deer, rabbits, foxes) or as a permanent habitats (various species of mice, hog – *Cricetus cricetus*). They are an important habitat for lizards as well.

Plants number is about 3–4 times higher than on the neighbouring agricultural terrains (Raskin *et al*., 1992), the baulks which are located on chalky and skeletal soils have the highest species diversity.

Baulks increase the diversity of the natural-cultural landscape, especially those which are covered by various species of shrubs (*Prunus spinosa, Crataegus monogyna*, *Salix* sp. *Cornus* sp., *Rosa* sp.), but also old trees (Fig. 8). In these baulks we can find a

wide range of species, starting from predatory insects, parasites, spiders to insectivorous birds, which all contribute by their presence and activity to higher yields on the neighbouring cultivated terrains. Besides the useful flora and fauna for which baulks have a sheltering role, the ones with a more complex structure can house even rare or endangered species. For example such species of butterflies found in these structures are: *Iphiclides podalirius, Satyrium acaciae, S. ilicis, S. pruni, Eriogaster catax, E. lanestris* etc.

Figure 8. A baulk completely covered by bushes and shrubs in Ieud, Maramureş.

Within a monotonous agroecosystem, baulks can represent a complex network of areas with spontaneous vegetation, linking also neighbouring areas with natural or semi-natural vegetation, thus working as authentic ecological corridors that are enriching the patchiness of the landscape. By this, the flux of energy and information within the landscape is being facilitated (Fig. 9 a, b).

We can consider the aesthetical effect to be of meaningful importance as well, especially by its role in fragmenting the monotonous sight of crops before and after harvesting, but also in structuring various relief forms like hills or mountains (Fig. 10).

Although the sole and primary role of these structures for the local community was a strictly social – administrative one, still the locals have done some empirical observation upon the importance of them for some animals. Some locals have observed that, along with chemical fertilizers and mechanical agriculture which had the major role in the drastic reduction of the density of wild rabbits in their area, the destruction of baulks during the socialist agriculture was also of a major importance, since these worked a shelter in the way of intensive agriculture (unpubl. results).

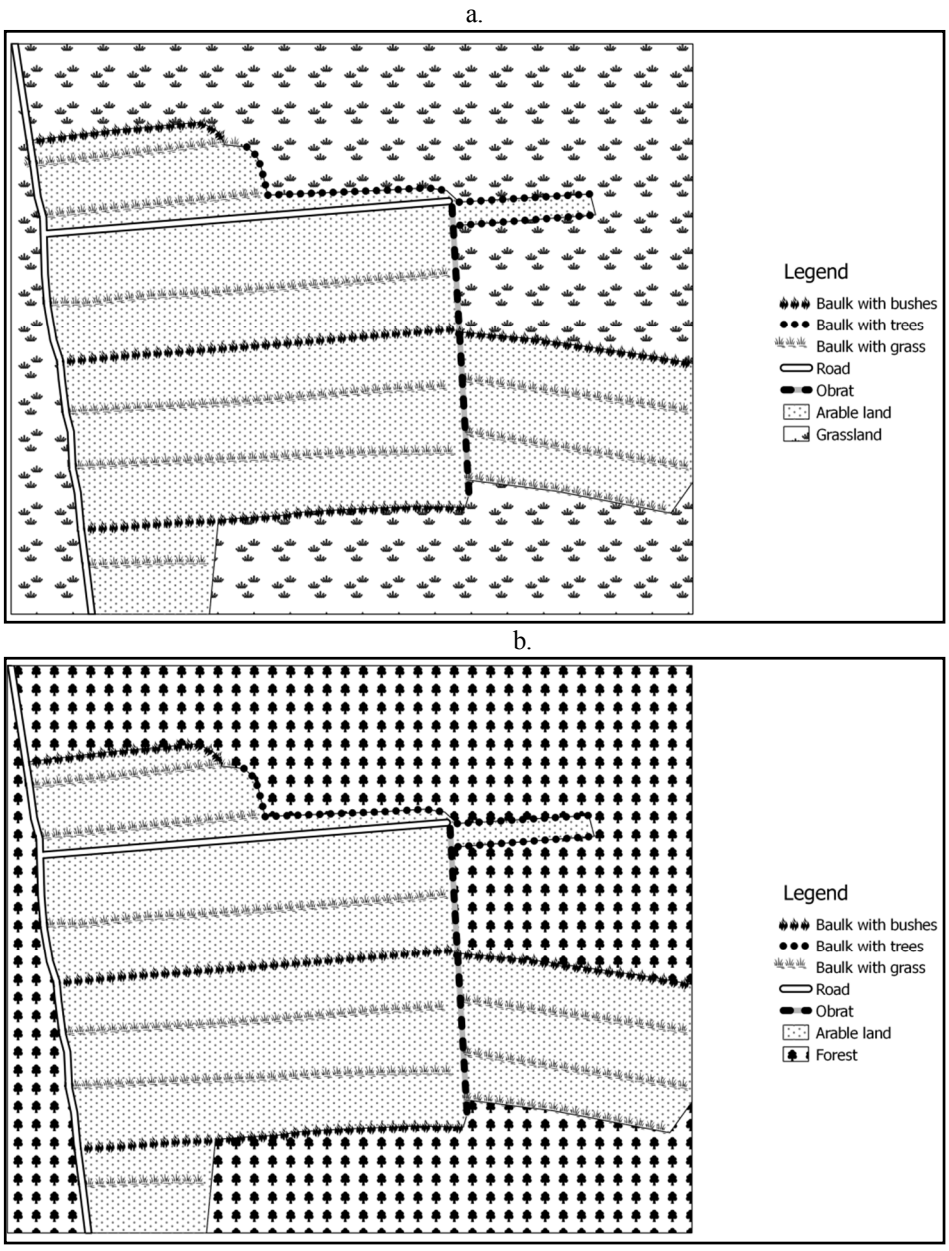

Figure 9. A hypothetical schema illustrating how various types of baulks create a complex network of spontaneous vegetation within a crop field, in a lowland region. The network of baulks and obraţ structures are linked directly to a grassland (a) and a forest (b).

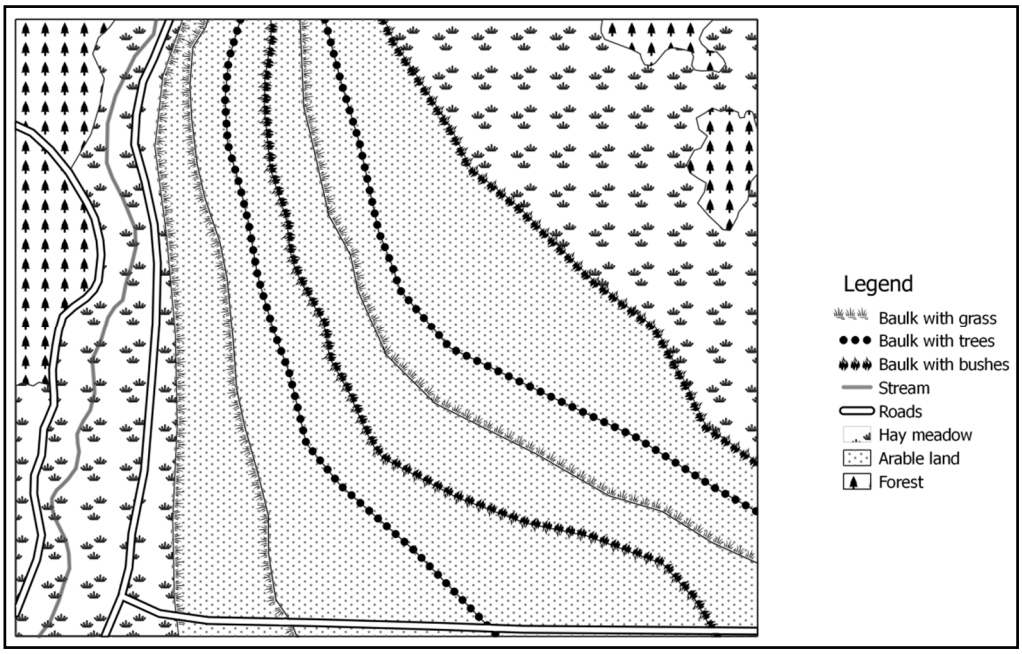

Figure 10. A hypothetical schema illustrating how various types of baulks create a complex network of spontaneous vegetation on a terraced relief unit.

Some other locals have asserted the importance of these structures in the biology of some bird species, since some species use these as shelter, nesting place, or feeding habitat. These structures have been regarded of great importance also for micro-mammalians, although those are seen rather in a negative way (i.e. sheltering the pests, damaging the crops) (unpubl. results).

Conclusions

Baulks are a structural element of the traditional natural-cultural landscapes, and a common trademark of traditional subsistence agriculture. Nowadays they are in decline or have completely disappeared in many regions throughout Romania due to shifts that have affected human society and especially farming practices.

In Romania we can find baulks under several different local names, admeasurements, and shapes, from the ones with no vegetation (simple furrows), through those covered only with grassland vegetation and to the ones with bushes, scrubs or even trees, depending on the geographical conditions and the management system of the local community.

We have distinguished two forms of baulks which are geographically determined. The first one includes baulks developed horizontally on the backfall of more pronounced relief units (hills or mountains), which practically are separating the cultivated terraces, while the second form is found on small hills and planes, separating agricultural terrains.

Their initial role, that is also the one which generated their appearance, is that of marking the border separating different properties, while their management was done according the needs of the local communities, but mainly of the two neighbours.

These structures are highly important in the conservation of local biodiversity, especially in agroecosystems. They constitute authentic shelters for the local fauna and flora against the agricultural practices from the neighbouring cultivated terrains, working as ecological corridors that are building a complex network of natural or semi-natural vegetation, with beneficial effects even for some endangered or rare species.

Therefore, baulks have an extraordinary socio – cultural, ecological and scenic value. Their conservation and maintenance is an obligation of those who understand the uniqueness of and value of the natural – cultural landscape of Romania, in broader national and especially European context.

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=== REVIEW ===

Human Papilloma Virus infection and cervical cancer in Romania

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SUMMARY. Infection with human papillomaviruses (HPV) is a major public health burden worldwide and is associated with a variety of epithelial lesions, including benign warts and several types of anogenital tumors, particularly cervical carcinoma.HPV can be grouped into cutaneous types and mucosal types based on their preferred tissue tropism. Cutaneous types are typically found in the general population and cause common warts. Mucosal HPV is further classified into high-risk and low-risk types, based on their association with cervical cancer. The most common low-risk types are HPV 6 and 11, detected most often in benign genital warts. HPV 16, 18, 31, and 45 are predominant types found in cervical squamous cell carcinoma. HPV 16 is the most prevalent type in cervical cancer (55%), followed by HPV 18 and HPV 45. Epidemiological evidence has convincingly demonstrated that infection with HPV is the greatest risk factor, its role in the progression of the precursor lesions to cervical cancer is well established. HPV is exclusively epitheliotropic, and their replication is linked to the differentiation process of the host cells. Normal squamous epithelial cells grow as stratified epithelium, with those in the basal layers dividing as stem cells of transient amplifying cells. After division, one of the daughter cells migrates upward and begins to undergo terminal differentiation while the other remains in the basal layer as a slow-cycling, self-renewing population. Productive papillomavirus infection begins when infectious virions gain access to cells of the basal layer, probably through micro-wounds. The viral genome is maintained in these cells at low copy number. These infected cells from the reservoir for the development of a productive wart. Early HPV genes E1 and E2 support viral DNA replication and its segregation such that the infected cells can be maintained in the lesion for a long period. As infected daughter cells migrate towards the epithelial surface, viral late gene products are produced to initiate the vegetative

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phase of the HPV life cycle, resulting in the high-level amplification of the viral genome. In the outer layers of the epithelium viral DNA is packaged into capsids and progeny virions are released to reinitiate infection. Given the worldwide burden of HPV infection (anogenital warts and neoplasia of several sites), prevention of infection could provide relief from an important public health threat. With the introduction of cervical screening in developed countries, the number of deaths from cervical cancer has declined dramatically, but in developing countries it still remains the number one of female cancer.

Keywords: cervix cancer, Human papilloma virus, koilocyte

The Human papillomavirus (HPV) infection is now a well-established cause of cervical cancer and there is growing evidence of HPV being a relevant factor in other anogenital cancers (anus, vulva, vagina and penis) and head and neck cancers (Anic and Giuliano, 2012, Deng *et al*., 2015). HPV types 16 and 18 are responsible for about 70% of all cervical cancer cases worldwide (Gross, 2014).

Romania has a population of 9.54 million women aged 15 years and older who are at risk of developing cervical cancer. Current estimates (*http://www.hpvcentre.net/statistics/reports/ROU.pdf, 2015*) indicate that every year 4343 women are diagnosed with cervical cancer and 1909 die of the disease.

AIDS, the plague of our age, demands approximately 1200 in Romania yearly. It is not known widely, but cervix cancer due to infection with human papilloma virus (HPV) demands more than 2000 in Romania every year (*http://www.hpvcentre.net/statistics/reports/ROU.pdf, 2015*). According to the survey of the WHO more than 4300 women are infected with HPV virus in Romania yearly. In our country HPV is the third most frequent cancer among women and the most frequent cancer type affecting women between ages 15 and 44. In Romania 9.54 million women are above age 15, they have the highest risk of cervical cancer.

This virus has been known in medicine research for 100 years, and earlier it was thought that it caused warts and skin growths in the epithelium. Mutations of the virus have been found in South-American Indian tribes too, and it justifies that the virus appeared in the early age of human race. HPV infection can incubate for a long time without causing any symptoms, or it can cause epithelial lesions in sexual organs and it can cause warts on different parts of body. The correlation between HPV and cervical cancer was shown by Harald zur Hausen, German scientist, for which he was awarded the Nobel Prize for medicine in 2008. He started his research in 1980 and his

suspicion was awakened by observations that cervical cancer was higher in prostitutes than in the average population. Harald zur Hausen had found that cervical cancer does not occur without HPV cervical cancer *(http://www.nobelprize.org/nobel_prizes/medicine/laureates/2008/).*

HPV infection occurs frequently all over the world and in certain countries (US states) men are virus carriers in similar rate as women (Anic and Giuliano, 2012). In other countries (mostly in European countries) the majority of virus carriers are women. The virus is transmitted through sexual contacts, by certain body fluids from vagina and rectum, or with mouth contact. Viruses foothold in the mucosa of motioned genitals. The probability of infection is increases with injured mucosa (for instance injuries in wall of vagina). In their lives every second woman goes through HPV, 60 percent of which is high risk infection.

Human papillomavirus (HPV) is well known as the major etiological agent for anogenital cancer. In contrast to cervical cancer, anal cancer is uncommon, but is increasing steadily in the community over the last few decades (Moscicki*et al.,* 2012, Stanley *et al.*, 2012).

Given the worldwide burden of HPV infection (anogenital warts and neoplasia of several sites), prevention of infection could provide relief from an important public health threat. With the introduction of cervical screening in developed countries, the number of deaths from cervical cancer has declined dramatically, but in developing countries it still remains the number one of female cancer.

HPV16 genome organization

HPV belongs to the *Papovaviridae* family, it is dezoxyribovirus. Its genome is formed by 8000 bases, coding proteins E1-E7 and L1-L2 (Fig. 1).

Proteins L1 and L2 pack the DNA of the virus, and participate in forming shell pack (capsid). The E1-E7 proteins ensure the function of virus. Since viruses incorporate in DNA of host cell, it is difficult to identify them. This is why a lot of virus infections are difficult to treat. While they multiply in host rapidly, they gain mutation. The structure of next generation differs, so there is no vaccination to ensure long term protection against these viruses (Hamkar and Delforoush, 2009, Lin *et al.,* 2007).

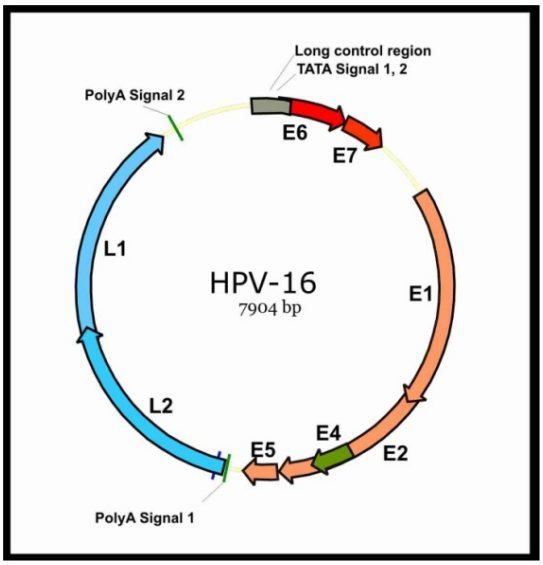

Figure 1. HPV-16 genome organization. The genes have the following functions: L1-L2 capsid protein coding genes having roll in connecting virus to the DNA of the host cell. The vaccination against HPV is connected to this protein too. E1 – it is responsible for replication of DNA, it ensures connection to DNA of host cell. E2 – protein controlling transcription, its inactivity enhances activity of E6-E7. E3 is small in size. It can miss. Its function is not known. E4 is responsible to detaching from host cell. It unseals the cell frame. E5 destabilizes cell membrane. It makes intrusion into the host cell easier. The E5 protein of HPV type 16 inhibits generating MHC1 protein. This protein would indicate modified cell presence for immune cells. In their absence immune cells are not able to recognize the cell with the changed function. E6-E7 hinders cell death. Thus they ensure replication of virus DNA for long and help generating new viruses. They are responsible for generating malignant tumors. They inhibit proteins from hindering tumors generation. As a consequence, the structure and function of host cells change. Instead of eliminating these cells, they divide and generate new mutations, as a consequences new tumor cells come into existence. E8 can be missing from infectious viruses. E8 function is similar to E5 function (wikipedia.org/wiki/Human_ papillomavirus)

HPV subtypes

At least 100 versions of HPV are known. According to epidemiological data (occurrence in precancer states and in cervix cancer) and biogenetical similarity, low, transition and high risk types are distinguished. The HPV viruses can be distinguished where they foothold - anogenetical, oral, mouth mucosal, pharynx, larynx and in skin- (Gross, 2014, Szentirmay *et al.,* 2005).The HPV viruses are marked with Arabic numbers.

The most dangerous, high risk viruses are 16, 18, 31, 33, 35, 39, 45, 51and 52 (Chaturvedi *et al.,* 2005, Gross, 2014, Fotopoulos and Pavlidis, 2015, Muñoz *et al.,* 2003, 2010).The distribution of viruses causing tumors of the genital organs (cervix, vagina, mouth cancer) is the following: HPV-16 occur in 54 %, HPV 18 in 17.2%, HPV 45 in 6.7%,HPV 31 in 2.9%, HPV 33 in 2.6% (Smith *et al.,* 2007). The presence of the virus can be detected in new born babies of infected mothers, as the virus can get from the mucus of birth channel into the oral cavity where viruses can cause laryngeal or pharyngeal tumor.

Less aggressive types (HPV 6, 11, 42, 43 and 44) can cause warts (Fig. 2) or benign tumors, however large and very disturbing skin outgrowth in the oral cavity or around genital organs (Fig. 3).

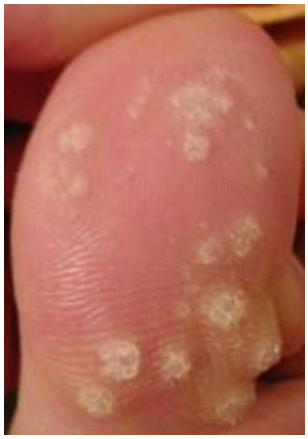

Figure 2. HPV infection of the skin, warts can be observed on the skin surface *(http://en.wikipedia.org/wiki/Wart).*

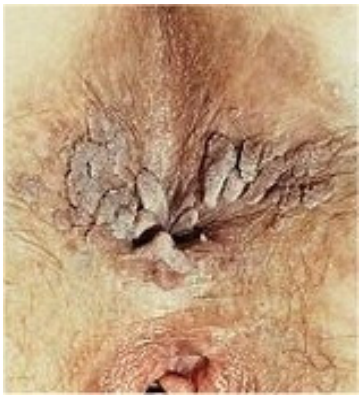

Figure 3. Anal warts *(http://en.wikipedia.org/wiki/Genital_wart)*

HPV detection methods

The anogenital virus infection can be detected by Papanicolau test. Characteristic cells can be seen in vaginal smear in case of infections (Fig.4). The core of infected cells is higher and a homogeneous light edge can be observed around of the core. It is surrounded by deep cell plasma. Infected cells generally have several cores. These cells are called koilocytes. The evaluation is performed with an optical microscope and it requires a lot of practice. Pink cells can be seen with two cell cores in the middle on the left of the photo below (Fig. 4).

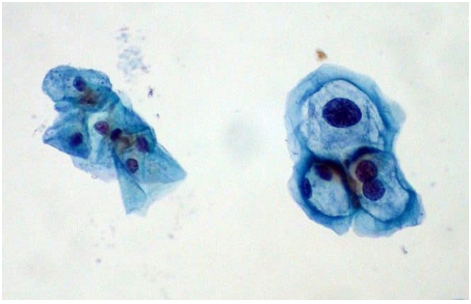

Figure 4. Papanicolau smear with group of normal cervical cells on left and HPV-infected cells showing features typical of koilocytes: enlarged nuclei and hyperchromasia *(http://commons.wikimedia.org/wiki/File:ThinPrep_Pap_smear_HPV.jpeg)*

If the Papanicolau test is positive, a specialist may suggest further tests like HPV DNA test. The HPV DNA test is very accurate because does not only show one strain, but the most common strains, if a multiplicity of infection has occurred (Wright and Schiffman, 2003). In the test a DNA extract is prepared in smears for the detection of various strains of HPV test strip.

This nucleic acid hybridization method utilizes a DNA cocktail specific for intermediate/high risk serotypes (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). Results are reported as not detected or as detected for high-risk HPV serotype. A not detected result is consistent with the absence of high-risk HPV DNA serotypes, a level of HPV DNA below the detection limit of the assay, or presence of a serotype other those listed above. A detected result indicates the presence of a one or more of these high-risk HPV serotypes (Saslow *et al.,* 2012).

Viral infection and replication

Most HPV viruses that infect a part of the body surface are covered with multilayer squamous mucous membrane (e.g. cervix, vagina, vulva, mouth, pharynx, larynx, esophagus, urethra and anus area). Papillomaviruses replicate and assemble

exclusively in the nucleus. Virus infects thekeratinocytes in the basal layers of a stratified squamous epithelium.

The viral DNA which has entered in the epidermis integrates into the germ layers dividing cells DNA and its replication starts in parallel tumble regulating epithelial cell proliferation operations. Towards the surface of the epidermis in the keratocytes the synthesis of viral components and assembly starts, and on the surface of the cell disruption newer viruses appear, and these new infected harness and an increasing number of virus are produced (Fig. 5).

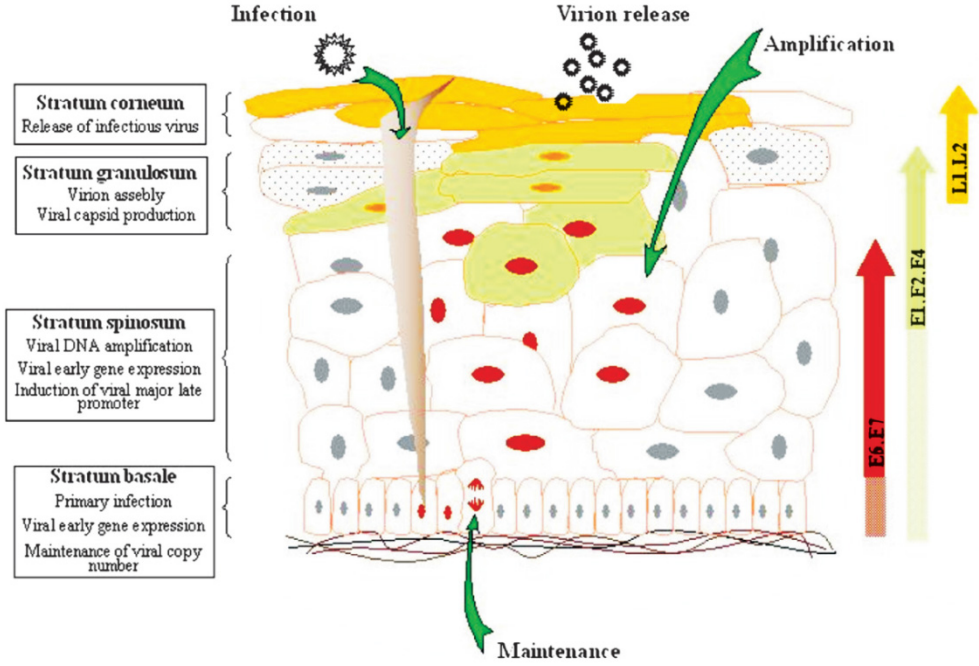

Figure 5. Stages of viral infection. The germ layer-dividing cells are already in the virus which is shared with the host cell. While wandering cells migrating to the surface of the epithelia the virus generates their constituents. The surface epithelial cells which have reached new viruses are released (Grm *et al.,* 2009).

It has been shown that the virus strains which cause genital tumors of E6-E7 protein coding genes are responsible for the oncogenic activity (Ganguly and Parihar, 2009, Hebner and Laimins, 2006). HPV once incorporated into DNA, inhibits the expression of regulators of E2 gene transcription, resulting in increased activity of E6-E7 genes (Grm *et al.,* 2009, Prétet *et al.,* 2007). E6, and E7 are viral oncogenes and their expression induces cell immortalization and transformation.

The proteins derived from transcription of genes, linked to the tumor suppressor proteins (p53 and pRb) in cell cycle stop regulation (Zheng and Baker, 2006). Following infection, cell division becomes out of control. The immune cells are not able to recognize the tumor cells. More and more abnormal cells are formed, which eventually lead to the appearance of malignant tumors.

Viral infection do not last lifetime, it can take the body several months or years to get rid of the pathogen if the person does not become infected again. If the body cannot get rid of the infection, triggered by the above-mentioned mechanisms it can lead to cancer induction process, which is slow, and can last decades.

How to defend ourselves against HPV?

In sexual intercourse it does not spread with body fluids, but with skin contact, so condom does not protect against the infection. For those who are sexually active (rather women than men) regular check-ups can be a lifesaver. Infected women partners can be at risk, on the one hand they can cause cancer in males, on the other hand there is a risk of re-infection in both sexes. Frequent change of partners increases the potential for the spread of the highly infectious (high-risk) viruses, and that at the same time even more virus strains are absorbed into the body.

Conclusions

Since mortality caused by HPV is higher than that of AIDS, the HPV threat to society should be informed and HPV should be handled more seriously. Globally cervical cancer is the second most common female cancer after breast cancer. HPV is the main reason of cervical cancer.

The majority of men infected with HPV can have symptoms and no symptoms at all, but certain types can cause genital warts and cancer. Public attention and that of students should be directed at the risk and treatment of HPV, as well as at the importance of prevention and screenings.

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=== REVIEW ===

New insights into the interaction between cultivated potato and *Phytophthora infestans*

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SUMMARY. Late blight is the most destructive disease of potato. Due to the sexual and asexual reproduction, late blight has the capacity to evolve rapidly, progression that makes breeding for resistance very challenging. The favourable characteristics of vertical and horizontal resistance might be a good source for breeding resistant cultivars. Understanding the infection steps and defence response of plants is important for the next breeding programs. The goal of this review is to discuss some new insights into the interaction between pathogen and host and to point out new ways of transferring durable resistance genes to *Phytophthora infestans* into potato gene pool.

Keywords: effectors, late blight, resistance genes, *Solanum*

Introduction

Potato (*Solanum tuberosum* L.) is one of the most prominent cultivated plant of humanity, ranking third as a food crop after rice and wheat. Due to increasing consumption of potato, the importance of this crop intensified in the last years. The origin and the first domestication area of potato was the Andes Mountains, Peruvian region of South America (Pérez *et al*., 2001). Taking into account European evaluation data, Romania occupies the third place in the extension of potato cultivation area and the sixth place if we choose the volume of potato production as criteria. Romanian annual potato consumption shows a decrease tendency, in 2012 it was 98.3 kg /capita, which is 6.4% less than in 2006 (Vlad and Done, 2014). Romanians often called potato crop as the second bread, which proves its important role in alimentation (Baciu *et al*., 2009).

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Nowadays potato is one of the most chemically protected crop in the world. Every year more and more million dollars are spent for different treatments against fungi and herbivores.

In case of late blight disease the annual financial loss due to different crop protection methods and crop losses can be 3 billion dollars. With chemical control and targeted breeding, it is possible to reduce the annual yield loss to 16%, which is still insufficient (Fry, 2008).

Cultivated potato is a tuber-bearing autotetraploid (2n=48) species, which is a result of multiple successive hybridization of diploid species (Thermoshuizen, 2007). Potato has got a large number of related wild species, around 230, that all carry resistance genes for different diseases and pests (Spooner *et al*., 2014).

Potato breeders are always looking for new possibilities to obtain more resistant cultivars against different diseases. Wild *Solanum* species represent a rich reservoir of resistance genes that might be used in breeding programs, but the majority of them cannot be crossed with potato crop.

Two different kinds of resistance have been described against late blight and other diseases, vertical and horizontal, both interesting to increase crop protection by using resistant cultivars. Vertical resistance has a monogenic nature, is specific to pathogen strains, but isn't durable. The operating mechanism of vertical resistance is based on the gene for gene model, the pathogen avirulence genes are detected by matching resistance genes, the detection being either direct or indirect. In the case of incompatible interaction, the plant remains resistant. This type of resistance provide a protection just for a short period, because the pathogen, in our case the oomycete *P.infestans* evolves rapidly and overcomes the respective resistance gene. To date there are resistance genes discovered and characterized but most likely there are more to be discovered (Pérez *et al.*, 2001).

The hexaploid *Solanum demissum* was for the first time used as a source of vertical resistance against late blight, due to the identified eleven resistance genes (R1-R11). These genes confer a race specific resistance since the virulent new strains of *P.infestans* rapidly overcome this type of resistance (Jo *et al*., 2011; Saldana *et al*., 2011). Nowadays more than 50% of world potato cultivars contain *S. demissum* germplasm (Pérez *et al*., 2001).

Another solution could be the accumulation of resistance genes in *Solanum tuberosum*, the multiple R genes can confer a broad spectrum resistance to various diseases (Tan *et al*., 2010; Hajianfar *et al*., 2014). The breeders recognizing the unfavourable characteristics of vertical resistance, for example the non-durability, try to identify the genes involved in the horizontal resistance and to include this type of resistance in the new cultivars.

Horizontal resistance provides a general protection against pathogens, more durable than vertical resistance, but isn't specific and involves more genes in the defense response (Saldana *et al*., 2011). Introducing a horizontal resistance mechanism

instead of R genes into cultivated potato could be a promising method. Difficulties for breeders represent the polygenic nature and the linkage with other characteristics of plants, for example the late maturity of the host plant. The above mentioned new type of resistance confers a general protection against all races of *P.infestans*.

Screening after new resistance sources from wild species continues even current days, in the latest study researchers found some race nonspecific resistance genes in *Solanum chiquidenum* and *S. multiinterruptum*. The newly tested wild species show another or unknown type of response again *P. infestans*, probably in these cases the host-pathogen interaction is different than that with *S. demissum* (Pérez *et al.*, 2001).

Late blight of potato

Late blight of potato caused by the oomycete *Phytophthora infestans* (Mont.) de Bary is recognized worldwide as the most devastating disease of potato. This pathogen left his mark in human history by the great Irish Potato Famine in XIX century. The huge loss of potato yield caused one million people death and more than one million people emigration from Ireland (Goss *et al.*, 2014). *P. infestans* is a hemibiotrophic oomycete, which mean that in the early stage of infection requires a living tissue, this period could be for couple of days to weeks (Termorshuizen, 2007). This oomycete has a capacity to develop resistance to new fungicides or resistant potatoes, quickly alters genetically in consequence increases the virulence capacity.

P. infestans is a heterothallic oomycete, with two mating types, respective A1 and A2, therefore it is able to reproduce both asexually and sexually. Oospores resulting after sexual reproduction are more resistant to abiotic stresses than asexual reproductive forms, they remain infectious even four years (Turkensteen *et al.*, 2000). Sexual reproduction increases the genetic variation of oomycete, which could generate new strains, new genotypes resulting in quicker adaptation and more powerful attacks (Sujkowski *et al.*, 1994).

In *Stramenipiles* regnum *P. infestans* has the biggest genome, 240Mb, which can be divided into two parts, based on gene contents. Approximately, 25% of the genome represents the gene dense region, and the other 75% the gene-sparse region. In the first region the housekeeping genes are present and the second region contains the effector or other genes, which play an important role in the virulence of the oomycete. Another difference between above mentioned two regions is: in contrast with gene dense region, the gene–sparse region includes large number of repetitive sequences, which are dynamic and serve as site for evolutionary processes (Jiang and Tayler 2012). This region promotes an increasing genetic variety of genes, which has a role in pathogenicity and host specificity (Vleeshouwers, 2011).

Life cycle *of P. infestans*

Since the appearance of A2 mating type, the sexual life cycle near the asexual life cycle of *P. infestans* has been revealed. The features of the spores represent a cornerstone for the success of oomycete, this survival structures allowing space and time dispersion to *P.infestans* (Judelson and Blanco 2005).

The asexual life cycle begins with the landed sporangia on the leaf surface, and can germinate in two different modes, adapting to the weather conditions. In dry and hot conditions the sporangium directly germinates via germ tube, otherwise in humidity and low temperature the sporangium releases biflagellate zoospores. These motile zoospores swim and encyst in the host surface, and in order to penetrate they secrete enzymes to digest the cuticle (i.e. pectate lyases), the cell wall components (cellulases) and additionally produce suppressors, which keep down the plant defence response (proteinase inhibitors) (Judelson and Blanco, 2005; Danies *et al*. 2014).

For the sexual reproduction, the presence of both mating types in the same location is required. This life cycle begins with the formation of oogonium and antheridium. The formed oospore can germinate either hyphae tube or sporangium. Important traits of oospores in contrast with zoospores are: they are more resistant to environmental changes, they can survive until next year and can infect the new culture of potato. The development of these two different germination modes of spores in both asexual and sexual reproduction, represent an evolutionary advantage, ensuring the possibility of colonization in any environmental conditions.

Interaction of late blight with the host plant

Plant pathogens use diver's strategies to penetrate into the plant via water pores, intercellular spaces or trough wounds, depending on the level of perceive system development of host plants. Oomycete can invaginate haustoria into the plasma membrane, which form closer interface for the next interaction steps with plant tissue (Han *et al.*, 2013).

At the beginning of the infection all pathogens confront with the first layer of plant defence, with the basal immunity system. Transmembrane pattern recognition receptors (PRRs) of plants detect the highly conserved microbial molecules, called PAMPs, which could be peptides, derived from bacteria or polysaccharides (chitin or beta-glucans) in the case of fungi or oomycete. As a result of the confrontation the PAMP triggered immunity (PTI) is switching on (Rouxel and Balesdent, 2010).

The successful pathogens have a well-developed ability to suppress the PTI by the effector molecules, which are products of oomycete avirulence (*avr*) genes. These effectors manipulate the host cell structure and function, in this way facilitating the infection and triggering defence responses (Kamoun, 2006). In this defence level the MAP kinase signalling will activate the pathogen-responsive genes transcription, the strengthening of cell wall and the Reactive Oxygen Species (ROS) production (Chisholm *et al*., 2006).

If an effector is recognized by a host cell resistance protein (R), the effector triggered resistance (ETI) will be activated, this is more rapid and vigorous compared with PTI (Jiang and Tyler, 2012). This type of interaction complies with the 'gene for gene' theory and lead to the hypersensitive response (HR), programmed cell death (PCD) at the site of infection. The collapse of the infected tissue creates a physical barrier to prevent the proliferation and spread of the pathogen.

Resistance and avirulence proteins

Resistance proteins are part of the second layer defence system, the majority of them contain Nucleotide Binding Site domain (NBS) and Leucine-Rich Repeat (LRR) domain. There are two subgroups of these proteins based on the amino terminal, either Coiled Coil (CC) or toll/interleukin receptor (TIR) domains.

In the *Solanum* species all resistance genes encode CC-LRR-NBS intracellular proteins against *P. infestans*.

In the construction of NBS domain, protein motifs kinase 1a or P loop, kinase 2a and kinase 3a, which are involved in the binding and hydrolysis of ATP or GTP take part. Each domain has a specific role in the interaction. NBS domain works as a molecular switch regulating the signal transduction (Tameling *et al.*, 2006). The LRR domain is involved in a specific recognition of effector molecules and represents a platform for upstream activators (Belkhadir *et al*., 2004; Tameling and Takken, 2008).

Another classification criterion of resistance genes is the evolution pattern, two types are known: the fast evolving (Type I) and the slowly evolving resistance genes (Type II). The only difference is attributed to the frequency of sequence changes.

Up to 2013 in total 68 functional resistance genes against *P. infestans* were identified in *Solanum* species (Rodewald and Trognitz, 2013).

Solanum resistance proteins are classified into seven families based on the different resistance specificities to *P. infestans* (Vleeshouwers *et al.*, 2011).

Effector proteins

Oomycetes, like *P. infestans* secrete hundreds of effector molecules, which target the host cells. Their primary role is the virulence, the secondary role is to elicit innate immunity in plant. In function of acting site, the effectors are grouped in cytoplasmic and apoplastic effectors. Effectors also could act as elicitors or toxins.

Oomycete apoplastic effectors interact in the extracellular space with plant cell wall, host's proteases and defence–response networks. Based on their activity they can be divided into: extracellular toxins, hydrolytic enzymes and enzyme inhibitors. Many apoplastic effectors were identified by biochemical isolation or bioinformatics methods (Oh *et al*., 2009).

Hydrolytic enzymes can digest the carbohydrates from apoplast and the cell wall degrading enzymes promote the oomycete penetration into host plant. Degradation

of pectin and β-glucan is realized by endo-polygalacturonase and β-glucanases secreted by oomycete (Nowicki *et al*., 2012; Jiang and Tyler, 2012).

When potatoes are attacked by *P.infestans*, as a response, they begin to excrete pathogenesis related proteins (PR) like glucanases, proteases and chitinases, therefore the oomycete secrete enzyme inhibitors like serine protease inhibitors (EPI1 and EPI10), cysteine protease inhibitors (EPIC1 EPIC2), etc. A set of elicitins (INF1, INF1A and INF2B) genes were identified in *P. infestans* genome, which encode some small, cysteine rich proteins and have the role to induce a hypersensitive response of the host plant.

The stability of effector molecules in the apoplast is due to disulphide bridges in their structure. Another member of this group is represented by PcF-like SCR74 and SCR91 toxins, which are secreted in the early stage of infection. They have two identified biological activity, the elicitation of the phenylalanine ammonia-lyase (PAL) activity and the promotion of leaf whitening (Liu *et al.*, 2004; Kamoun, 2006; Orsomando *et al*., 2011). PiNPP1 takes part in Nep1-like family, it is induced in the later stages of infection, during the necrotrophic phase (Kamoun, 2006; Kelley *et al*., 2010).

After translocation into cytoplasm, the cytoplasmic effectors of *P. infestans* target the different subcellular compartments of host cell, entering into host cell by lift draft mediated endocytosis (Nowicki *et al.,* 2012)

Similarly with resistance genes, avirulence genes are rapidly evolving and highly diverse genes, which encodes modular proteins. Based on protein domain characteristics, two types of cytoplasmic effectors, RXLR and crinkler (CRN) effectors were distinct (Jiang and Tyler, 2012).

RXLR effectors are products of *avr* genes, their name derived from conserved amino terminal motif, which have a role in the translocation into host cell. The amino terminal part of effector represents the sign for secretions and the carboxyl terminal part plays the effector side of protein (Morgan and Kamoun, 2007). Two additional protein motifs are important to be present, the dEER motif, which is required to entry into host cell, and W, Y, L motifs in the carboxy-terminal of effector with a role in the PCD suppression. Crinkler effectors belong to modular proteins family, like RXLR effectors, are rapidly evolving and can produce plant necrosis if they overexpress. The crinklers can enter into the nucleus and inhibit the PCD (Kamoun, 2006; Jiang and Tyler, 2012).

Other cytoplasmic effectors targets molecules, which have a key role in the recognition of effectors (Saunder *et al*., 2012).

Pseudogenesis and mutation lead to allelic variation of these genes. They could differ in the expression time, *Avr1* express in the early stage while *Avr3a* in the later stages of infection (Vleeshouwers *et al*., 2011). Some effectors could be important for potato breeders, for example the Avr3a, which could suppress the PCD induced by INF1 (Vleeshouwers *et al*., 2011).

These examples demonstrate, that effectors could act at a different level in plant, gene expression level, protein modification level or regulation level, for promoting plant susceptibility. Understanding their acting mechanism, could be helpful to design new, more targeted breeding programs.

Interaction between oomycete and plant cell is a very complex process, each part of this pathosystem develop their "attacker arm" and their defensive system (Fig. 1). This figure represents schematically the main steps of interaction. Oomycete secrete two types of effectors: intracellular effectors (IE) and cytoplasmic effectors (CE), which enter in the plant cell by endocytosis (1-3). After recognition of pathogen associated molecules (PAMPs) by pattern recognition receptors (PRR), will activate a PAMP triggered immune response (PTI), which will turn on other defensive responses, like Reactive Oxygen Species (ROS) production (4). Infected plants cells secrete proteases (P) to interact with intracellular effectors (5), after that as a response the oomycete secrete protease inhibitors (6). Cytoplasmic effectors, or RXLR effectors, are recognized by resistance proteins (R) and will activate the Effector Triggered Response (ETI) (7). RXLR effectors also can inhibit the PTI, or the ETI (10). If the interaction is incompatible, the Programmed Cell Death (PCD) will be triggered, that means a hypersensitive response (HR) and the infection keep back. Another type of cytoplasmic effectors, the crinklers (CRN) can enter into nucleus and inhibit the PCD (9).

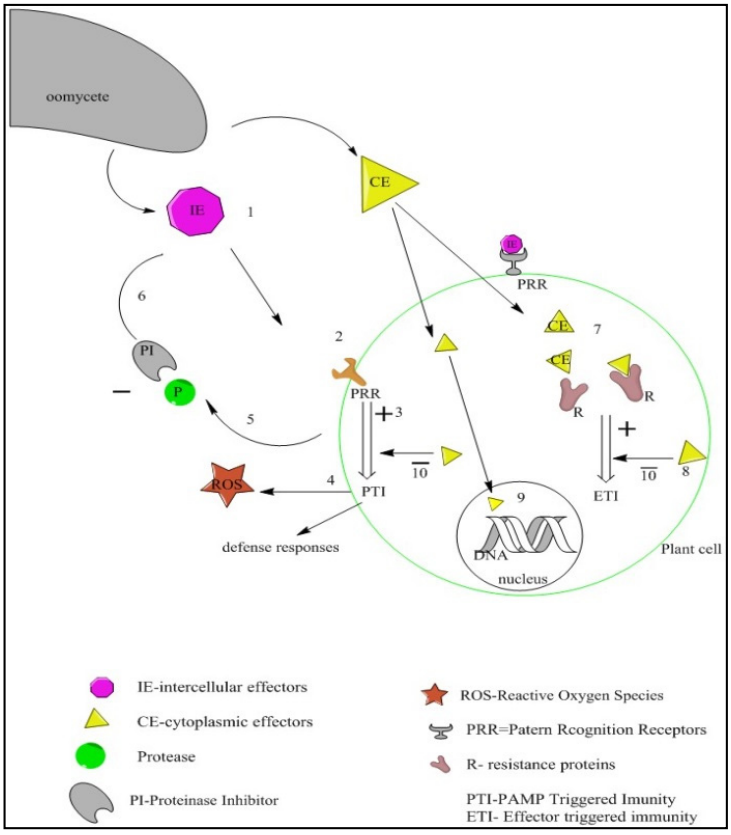

Figure 1. Schematic representation of the oocycete – plant cell interaction

Theories explaining the interaction between pathogen and host

Vertical resistance is based on the 'gene for gene' model. The pathogen avirulence genes are detected by matching resistance genes. In the case of incompatible interaction, the plant remains resistant. This type of resistance provides protection just for a short period, because the pathogen, in our case the oomycete *P.infestans,* rapidly evolves and overcomes the respective resistance gene. Several breeding programs obtained resistant potato cultivars with one or more resistance genes from *Solanum demissum*, but the "super" races of *P. infestans* rapidly overcomes this resistance.

A total of four different theories are known, which explain the interaction between resistance gene and *avr* gene products. The first theory considers a classical receptor-ligand interaction, which predicts direct interaction of effectors with receptor proteins. The second model assumes on indirect interaction, effectors interact with specific receptors, which can activate the resistance proteins and initiate a defence reaction.

Nowadays the most accepted model is 'guard' model, the third and fourth models, which presupposes the association of resistance proteins with the target of effector molecules. There are two theories for this type of interaction, the first is based on the activation of resistance proteins by conformation changes of the effector target molecule, which are physically connected. The second theory is based on the affinity changes of the effector target molecules. Direct interaction with resistance gene products only occurred after the effectors interact and change the affinity of targeted molecules (van der Hoorn and Kamoun, 2008; Maekawa *et al.*, 2011).

Conclusion

Late blight of potato is still remaining one of the greatest damage causing disease of potato. To prevent or to control these pathogen more insights into the biology and life cycle of the *Phytophthora infestans* are needed.

The most used methods to transfer resistance into cultivated potato, represent the crossing with wild species, they representing the source of resistance genes.

In some cases when it is not possible to obtain viable hybrids, in natural way or this hybrids, besides the resistance to late blight, have another unfavorable trait, breeders used other methods, for example identifying and cloning broad spectrum resistance genes (Song *et al.,* 2003; Brylińska *et al.,* 2015).

Knowing that *P. infestans*, is a rapidly evolving species, hybrids which contain, just one resistance genes will be rapidly overcome by the pathogen. Pyramiding of resistance genes, could be a better way to obtain durable resistance to late blight (Tan *et al*., 2010).

Based on the current knowledge, about the interaction of *Phytophthora infestans* and potato, the best method for obtaining durable resistance could be transferring both type of resistance, resistance genes and quantitative trait loci, which take part in horizontal resistance, and confer race-nonspecific resistance (Bormann *et al*., 2004).

A faster solution for breeders could be somatic hybridization combined to new tools of genomics, proteomics and next generation sequencing, which have the advantage to combine characteristics of wild *Solanum* species with beneficial proprieties of cultivated potato.

Via somatic hybridization more genes, for example quantitative trait loci, part of horizontal resistance, can be transferred without the short comes of transgenesis which may transfer fewer genes and is yet not accepted by consumers

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