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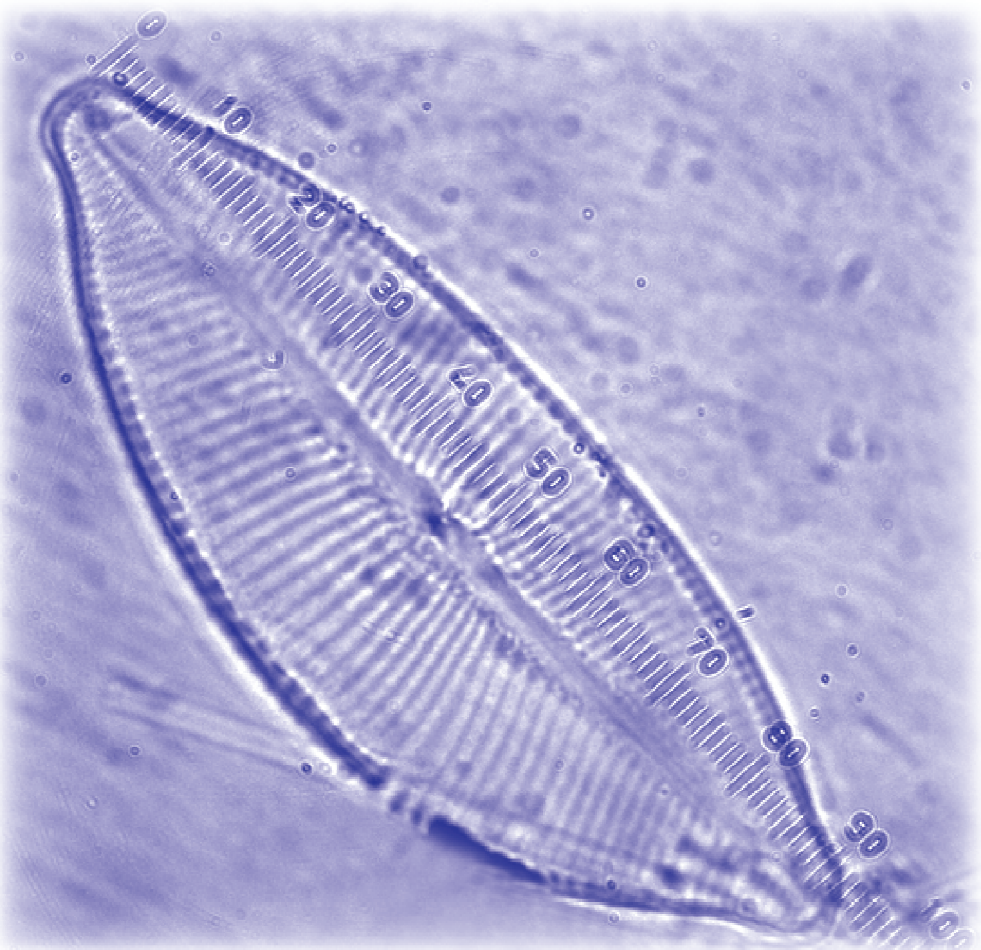

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Original pictures on front cover: The diatom *Cymbella ehrenbergii* Kützing 1844 from Lake Iezerul Ighiel (Transylvania, Romania) © Anca Ciorca

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Rhizoremediation of poly aromatic hydrocarbon content of a model waste diesel engine oil-polluted soil by some local lawn plant species in Benin City, Nigeria

Beckley Ikhajiagbe[1,✉], Geoffery O. Anoliefo[1] and Alphonsus E. Imoni [2]

SUMMARY. This study investigated the effect of 10 local lawn plant species namely *Eleusine indica, Paspalum vaginatum, Stenotaphrum secundatum*, *Cynodon dactylon*, *Cymbopogon citratus*, *Axonopus compressus*, *Sporobulus pyramidalis*, *Cyperus rotundus*, *Chrysopogon aciculatus* and *panicum maximum* in the rhizoremediation of a waste engine oil-polluted soil for a period of three months. Soil, weighing 20 kg was thoroughly mixed with waste engine oil to obtain a constant 5% w/w concentration of waste engine oil in soil. After 4 weeks, the ten lawn plant species were sown in the bowls. The plants' response to stress occasioned by the oil pollution was studied using leaf number as well as occurrence of chlorosis and necrosis; whereas rhizospheric soil samples were analyzed for poly aromatic hydrocarbon contents and microbial composition. PAH concentrations of some of the soil sown with some of the grasses were reduced indicating that remediation took place although not completely. The soil sown with *Eleusine indica* had the highest total remediation efficiency which was 90.61% after eight weeks of sowing. The plant-associated microbial community was examined in all the lawn plant species. The assessment of the influence of grass on the abundance and activity of microorganisms in the rhizosphere showed a buildup of microbial communities over the period and this helped in the remediation of the contaminated soil. *Eleusine indica* had the highest heterotrophic bacteria count of 5.6 x 10^5cfu/g, while the percentage of hydrocarbon degrading bacteria was highest in soil sown with *Stenotaphrum secundatum.* Of all the local lawn plant species used in the research, *Eleusine indica* was observed to be a suitable candidate for in situ rhizoremediation potential.

Keywords: *Cynodon dactylon, Eleusine indica,* lawn grasses, rhizoremediation, polyaromatic hydrocarbon.

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Introduction

The environment is currently plagued by various forms of contaminants ranging from heavy metals, pesticides, waste water, as well as oil wastes. One of the most common sources of oil pollution is waste oil. Pollution due to waste engine oil has been a worldwide problem, and the estimated number of contaminated sites is significantly increasing especially in developing countries (Mougin, 2002; Kaimi *et al.,* 2006). This has been shown to have harmful effects on the environment and human beings at large due to the presence of poly aromatic hydrocarbons and other contaminants. Soil contamination by waste engine oil is increasing rapidly as a result of the global increase in the usage of petroleum products (Mandri and Lin, 2007).

It is usually difficult to accurately quantify the details regarding the degree of hydrocarbon contamination in the terrestrial environments. This is primarily due to the accidental spills either around factories or petrol station. In other to enhance environmental development increasing efforts have been going on in order to remediate and recover contaminated sites by using "green" technologies. It is hoped that would either to serve reverse the risk of adverse health or environmental effects, or to create an enabling avenue for the redevelopment of contaminated sites (Vidali, 2001). Many plants have been identified to have the capability for site clean-up and reclamation; among these are those identified to have abilities to enable rhizoremediation of contaminants, particularly petroleum hydrocarbons (Reilley *et al.,* 1996). Some studies (Qiu *et al.*, 1997; Gunther *et al.*, 1996; Chen *et al.*, 2003) have shown that tall fescue (*Festuca arundinacea*) and switch grass (*Panicum virgatum*) could both degrade about 1.6 and 8.7% pyrene for a period of 190 days of incubation.

Rhizoremediation refers the degradation of soil organic compounds by the activities of microorganisms through rhizospheric influence, in the soil or ground water immediately surrounding the plant roots. The plant roots exude acids, minerals, vitamins and enzymes that enhance microbial activities within the root zone, thereby facilitating degradative processes within this region. As a result of the hydrophobicity of these contaminants, they cannot enter the plant, and as such are favourably remediated by rhizoremediation. Kuiper *et al.* (2004) reported that the by-products of rhizoremediation products like alcohols, acids, carbon dioxide and water, are usually less persistent and toxic in the environment than their parent compounds. Many researches on hydrocarbon remediation of contaminated sites have relied on grasses for the extensive root system. (Gunther *et al.,* 1996; Qui *et al.*, 1997; Xia 2004). The most extensively characterized fibrous root systems belong to the grass family, *Poaceae*. Grass roots cover an extensive surface area when compared to other plant types. The potentiality for many grasses for hydrocarbon rhizoremediation may be as a result of their highly branched, fibrous root systems which are able to house large numbers of microbes and greatly influence the soil environment (Anderson *et al.*, 1993). However, the plant selection is key to phytoremediative success. Selected plants are usually those that are tolerant to hydrocarbon contaminated sites (Tesar *et al.,* 2002; Anoliefo *et al.,* 2006).

Studies (Adam and Duncan, 1999; Pichtel and Liskanen, 2001; Dominguez-Rosado and Pichtel, 2004) have shown that grasses are tolerant to various hydrocarbons, most especially aliphatic hydrocarbons. Further, previous studies by the authors have demonstrated the capability for some local lawn plant species like *Eleusine indica* to tolerate oil-contaminated soils (Anoliefo *et al.*, 2008; Ikhajiagbe and Anoliefo, 2012; Ikhajiagbe *et al.,* 2013).

In the present study, a total of ten (10) local lawn plant species, including grasses and sedges, would be selected for use, including *Eleusine indica, Paspalum vaginatum, Stenotaphrum secundatum*, *Cynodon dactylon*, *Cymbopogon citratus*, *Axonopus compressus*, *Sporobulus pyramidalis*, *Cyperus rotundus*, *Chrysopogon aciculatus* and *panicum maximum*. These plants were employed in this research as some of them have been identified by previous researches (Adam and Duncan, 1999; Pichtel and Liskanen, 2001; Dominguez-Rosado and Pichtel, 2004; Kuiper *et al.,* 2004; Anoliefo *et al.*, 2006; Anoliefo *et al.*, 2008; Ikhajiagbe and Anoliefo, 2012; Ikhajiagbe and Chijioke-Osuji, 2012; Ikhajiagbe *et al.*, 2012; Ikhajiagbe *et al.*, 2013) as plants that may have capabilities for remediating oil-contaminated soil. The aim of this study was to evaluate the potential of native lawn plant species in Benin City in the rhizoremediation polyaromatic hydrocarbon fractions of a waste engine-oil-polluted soil.

Materials and methods

Collection and preparation of materials for the experiment

The study area for this research was a plot of behind the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria. Site demarcation and clearing of the plot of land in which the experiment was conducted was carried out. Thereafter, top soil (0 – 10 cm) of known physiochemical properties was collected in the morning at about 8.00 am from an area of 1 m^2. With a weighing balance, 20 kg each of soil was weighed and transferred into large perforated bowls (58.43 cm in diameter) with 8 perforations made using a 2-mm diameter nail at the bottom of each plastic bowl.

Waste diesel-engine oil (WEO) was obtained from a mechanic workshop and stored in jerry cans, and transferred to the site of the experiment. Samples were then analyzed for poly aromatic hydrocarbon composition before use.

Soil pollution using waste engine oil

Measured quantity of soil in each the 58.43 cm diameter bowl (12 kg) was carefully mixed with WEO at 5% w/w concentration. The soil was thereafter left for a period of 4 weeks for attenuation.

Cultivation of the plant/duration

After the period of attenuation, the lawn plants, consisting of both grasses and sedges, were transferred from their nursery beds and sown into the bowls containing the hydrocarbon polluted soil at a depth of 5 cm and covered with the soil. The lawn plants were constantly observed for vegetative parameters. They were also watered daily, on a 2-day interval depending on the prevailing environmental conditions. At the end of the study, soils were taken to the laboratory for analyses.

Results and discussion

Table 1 below shows soil PAH content at the beginning of the experiment. The total PAH content of oil-polluted soil after 3 months of oil pollution showed total PAH content of soil as 1025.15 mg/kg immediately after contamination by WEO (Table 2). After 3 months of WEO pollution, *Eleusine indica* had the lowest total PAH content (96.31mg/kg), *Paspalum vaginatum* (126.52mg/kg) *Stenotaphrum secundatum* (229.54mg/kg), *Cynodon dactylon* (250.33mg/kg), and *Panicum maximum* (410.68 mg/kg) which was the highest total PAH. Also, soil-polluted soil sown with *Eleusine indica* recorded the highest bioremediation efficiency (90.61%), followed by *Paspalum vaginatum* (87.66%), and then *Panicum maximum* with the lowest bioremediation efficiency (59.94%).

At the end of the study, there was an 11.46% decrease in the number of leaves recorded for *Panicum maximum,* compared to a 13.79% increase for *Chrysopogon aciculatus* (Table 3). There were no leaves recorded for *Sporobolus pyramidalis* from the 3rd week after sowing in oil-polluted soil. the plant lost all its leaves eventually. There was above 50% increase in number of leaves of *Cyperus rotundus*. *Paspalum vaginatum* lost over 50% of its leaves. Percentage of the total number of leaves per plant stand that showed evidence chlorosis was 92.94% in *Panicum maximum*, 91.77% in *Cyperus rotundus,* and 100% in *Cymbopogon citratus.* The plant with the least number of chlorotic leaves was *Eleusine indica* with only 44.54% of its total number of leaves showing evidence of chlorosis for the entire 8-week period. *Eleusine indica* was the plant with the least number of leaves showing evidence of necrosis.

The result of the current study thus demonstrated the removal of hydrocarbons in soil by some of the local lawn plant species used. Quite a number of studies on hydrocarbon rhizoremediation have focused on the assessment of individual species in relation to unplanted soil (Hutchinson *et al* 2003; Kuiper *et al.,* 2004). The assessment of the lawn plant species *Eleusine indica, Paspalum vaginatum, Stenotaphrum secundatum*, *Cynodon dactylon*, *Cymbopogon citratus*, *Axonopus compressus*, *Sporobulus pyramidalis*, *Cyperus rotundus*, *Chrysopogon aciculatus*

and *panicum maximum* was done by measuring the effect of the contaminants on the continued growth of the plants in the contaminated soils, using leaf morphology as a yardstick.

Table 1.

Chemical composition of waste engine oil and top soil used for the experiment

Parameters	WEO (mg/kg)	Soil (mg/kg)
pH	6.15	5.98
Electrical Conductivity (µs/cm)	NA	309
Total Org. Matter (%)	NA	0.61
Total Nitrogen (%)	NA	0.16
Exchangeable Acidity (meq/100 g soil)	NA	0.24
K (meq/100 g soil)	NA	1.40
Ca (meq/100 g soil)	NA	12.20
Mg (meq/100 g soil)	NA	9.95
P (mg/kg)	NA	153.00
Clay (%)	NA	7.9
Silt (%)	NA	13.9
Sand (%)	NA	78.2
Naphthalene	25.95	0
Acenaphthylene	7.62	0
2-bromonaphthalene	28.32	0
Acenaphthene	21.25	0
Fluorene	42.33	0
Phenanthrene	4.20	0.85
Anthracene	19.65	0
Fluoranthene	33.21	0
Pyrene	24.09	0
Benzo(a)anthracene	41.09	0
Chrysene	116.04	0
Benzo(b,j,k)fluoranthene	38.05	0
Benzo(a)pyrene	118.24	40.28
Indeno(1,2,3-cd)pyrene	131.05	5.24
Dibenzo(a,h)anthracene	34.22	12.25
Benzo(g,h,i)perylene	59.66	19.24
Copper, Cu (mg/kg)	0	42.52
Manganese, Mn (mg/kg)	0	8.54
Nickel, Ni (mg/kg)	0	0.2
Vanadium, V (mg/kg)	0	0.1
Chromium, Cr (mg/kg)	0.08	16.85
Lead, Pb (mg/kg)	0	19.96

Sensitivity of analytical equipment used ≤0.001mg/kg. NA: Not Available

Some of the plants grew successfully in the 5% w/w concentration of the waste engine oil contaminated soil. *Eleusine indica* showed promising behaviour in 5%-WEO-contaminated soil. In the present study, *Eleusine indica* recorded the greatest overall removal of PAH content of the 5%w/w waste engine oil polluted soil with an efficiency of 90.61% and fastest rate of removal of PAH content of all the lawn plant species assessed, while the overall lowest removal of PAH content was *Panicum maximum,* with an efficiency of 59.94%. *Paspalum vaginatum* was the second highest lawn plant species that was remediated with an efficiency of 87.66%.

Table 2.

Polyaromatic hydrocarbon contents of oil-polluted soil three months after exposure to rhizoremediation by various lawn plant species

PAH	Immediately after soil contamination	3 months after pollution/sowing									
		GG	PG	NG	AG	BG	PS	LG	BH	CG	CT
Naphthalene	31.02	BDL	BDL	0.56	BDL	BDL	BDL	BDL	13.75	21.35	26.52
Acenaphthylene	19.74	16.26	BDL	BDL	BDL	BDL	BDL	BDL	13.78	BDL	9.21
2-bromo-naphthalene	35.21	17.88	22.71	29.25	30.55	6.25	3.65	20.05	14.95	22.26	23.12
Acenaphthene	37.41	16.18	21.30	23.51	BDL	BDL	0.62	19.33	13.96	21.25	32.25
Fluorene	45.22	16.59	22.04	26.32	29.90	11.35	16.25	20.13	14.55	21.82	21.52
Phenanthrene	5.66	1.32	1.01	0.95	0.92	BDL	1.66	BDL	1.17	BDL	0.92
Anthracene	29.24	17.39	22.94	21.62	31.14	3.65	9.85	21.07	15.27	22.99	15.62
Fluoranthene	42.53	16.82	BDL	0.04	BDL	BDL	9.65	20.13	BDL	BDL	0.51
Pyrene	38.22	18.84	21.78	23.20	29.71	12.65	25.21	19.56	14.60	21.80	19.25
benzo(a)-anthracene	53.87	22.54	25.39	22.35	BDL	0.31	BDL	22.029	16.55	26.51	26.85
Chrysene	123.54	4.86	BDL	0.62	BDL	BDL	BDL	BDL	1.11	BDL	0.96
benzo(b,j,k) fluoranthene	59.44	11.79	15.82	6.52	2.79	0.62	BDL	22.39	6.89	22.56	16.89
benzo(a) pyrene	198.42	113.81	96.12	68.54	53.30	36.20	23.21	46.95	47.85	57.89	63.52
indeno(1,2,3-cd)pyrene	169.54	34.53	62.04	58.26	3.59	0.05	3.26	1.57	17.68	2.64	12.51
dibenzo(a,h) anthracene	63.48	46.85	21.46	19.52	4.49	3.61	3.94	8.67	28.36	9.52	9.66
benzo(g,h,i) perylene	72.61	55.02	32.07	24.36	43.15	21.62	29.22	30.95	29.86	32.21	29.65
Total PAH	1025.15	410.68	364.68	325.62	229.54	96.31	126.52	252.83	250.33	282.80	308.96
Efficiency (%)	-	59.94	64.43	68.23	77.61	90.61	87.66	75.34	75.58	72.41	69.86

BDL: below detectable limit of 10^{-4}mg/kg. GG - Guinea grass (*Panicum maximum*); PG - Port Harcourt Grass (*Chrysopogon aciculatus*); NG - Nut grass (*Cyperus rotundus*); AG - St. Augustine grass (*Stenotaphrum secundatum*); BG - Bull grass (*Eleusine indica*); PS - Paspalum (*Paspalum vaginatum; LG* - Lemon grass (*Cymbopogon citratus*); BH - Bahama grass (*Cynodon dactylon*); CG - Broadleaf carpet grass (*Axonopus compresus*); CT - Cat's tail grass (*Sporobolus pyramidalis*)

Table 3.

Percentage development of selected parameters of test weeds sown in oil-polluted soils after 8 weeks of exposure

Lawn plants	Percentage change compared to original parameter before sowing in oil-polluted soil (%)		
	No. of leaves compared to NLS	Leaves with chlorosis	Leaves with necrotic lesions and spots
GG	-11.46	92.94	25.98
PG	13.79	85.89	10.54
NG	62.00	91.77	18.65
AG	1.97	89.13	26.54
BG	-22.99	44.54	2.54
PS	-52.38	50.00	5.36
LG	-23.29	100	39.32
BH	20.70	74.82	39.08
CG	18.39	91.18	23.87
CT	-100	0	0

Negative values indicate percentage decreases from original values, whereas positive values indicate percentage increase from original value. GG - Guinea grass (*Panicum maximum*); PG - Port Harcourt Grass (*Chrysopogon aciculatus*); NG - Nut grass (*Cyperus rotundus*); AG - St. Augustine grass (*Stenotaphrum secundatum*); BG - Bull grass (*Eleusine indica*); PS - Paspalum (*Paspalum vaginatum; LG* - Lemon grass (*Cymbopogon citratus*); BH - Bahama grass (*Cynodon dactylon*); CG - Broadleaf carpet grass (*Axonopus compresus*); CT - Cat's tail grass (*Sporobolus pyramidalis*). NLS – number of leaves recorded per plant just before being transplanted into oil-polluted treatments.

Acenaphthylene, naphthalene, fluoranthene and chrysene were observed to be significantly degraded in at least 5-7 of the lawn plant species used in this experiment at 3 months after pollution with oil.

There was also a total (100%) remediation of naphthalene content in *Panicum maximum, Chrysopogon aciculatus, Stenotaphrum secundatum, Eleusine indica, Paspalum vaginatum* and *Cymbopogon citratus*. Acenaphthylene was completely (100%) remediated in *Chrysopogon aciculatus, Cyperus rotundus, Stenotaphrum secundatum, Eleusine indica, Paspalum vaginatum, Axonopus compressus* and *Cymbopogon citratus.* Fluoranthene was also totally (100%) remediated in *Chrysopogon aciculatus, Stenotaphrum secundatum, Eleusine indica, Cynodon dactylon,* and *Axonopus compresus.* Complete (100%) remediation of chrysene was observed in *Chrysopogon aciculatus, Stenotaphrum secundatum, Eleusine indica, Paspalum vaginatum, Cymbopogon citratus* and *Axonopus compresus*.

In the present study, two rings PAHs were mostly degraded than three ring PAHs compounds. Acenaphthylene was the overall highest degraded PAH. Seven out of the ten lawn plant species were 100% degraded of WEO, while Benzo (a) pyrene was the overall least degraded PAH.

Hydrocarbon compounds can be broken down and degraded by soil biota, especially by soil microorganisms. This greatly depends on the properties of the pollutants and the activity of soil microbial oil degraders. Generally, hydrocarbons with straight and few chains degrade more readily than those with highly condensed ring structures (ATSDR 1999). Results of microbial composition of soil showed the presence of *Achromobacter species, Clostridium perfringens, Micrococcus luteus, Bacillus pumilis, Bacillus subtilis, Enterobacter aerogenes, Pseudomonas species* and *Pseudomonas aeruginosa* (Table 4). In the rhizospheres of all the ten local lawn plant species used, *Bacillus pumilis,* followed by *Bacillus subtilis,* was the most predominant bacteria species present; while *Enterobacter aerogenes* was the least predominant in all the samples of soil assayed. However, immediately after soil contamination, total heterotrophic bacteria count was 4.3 x 10^5cfu/g with a percentage hydrocarbon degrading bacteria count of 53.49%. *Eleusine indica* when assayed had the overall highest total heterotrophic bacteria count and overall highest hydrocarbon degrading bacteria count which were 5.6 x 10^5 cfu/g and 3.5 5.6 x 10^5 cfu/g respectively while the percentage of hydrocarbon degrading bacteria was 62.5% indicating that it has a good relationship with the microorganisms present in the polluted soil when compared to other lawn plant species used and therefore aided in the remediation of PAH content of the soil. Meanwhile, *Panicum maximum* had the overall lowest total heterotrophic bacteria count of 3.9 x 10^5 cfu/g and the percentage of hydrocarbon degrading bacteria of 52.78% of soil assayed for.

Heterotrophic bacteria count immediately after soil contamination was 4.3 x 10^5 cfu/g, while for *Panicum maximum, Chrysopogon aciculatus, Cyperus rotundus, Stenotaphrum secundatum, Eleusine indica, Paspalum vaginatum, Cymbopogon citratus, Cynodon dactylon, Axonopus compressus and Sporobulus pyramidalis* were 3.6 x 10^5 cfu/g, 3.9 x 10^5 cfu/g, 4.2 x 10^5 cfu/g, 4.3 x 10^5 cfu/g, 5.6 x 10^5 cfu/g, 4.0 x 10^5 cfu/g, 3.6 x 10^5 cfu/g, 4.6 x 10^5 cfu/g, 4.2 x 10^5 cfu/g and 3.9 x 10^5 cfu/g respectively. Similarly, *Aspergillus niger* followed by *Penicillium* species were the most abundant fungi present in the WEO-polluted soil, while *Fusarium species* and *Aspergilus flavus* were the least predominant. Yamazaki *et al.* (1988) earlier reported that *Aspergillus niger* converts terpene B- myrcene to dihydroxylated derivatives. According to Yogambal and Karegoudar, 1997, *Aspergillus niger* has the ability to cleave to the rings of naphthalene, anthracene, and phenanthrene. Numerous genera of fungi have the ability to oxidise naphthalene. Bacteria have also been reported (Chen *et al.*, 2003) to have mineralized benzo(a)pyrene.

Table 4.

Microbial counts and parameters of test weeds sown in oil-polluted soils

	Immediately after soil contamination	3 months after pollution/sowing									
		GG	PG	NG	AG	BG	PS	LG	BH	CG	CT
Bacteria											
Achromobacter sp.	+	-	+	+	-	-	+	+	+	-	-
Clostridium perfringens	+	-	+	+	+	-	+	-	+	+	-
Micrococcus luteus	+	+	+	+	+	-	+	-	+	-	-
Bacillus pumilis	+	+	+	+	+	+	+	+	+	+	+
B. subtilis	+	+	+	+	+	+	+	+	-	-	+
Enterobacter aerogenes	-	+	-	-	-	+	+	-	+	+	-
Pseudomonas sp.	+	-	-	+	-	+	+	+	-	-	+
P. aeruginosa	+	-	+	+	-	+	-	+	+	-	+
Heterotrophic (x 10^5 cfu/g)	4.3	3.6	3.9	4.2	4.3	5.6	4.0	3.6	4.6	4.2	3.9
Hyd. Deg. (x 10^5 cfu/g)	2.3	1.9	2.1	2.3	2.9	3.5	2.6	1.9	3.2	2.2	2.8
% Hyd	53.49	52.78	53.85	54.76	67.44	62.5	65	52.78	69.57	52.38	71.79
Fungi											
Aspergillus niger	+	+	+	+	+	+	+	+	+	+	+
A. Flavus	-	+	-	+	-	+	-	+	-	-	-
Penicillium sp.	+	+	+	+	+	-	+	+	+	+	+
Fusarium sp.	-	+	+	-	+	-	-	-	-	-	-
F. solani	-	-	-	-	+	+	+	-	-	-	+
Rhiszopus stolonifera	+	+	+	-	+	-	-	-	+	+	+
Geotrichum sp.	+	-	-	+	+	+	+	+	+	-	+
S*accharomyces* sp.	+	+	+	+	-	+	-	+	-	+	-
Heterotrophic (x 10^5 cfu/g)	2.3	3.3	2.6	2.4	3.0	2.9	2.0	1.9	2.6	3.5	2.8
Hyd. deg. (x 10^5 cfu/g)	1.5	2.0	1.3	1.8	1.9	2.0	1.8	1.3	1.2	2.8	1.2
% Hyd	65.22	60.61	50	75	63.33	68.97	90	68.42	46.15	80	42.86

Conclusions

The success of rhizoremediation depends on how efficient the root-soil contact is in order to produce the desired contaminant degradation. The outcome of this experiment clearly indicates that the rate of hydrocarbon degradation in

rhizoremediation application presents a better alternative for dealing with hydrocarbon contaminated soils compared with the rates of degradation but a longer duration of time should be employed to get a more better and positive result.

In the current study, *Eleusine indica* enhanced the removal of polyaromatic hydrocarbon content of waste engine oil polluted-soil and this was done without the need for nutrient addition. Alternatively, nutrient addition to a rhizoremediation system involving local grass species may increase biodegradation efficiencies. More intensive research involving the local grasses used in this research, as well as the sedge, should be carried out and tested for their rhizoremediation capabilities. Some of these grasses (*Poaceae* family) should be used in this application over a longer period of time with or without the use of any amendments as this is likely to increase the rhizoremediation efficiency. This is because according to researches already carried out, grasses belonging to this family are very good candidates for rhizoremediation.

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The *Robinia pseudoacacia L.* seed germination and plantlets growth in septic or aseptic conditions under led light of different wavelenghts

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SUMMARY. The lighting of plant vitrocultures using white LEDs expecially the ones of ultrabright type is recomended because – as resulted from the experiments performed in this study, under septic or vitroculture conditions, with *Robinia pseudoacacia L.* seeds and with plants resulted from these – it was shown that compared with red, yellow, green and especially blue light, under white light the organ growth and assimilating pigments synthesis was stimulated. Furthermore the endocellular substances metabolisation was also accelerated. Among other types of light emited by LEDs, only the yellow light has stimulated the increase in the values of the parameters that were investigated. This has favored, in the 40st day of germination the growth of the stemlet in the septic cultures even as high as 51% compared to the control (100% , plantlets under white light LEDs).

Keywords: germination, growth LEDs lighting, plantlets, *Robinia pseudoacacia*.

Introduction

Plant vitrocultures, mainly the ones from the plant micropropagation industry require to set up growth chambers in which the phyto-inoculi or vitroplantlets regenerated „*in vitro*" or the one transferred „*ex vitro*" (to acclimatize them for the septic environment), need to be exposed to light.

The artificial light for lighting such cultures, is installed in growth chambers in which the environmental conditions are automatically adjusted. Most frequently, the lighting in such chambers is made using white light from fluorescent tubes.

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These types of lamps, however, warm the room and as such, an environmental control system has to be used. The cooling of the growth chambers increases the electricity consumption and the maintenance costs of the chambers.

It is also worth to be mentioned that in modern horticulture the planting material is obtained using micropropagation techniques. The nature of the light wavelenght has different influence on the various physiological processes of the plants, depending on the species and developmental stage and also on the organ studied.

The spectral composition of the light is important for the synthesis of different substances or for the various biological processes that occur in plants (Burzo *et al*., 1999).

Solar light consists of a large number of visible electromagnetic radiation with a wavelenght between 400 and 700 nm (red, orange, yellow, green, blue and violet) and also invisible radiation – ultraviolet (UV) light under 400 nm and infrared radiation - above 700 nm (Tarhon, 1992).

Artificial light sources emit differently the spectral wavelenghts, the light being different from the natural one which is an equal blend of all spectral components.

At this moment, in different areas of activity the LED lighting is widespread. It is used as spots, signaling devices, panels of various sizes etc.

LED lighting fixtures are made of lamps consisting of semiconductor diodes that became luminescent when connected to a power supply. Such lamps are 5 -7 mm in size and can be grouped to form light sources with different intensities. They do not heat up and have very low energy consumption (http://ro.wikipedia.org/). The LEDs can be placed in the immediate vicinity of the flasks with the phyto-innoculi, allowing the increase of density of the shelves in the growth chambers for efficient space usage.

Tennessen *et al*. (1995) have studied the effect of LEDs lighting on the photosynthesis in *Solanum tuberosum* vitrocultures under continuous lighting compared to intermittent lighting. The photosynthetic capacity of the vitroplantlets was similar in both cases.

Regarding the distance at which the LEDs can be placed above the vitrocultures, Jao and Fang (2004) studied the *Solanum tuberosum* explants where the lamps were placed 1 cm above the vitrocultures. The lighting duration was as follows: 8 hrs light/16 hrs dark; 12hrs light/12 hrs dark and 16 hrs light/24 hrs dark. The best results were obtained using the last lighting regime. In relation to the use of LEDs for lighting, compared with fluorescent light – placed 30 cm above the cultures – a cost saving of about 17% was estimated.

A series of studies regarding the effect of LED light on plants were performed with *Marigold* and *Salvia* (Jeong *et al*., 2002), *Fragaria* (Nhut *et al.*, 2003), *Chrisantemum* (Petruş and Cachiţă, 2004), *Zantedeschia jucunda* (Jao *et al.*, 2005), *Pisum sativum* (Topchiy *et al.*, 2005), or at seed germination of *Raphanus* and of *Daucus* (Sommer and Franke, 2006), of *Sequoia sempervirens* (Pop and Cachiţă,

2007; 2009; 2013), of *Solanum* (Pop *et al*., 2011), or of *Pinus nigra*, *Brassica oleacea* and *Beta vulgaris* (Matioc-Precup and Cachiţă, 2013), or of kernels of *Sorgum* (Stana and Cachiţă, 2012) or of *Hordeum vulgare* (Matioc-Precup and Cachiţă, 2013). In the majority of the cases, positive results were obtained regarding the growth parameters.

The purpose of the study presented in this work was to investigate the influence of the lighting with LEDs of different colors (red, yellow, green or blue) on the *Robinia pseudoacacia L.* seed germination and on the plantlets growth under septic or aseptic regime for 40 days and exposed to a mono color light of a certain type, for 16/24 hours.

Material and methods

The *Robinia pseudoacacia L.* is a dicotyledonous (dicot) species. The plantlets have epigeal cotyledons that after raising above the ground, turn green and begin photosynthesis as long as they do not enter into senescence and fall (Fig. 1).

In the present work, the experiments were performed either with plantlets from *Robinia pseudoacacia L.* seeds germinated in colorless, transparent plastic containers (seeds placed on filter paper periodically moistened with tap water) or the germination and plantlet growth was done „*in vitro*" on agarised Murashige-Skoog (1962) growth medium without growth regulators.

Depending on the experiments, thesamples were analyzed either after 10 days from germination and after 14 or 40 days. Before „*in vitro*" inoculation, the seeds were disinfected with a „Domestos" solution for 20 minutes. After that, they were washed repeatedly with sterile water.

The inoculation in the culture containers was made in a hood with laminary sterile air flow. In both versions the lighting of the samples was done either with white LEDs (version V_0) or red (version V_1), yellow (version V_2), green (version V_3) or blue (version V_4). From the germination samples, a batch was exposed to natural light (Version V_0) by putting the containers in a northward facing window. The number and density of the LEDs on the lightins panel was choosen to give a light intensity of 2000 lux. The adopted photoperiod in all experiments was 16 hrs light/24 hrs. The LEDs were of ultrabright type and the wavelenghts were 700 nm for the red light, 550 nm for the yellow light, 500 nm for green and 450 for the blue light (Tab.1).

In the 14^{th} day of germination, cotyledons were taken from the plantlets to perform ultrathin transversal sections which were processed according to the specific procedures for this technique (Cachiţă and Crăciun, 1990). Firstly, the plant tissues were fixed in a 2.5% glutaraldehyde solution in 0.15 M phosphtate buffer followed by post-fixation in a 2% osmic acid solution in 0.1 M phosphate buffer. After that,

the samples were dehydrated in increased acetone concentration solutions and embedded in epoxy resin followed by encapsulation in gelatin capsules. The blocks were shaped under the stereomicroscope and ultrahin sections were obtained using a Leica UC6 ultramicrotome and collected on electrolytic grids and doubly contrasted with uranyl acetate and lead citrate. The examination of the sections was done on a Jeol 1010 transmission electron microscope (TEM) and the most representative electronmicrographs are shown in figures 2 and 3. Further, in the 40st day from germination, biometric measurements on the *Robinia pseudoacacia L.* plantlets size were done by measuring the lenght of the embryonary rootlet and stemlet (of the hypocotyl and epicotyl respectively) and by summing their lenghts, the size of the whole plantlet was obtained (Fig. 4 A and B).

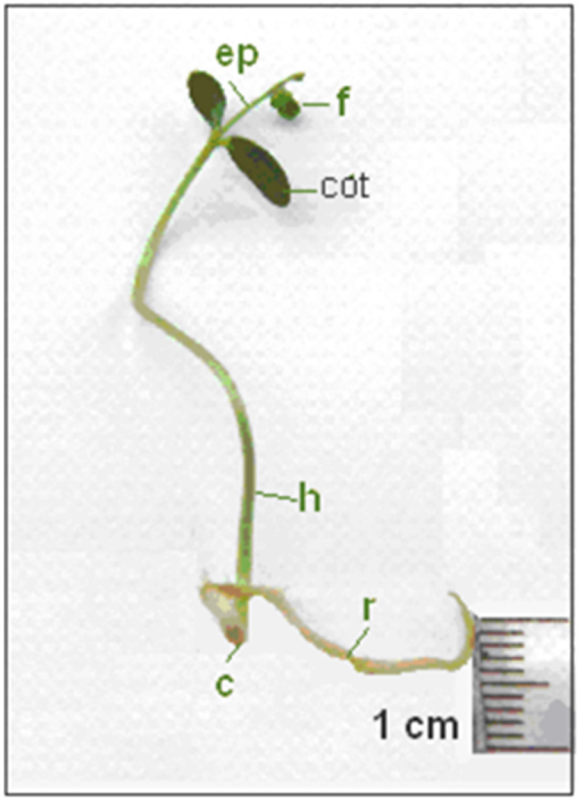

Figure 1. The aspect of a 40 days from germination *Robinia pseudoacacia L.* plantlet (abbreviations: c – root-stem transition zone; cot – cotyledons; ep – epicotyl; f- leaflet foliole; h – hypocotyl; r – embryonary rootlet).

Such measurements were performed both on the plantlets kept in plastic containers and grown on aseptic medium. The biometric measurements were done on 50 plantlets for each experimental version, the average of these values/version were included in the percentage calculations, reporting the values to the reference average evaluated as 100% recorded for the control (V_0) – the sample exposed to white LEDs.

Table 1.

Experimental versions performed to determine the percentage of germinated seeds in the 10th day

Versions	**Percentage of germination**
Natural light (V_{00})	99%
White LED (V_0)	98%
Red LED (V_1) 700 nm	91%
Yellow LED (V_2) 550 nm	96%
Green LED (V_3) 500 nm	99%
Blue LED (V_4) 450 nm	95%

The content in the assimilating pigments, was also determined in the 40st day from germination in the biomass made of a homogenate prepared from the "above the ground" organs of a plantlet – the whole green mass resulted from grounding the hypocotyls, cotyledons, epicotyl and leaflets, where they were developed.

From the fresh plant material, the pigments were extracted with a dimethylformamide solution and the extract measured using a „Spekol 11" spectrophotometer at the following wavelenghts: 664 nm to determine the content in chlorophyll **a**, 647 nm to determine the content of chlorophyll **b**, and 480 nm to determine the content in carotenoid pigments.

From the fresh biomass 50 mg per sample were taken and placed in 5 ml dimethylformamide (DMF) (Moran and Porath, 1980); the mixture was kept in a refrigerator fo 72 hrs at 4ºC. After that, the supernatant was decanted and from this solution the extracted assimilating pigments content was determined (green pigments and carotenoid pigments). By summing the values obtained for each pigment, the total amount of assimilating pigments was obtained.

For each experimental version five measurements were performed.

The data from the spectrophotometer was matematically processed according to a formula proposed by Moran and Porath (1980):

- chlorophyll a (mg/gPM) = 11,65 A664 – 2,69 A647 · v/s;
- chlorophyll b (mg/gPM) = 20,8 A647 – 3,14 A664 · v/sp;
- carotenoids (mg/gPM) = (1000 A480 – 1,28 clorof. a – 56,7 clorof. b) / 245 · v/sp;

where: the numbers near letter ”A” are the wavelenghts in nm;
v – solvent volume in ml;
PM – plant material weight in mg used for extranction/sample.

From the obtained photometric data, average values were calculated and reported to the numbers obtained for the samples under white LEDs (Version V_0) - considered as reference 100%. These percentage values are represented as histrograms in figures 5 A and B.

It is to be emphasised that the experiments from this work consist of a step regarding the examination of the germination percentage evaluated in the 10^{th} day of germination and of studying the ultrastructure aspects in the 14^{th} day of germination under different light in septic regime. The experiments concerned with the determination of the plantlet organs growth and assimilating pigments content were performed on samples exposed to both septic and aseptic vitroculture conditions.

Results and discussions

As it can be seen in Table 1, the data regarding the percentage of germinated seeds– recorded in the 10^{th} day of germination – indicated that under natural light (Version V_{00}) and green light LEDs (V_3), 99% of seed germination was achieved and the LEDs white light induced 98% germination (V_0). The lowest germination percentage – under 91% - was recorded for the seeds under red light (V_1), 95% under blue light (V_4) and 96% under yellow light (V_2). From what is was presented, only the red light produced a 9% inhibition of the *Robinia pseudoacacia L.* seeds germination.

At 14 days from germination, the plantlet cotyledons from each version (V_0 – V_4) were detached to take fragments for fixation for electronmicroscopy studies and to perform transversal sections through this organ. The most representative electronmicrographs are shown in Figures 2 and 3.

From the examination of these images results that at this age, most of the cells had in their vacuolar content various highly electrondense, corpuscular deposits. This suggests the extent to which the endocellular reserve substances are metabolised or not by the growing embryo. It should be mentioned the fact that as the plantlets grow, the storage substances from the cotyledons are exhausted and finally the cotyledons turn yelow and fall.

From previous studies (Cachiţă and Crăciun, 1990) it resulted that in the plant cells vacuolar content there is a tendency to form corpuscules containing anthocyanins (colored in red in acid pH or blue in basic pH) or that different vital dyes administered from the exterior can be included.

Such corpuscles have a liquid crystall type structure (called liposomes or recently nanoparticles). The liquid crystalls can be generated from plant phospholipids, for example soy lecithin (Cachiţă, 1975, 1981 and Cachiţă and Gergely 1990). These formations are of great interest for researchers mainly for their use for medical or cosmetic purposes.

Therefore, in the case of the experiments performed in this work it was considered that in the vacuolar content of the cotyledon cells of *Robinia pseudoacacia L.,* same as in soy seeds, probably there are phospholipids that as the embryo grows, are gradually exhausted and the vacuolar content „clarifies". The cotyledons even if they are green and photosynthesise, their functions gradually stop, they turn yellow and fall.

As it can be seen in Figures 2 and 3, during the first 14 days of germination, in the vacuolar content of the *Robinia pseudoacacia L.* cotyledons cells, transformations of the compounds from the vacuoles occur. Thus, the epidermis of the cotyledons of the germinated plantlets grown under white LED light (V_0) or yellow (V_2) has cells that have in their vacuolar content a very fine suspension (Fig. 2A and 2 E) suggesting that these cotyledons function normally.

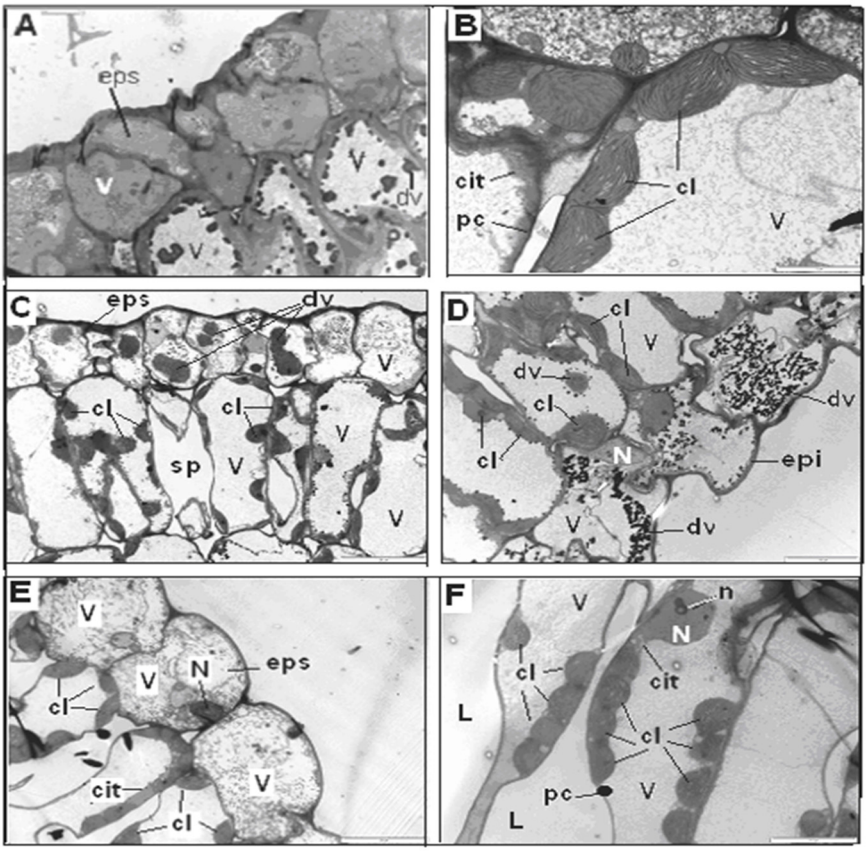

Figure 2. A – F. Transmission electronmicrographs showing the ultrastructure of the cells of *Robinia pseudoacacia L.* cotyledons from the 14 days old plantlets taken from the seeds germinated in plastic containers by placing on filter paper moistered with tap water and exposed to LED light: Fig. 2 A and B – white light – V0; Fig. 2 C and D – red light V1; Fig. 2 E and F – yellow light V2 (abbreviations: cl – chloroplasts; cit – cytoplasm; dv – vacuolar deposits; eps – upper epidermis; epi – lower epidermis; G – air gap; N – nucleus; n – nucleolus; pc – cellular wall; sp – intercellular space; V – vacuole)

The sub-epidermal and deeper cells at the same experimental versions also show the specific structures and the vacuolar content is clear; in the cytoplasm, chloroplasts with a normal aspect can be seen (Fig. 2 B and E – F).

In the case of *Robinia pseudoacacia L.* plantlets exposed to red light (Fig. 2 C and D) or green (Fig. 3 A and B), (versions V_1 and V_3), the epidermal cells of the cotyledons have in the vacuols quite large corpuscular deposits, electrondense, some of them being aggregates, or in section appearing as plates that occupy the whole surface of the vacuole (Fig. 3 B) have normal structures and have well defined chloroplasts.

In the case of the *Robinia pseudoacacia L.* plantlets exposed to blue light (version V_4, Fig. 3 C and D) both, the epidermal and underlying cells have in their vacuolar content very fine particles, in suspension; the chloroplasts are present but are small and look underdeveloped.

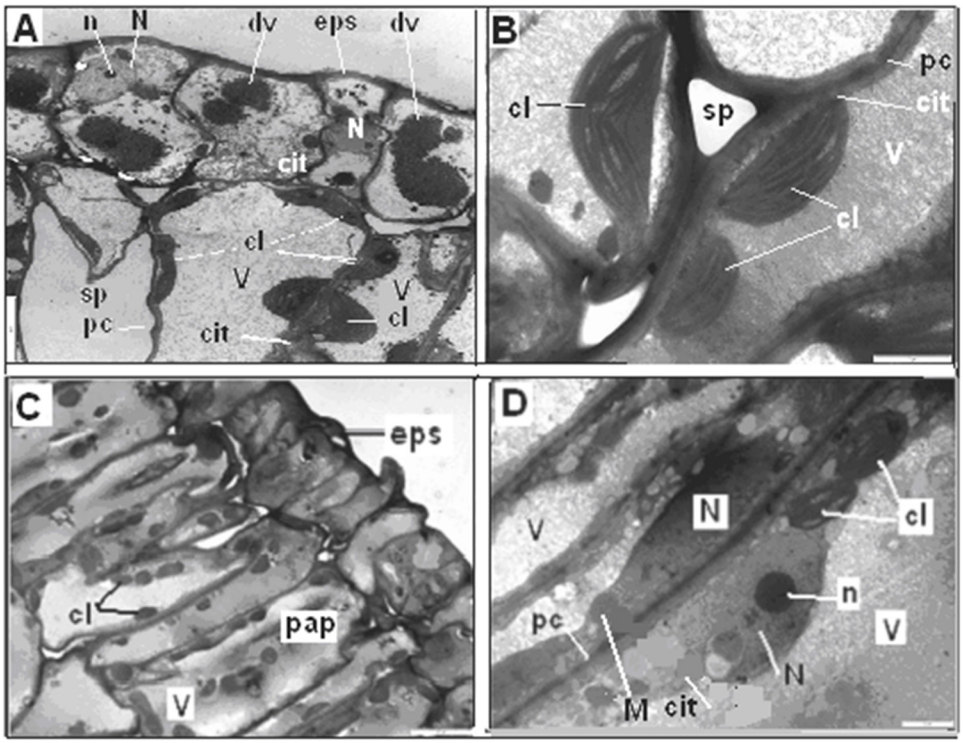

Figure 3. A – D. Transmission electron microscopy aspects identified in the transversal sections through the *Robinia pseudoacacia L.* 14 day old cotyledons from the seeds that have been germinated in plastic containers on filter paper periodically moistened with tap water and exposed 16hrs/day to LED light: version V_3 green LEDs (Fig. 3 A and B) and version V_4 blue LEDs (Fig. 3 C and D) (abbreviations: cl – chloroplasts; cit – cytoplasm; eps – upper epidermis; dv – vacuolar deposit; pap – palisade assimilating parenchyma; M – mitochondrion; mc –cotyledonary mesophyll; N – nucleus; n – nucleolus; pc – cellular wall; sp – intercellular space; V – vacuole).

The presented aspects led to the conclusion that the white and yellow LED lights have a positive influence on the structure, normalising processes and cotyledon functions. This is also reflected in the presented electromicrographs and in the driving of the *Robinia pseudoacacia* L. plantlet growth. This results from the biometric mesaurements done in the 40st day after germination and shown in Figure 4. Thus, the biometric measurements regarding the embryonary rootlet size, the hypocotyl and epicotyl (where was the case – Fig. 4) summed up give the size of the whole plantlet. Such measurements were performed on the plantlets grown both in plastic containers in septic conditions (Fig. 4 A) and „*in vitro*" in aseptic conditions (Fig. 4 B).

To the median value obtained for the plantlets exposed to white light (control 100%), version V_0, all the other averaged values for the different experimental versions (different color LEDs - versions V_1 – V_4), were reported.

The differences in growth recorded in the case of the measurements done in the 40st day of germination (Fig. 4 A and B) pointed out the fact that, depending on the size of the plantlets on which biometric measurements were performed, at the samples exposed to white LEDs (version V_0 – control 100%), exposing the plantlets (under septic culture regime) to yellow light (version V_2) has stimulated by 51% the growth in lenght of the stemlet and only by 5% of the rootlet and by 8% the elongation of this organ in the *Robinia pseudoacacia L.* vitroculture maintained in a similar antiseptic regime whereas the growth of the stemlet was only stimulated by 2.5%.

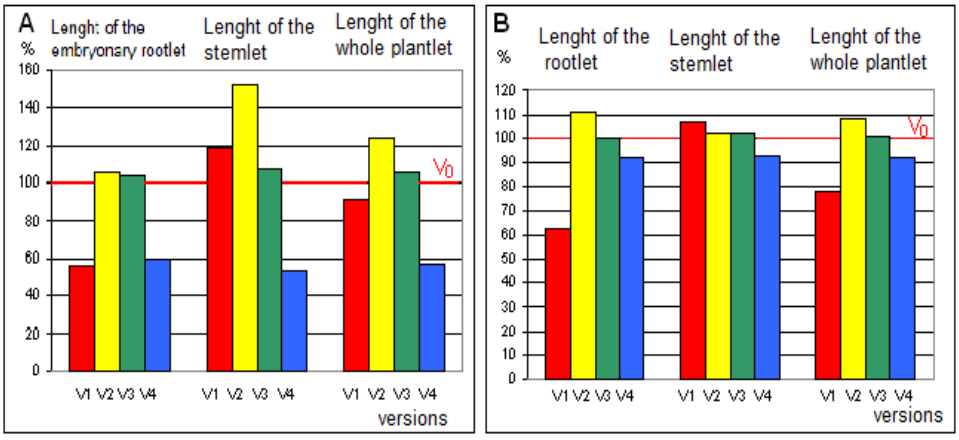

Figure 4. A and **B**. The growth of the *Robinia pseudoacacia L.* plantlets and organs from the germinated seeds after 40 days in plastic containers on filter paper moistered with tap water (4A) or vitro cultivated (4B) and exposed to LED with light of different wavelenghts (versions: V_0 -white light; V_1 - red light; V_2 – yellow light; V_3 – green light; or V_4 – blue light). The histograms show – in percentage values – the data recorded for different versions and organs compared with similarly marked parameters for the control (V_0) considered to be 100%.

In turn, the LEDs red light (version V_1) has inhibited by 45% the growth of the *Robinia pseudoacacia L*. plantlet embryonary rootlet that were maintained in an experimental aseptic regime and by 38% in the experiments done in vitro culture regime (Fig. 4 B). The growth of the stemlet size of the samples from aseptic conditions, under red light was stimulated by 20% whereas in aseptic conditions the results were closed to the control values (Fig. 4 B).

It is also interesting that the LEDs blue light has inhibited by 40% the growth in lenght of the embryonary rootlet (Fig. 4 A) and by 52% the growth in size of the stemlet in the samples from the experiments performed in septic regime. In turn, in the aseptic regime the LEDs blue light has inhibited only by 8% the growth of the *Robinia pseudoacacia L*. organs. This suggests that either a differential „filtration" of the radiation through the glass and culture medium occured or a countering of the positive or negative effects from the containers throught the components present in the culture medium.

In the experiment performed in septic conditions, the size of the whole stemlet was determined mainly by the size of the hypocotyl; the LEDs yellow light (V_2) has stimulated the elongation of this organ whereas the blue light (V_4) has strongly inhibited the growth of all organs and consequently the whole plantlet. The LEDs green light (V_3) has maintained the growth at the same level with the one observed in the plantlets exposed to white light (V_0) (Fig. 4 A).

In the case of a similar experiment performed in vitroculture regime (Fig. 4 B), the differences in growth compared with the control (V_0) were smaller only the inhibition of the rootlet growth in the presence of red light has reduced the size of the whole plantlet by 21% and the blue light by 8%.

Regarding the content in assimilating pigments of the hypocotyl, cotyledons, epicotyl and of the folioles (where they developed) (Fig. 5 A and B), it can be ascertained that in all the types of analysed pigments both green (chlorophyll **a** and **b**) and carotenoids, a strong decrease in their level was recorded – both in septic and aseptic regime – especially under green light. Thus, negative values were recorded down to - 50% for chlorophyll **a**, -72% for chloropyll **b** and -68% for carotenoid pigments, compared with the values obtained for the control (white light, 100%) in both septic (Fig. 5A) and aseptic regime (Fig. 5 B).

In the case of the carotenoid pigments under yellow light (V_2) a 9% increase was recorded. It is interesting that in the samples from the aseptic medium, the LEDs green light has reduced by about 60% the level of all pigments regardless of the type of the pigment, and in the septic regime the recorded values were – 72% for chlorophyll **b** and – 68% for the carotenoids. It was also surprising that the LEDs blue light has inhibited the growth of the *Robinia pseudoacacia L.* organs but in the same time has favored – compared with the red and green light- (and in the case of the samples from vitrocultures even with the values recorded for the yellow light) the synthesis and accumulation of green pigments in the stemlets and leaflets. The red light inhibited the accumulation of assimilating pigments in these organs (Fig. 5 A and B).

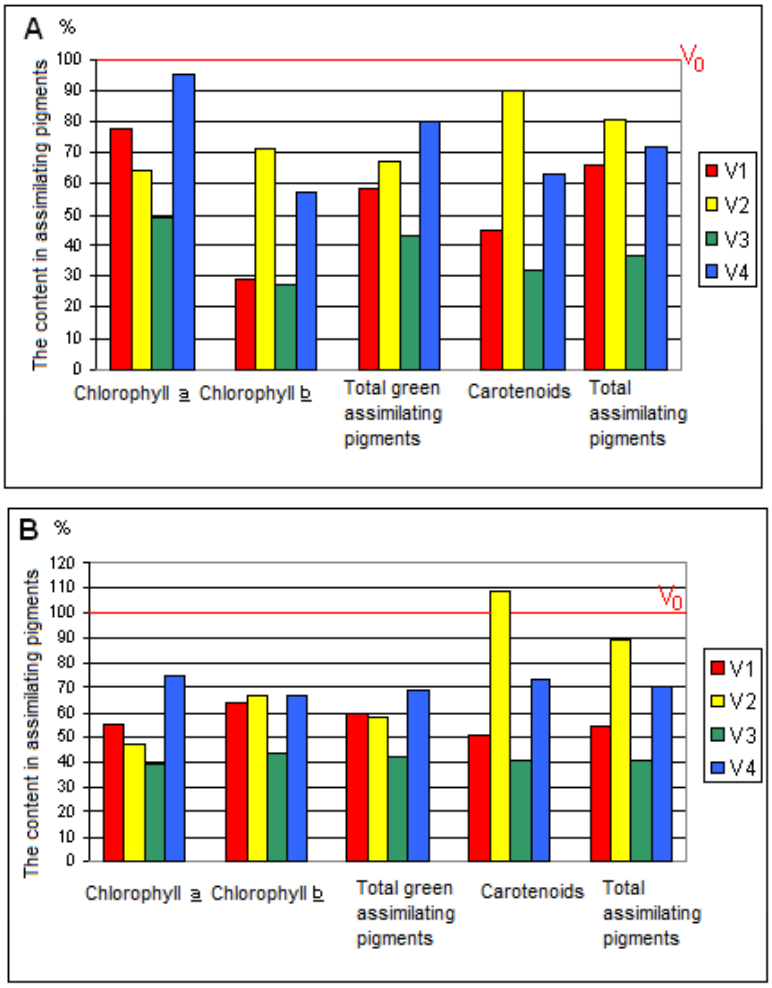

Figure 5. A and B. The content in assimilating pigments in the aerial organs of *Robinia pseudoacacia L.* plantlets (cotyledons, epycotyl and leaflets) from the stemlets of the plantlets from the seeds embryos at 40 days after germination; Fig. A – in plastic container son filter paper moistered with tap water, Fig. B – vitrocultivated and exposed 16 hrs/day to ultrabright LED with light of different wavelenghts. Experimental versions: V_0 – control – plantlets exposed to white light – values considered to be 100%; V_1 – plantlets exposed to red light; V_2 – plantlets exposed to yellow light; V_3 – plantlets exposed to green light and V_4 – plantlets exposed to blue light

Conclusions

Comparing the germination of *Robinia pseudoacacia L.*seeds – placed for germination in plastic containers on filter paper moistened with tap water- and the growth of the plantlets from their embryos for 40 days under 16hrs/day lighting with white, red, yellow, green or blue light LEDs, it was concluded that the white light was the most efficient to obtain the longest plantlets. Only under yellow light, the stemlets were 48% longer than those grown under white light. Same experiment performed *in vitro* culture regime has given far more uniform results regarding the growth of the plantlets under different light color LEDs lighting. This phenomenon is probably due to the filtration of light by the glass covering the vitroculture containers evening out the reaction of the plantlets. The LEDs white and yellow light, in the plantlets germinated in plastic containers, on filter paper moistened with tap water, at the cotyledons level, led to a faster consumption of the reserve substances from the epidermal cells and the cotyledonary storage parenchyma. A slowing down of these processes was observed mainly in the *Robinia pseudoacacia L.* cotyledons under red, green and blue light, in their cells persisting a blockage of the vacuolar content with black colored osmiophile substances that become strongly electrondense and generating corpuscle that are characteristic for the liquid crystall type formations of phospholipidic nature. The white light has also provided the highest level of green and carotenoid pigments in the „above the ground" part of the plantlets. The other wavelenghts have inhibited the formation and accumulation of assimilating pigments in the samples from both septic and aseptic culture regime.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, 2, 2015 (p. 31-38)

Deuterium depleted water effect on *Euphorbia canariensis* L. micropropagation

Adriana Petruş-Vancea [1,✉] and Andrei Fordon [1]

SUMMARY. It has been studied the effect of deuterium depleted water (DDW) (with 25 ppm D) on the culture of *Euphorbia canariensis* L., to transfer *ex vitro*. Distilled water (DW) (with 155 ppm D) from the culture medium of *Euphorbia* was replaced with DDW. Hypothesis of the present experiment was that presences of DDW in culture medium inhibit callus formation, stimulates rhizogenesis and optimizes *ex vitro* acclimatization of *in vitro* cultivated plantlets. After 18 months of *in vitro* culture - when the plantlets were transferred into soil - only those grown on medium prepared with distilled water showed an intense callusogenesis process at the level of the areolas and at the apical and basal poles. DDW stopped callus formation at this species and permitted rhizogenesis. Also, the chlorophylls *a*, *b* and carotenoids extracted in N, N-dimethylformamide - were increased in the presence of DDW in culture medium. At the end of the acclimatization, *ex vitro* survival rate of the plantlets from medium prepared with DDW, increased at 92%. *In vitro* plantlets cultivated on DW containing medium could not be transferred into a septic medium of life due to the lack of a radicular system.

Keywords: acclimatization, chlorophylls, deuterium, *Euphorbia canariensis*, micropropagation

Introduction

Euphorbiaceae species are an important source of dyes, oil, furniture, and rubber. From their latex extract are obtained diterpenoids used in pharmaceutical and chemical products that are useful for regulating blood vessel walls. *Euphorbia* is a genus of ecological, aesthetic and economic importance, due to its role in obtaining fuel and also as parent stock to other more sensitive species of the *Euphorbiaceae* family (Marco *et al.,* 1997; Jakupovic *et al*., 1998; Gaal *et al*., 2013).

In vitro multiplication and conservation techniques have economical and ecological importance. This type of cultivation and conservation is fast, efficient, modern and widely used. Germplasm cryopreservation is important for maintaining

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the genetic diversity of rare plants. Improved cell and tissue culture technologies would help producing the *in vitro* active compounds with better productivity, without decreasing natural resources (Kondamudi *et al*., 2009).

There are some opinions in the field of plant tissue culture suggesting that multiplication of *Euphorbiaceae* can and should be more developed (Balotis and Papafotiou, 2003; Toress, 2004; Bidarigh and Azarpour, 2013).

DDW has 25 ppm deuterium, comparatively with DW which has approximately 155 ppm D. Pacific Ocean water or Antarctic Region water has a lower deuterium contend (89 ppm) (Ignatov and Mosin, 2013). The authors studied the particularities of deuterium and the conditions of primary hydrosphere and tried to identify the origin of life and living matter.

Deuterium depleted water began to be used extensively for its effects to inhibit mitotic division of animal cancer cells, but it was also used to observe the influence on different plant species cultured *in vitro*. Deuterium depleted water (DDW) was known from our previous researches for having, in some cases, the effect of timing the growth (Cachiță *et al*., 2002; Butnaru *et al.*, 2004; Petruș–Vancea, 2011 a; Petruș-Vancea *et al*., 2013).

The chloroplast ultrastructure and assimilating pigments content at sugar beet *in vitro* formed leaflets was normal in the presence of deuterium depleted water in culture medium, because this treatment prevented hyperhydricity (Cachiță *et al.,* 2009).

At *Robinia pseudoacacia* var. Oltenica, deuterium depleted water rejuvenated the plant tissues by inhibiting basal callus formation, improving micropropagation, the method providing biological material in case of global climate change (Corneanu *et al.*, 2010).

In vitro studies on this species of *E. canariensis* are few (Tripon *et al.,* 2015), which is why we intend to realize this experiment. In the present study we propozed to identify the effect of deuterium depleted water on *E. canariensis* inoculs, to improve rhizogenesis process by stopping callusogenesis but also by stimulating the assimilating pigments synthesis for optimizing their adaptation to the septic medium of life after the *ex vitro* transfer.

Materials and methods

Initial *plant material* consisted in *Euphorbia canariensis* seeds germinated on standard basal medium Murashige-Skoog (1962) with Gamborg *et al*. (1968) ½ vitamins, solidified with agar-agar, without growth regulators and aminoacids, with 20 g/l sucrose (Tripon *et al*., 2015) instead of 30 g/l as in the original recipe.

Replication – using 0.5 – 0.7 cm inocula, respectively 3 nods – was made at 120 days from *in vitro* initiation (Fig. 1). The cuttings were placed on medium prepared with distilled water (control) (V_0) or with deuterium depleted water (DDW) (V_1) (Table 1).

Research design is presented in table 1.

After inoculation, the culture was incubating at 22-23 ˚C temperature, lighting with 1700 lx and 16/24h photoperiod.

Assimilating pigments measurement. Assimilating pigments measurement, respectively chlorophylls (Chl *a*, *b*) and carotenoids (Car), was made by extracting them from plantlet stem in N, N-dimethylformamide (DMF) 99.9%, according to the method developed by Moran and Porath (1980). Assimilating pigments quantitative values were obtained by using the absorption coefficients (Wellburn, 1994), measured at 664 nm for determination of Chl *a*, at 647 nm for Chl *b* content and 480 nm for carotenoids. Spekol 11 type, Carl Zeiss Jena spectrophotometer were used.

Table 1.

Experimental protocol concerning multiplication, conservation and acclimatization of *E. canariensis* (DW – distilled water; DDW – deuterium depleted water; G –Gamborg vitamins; MS –Murashige-Skoog basal medium).

Experimental steps	**Plant material**	**Culture Vessels**	**Culture medium**	**Culture and measurement period**	**Measured**
Step I *in vitro* initiation (first culture)	Seeds	7/2 cm size	MS ½ – G ½- DW	4 months	stem length nods number
Step II *in vitro* conservation	cuttings	7/2 cm size	V_0: MS – G – DW V_1: MS – G – DDW	18 months	roots length roots number total plantlets length (stem + roots) chlorophylls carotenoids
Step III *ex vitro* acclimatization	Plantlets with roots (from V_1)	pots placed in incubator	nutritive soil : sand 1:1	1 month	survival rate

Ex vitro acclimatization of *E. canariensis* was done at 18 months from *in vitro* culture subcultivation. Vitroplants were placed in individual containers. Since only vitroplants subcultured on media with deuterium depleted water (V_1) developed a vigorous root system, only they were transferred in the septic medium. Vitroplantlets without roots or a poorly developed root system, as those of control group (V_0), had no chance of survival in the *ex vitro* acclimatization. Lack of roots of these vitroplantlets was caused by the presence of callus at the base of inoculs. *Ex vitro* acclimatization conditions were identical to those held in *in vitro* incubating period cultures.

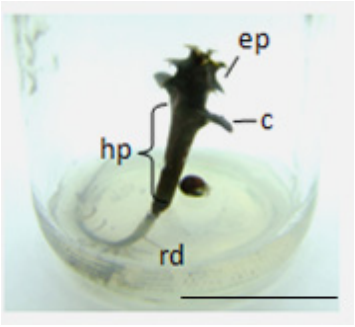

Figure 1. Aspect of *E. canariensis* seed plantlet, to 120 days from initiation (bar mean 1cm) (c – cotyledon; ep – epicotyl; hp – hypocotyl; rd – radicle).

Measurement results were mathematically (average, standard deviation and dispersion) and statistically (*t* test) processed using SPSS 16.0 for Windows Program. For each experimental variant three measurements were performed.

Results and discussions

At 18 months of in vitro culture, the total plantlets length did not have high growth (Table 2). At samples where distilled water was replaced with deuterium-depleted water (V_1) the rhizogenesis was intense.

Instead, the stem length was inhibited by the presence of deuterium depleted water in the culture medium (Table 2), as in the case of *Asparagus* species and *Beta vulgaris, Drosera rotundifolia, Cymbidium hybridum* (Petruş-Vancea, 2011 a, b).

Table 2.

E. canariensis plantlets statistical data processing at 18 months after inoculation on different culture media, namely: V_0 – MS culture medium prepared with distilled water (DW); V_1 - MS culture medium prepared with deuterium depleted water (DDW).

V_0-DW (control)				
Statistic data	**Roots length**	**Roots number**	**Stem length**	**Plantlets size**
X ± Sx	0.21±0.25	0.42 ±0.51	2.14±0.31	2.35±0.40
S^2	0.06	0.26	0.10	0.16
V_1-DDW				
X ± Sx	1.58±0.51	2.05±0.52	1.55±0.37	3.13±0.62
±d	+0.79	+1.16	-0.64	+0.15
S^2	0.26	0.27	0.14	0.39
P	**	**	*	**

Note: $\overline{x} \pm S_{\overline{x}}$ [average (cm) ± standard deviation], ±d [difference against control (cm)], s^2 – dispersion, p – significance: * - significantly ($p < 0.05$), ** - very significantly ($p < 0.01$).

In the present study, at 18 months of subcultures, only the plantlets grown on distilled water containing medium showed an intense process of callusogenesis at the level of the areolas. Callusogenesis process affected 27% of control lot inoculs (V_0). The deuterium depleted water (25 ppm D) stopped callus formation at this species and permitted rhizogenesis (Fig. 2 b and c), as in the case of sugar beet (Cachiță *et al.*, 2008; Petruş - Vancea and Cachiță, 2011).

Stopping callus formation is induced by DDW, because this type of water inhibits cell division, which is why it is used in the treatment of human cancer.

Callus lack at basal level of inoculum favored the formation of *in vitro* roots. A well-developed root system subsequently optimized adaptation of plantlets in septic medium, in *ex vitro* acclimatization stage.

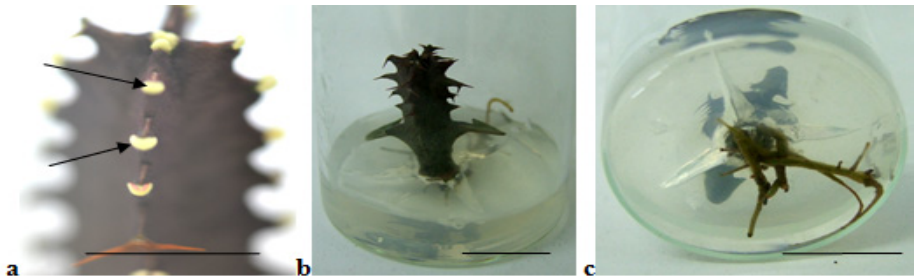

Figure 2. Growth of *E. canariensis* plantlets at 18 month on medium MS-G, prepared with distilled water (V_0 - control) (a) (arrows indicate callus formation at areola level), respectively on MS-G medium prepared with deuterium depleted water (V_1) (b and c) (bars indicate 1 cm).

Although some plantlets showed a brown skin, others were green as you can find in figure 3 (a and b), in the cross section we have seen that both groups had viable tissues (Fordon and Petruş-Vancea, 2015), even if the callus was stopped due to increased stem length. At other family, namely *Cactaceae*, this phenomenon also occurs under natural conditions of life, the appearance of necrotic stem doesn't show its lack of viability. Basically it is an adaptation to a harsh environment in which they live.

Assimilating pigments from stem, measured at 18 month of culture, showed that there was no significant difference between plantlets growth on DDW medium compared with those cultivated on medium prepared with DW, except carotenoids with a notable increase (74% percent) of its quantitative value at the lot containing DDW (Table 3). Also, the difference between the ratios Chl *a*/*b* was statistically insignificant at both lots of plants tested.

At 30 days of a*cclimatization*, post-acclimatization survival percent was calculated and the result was 92% (Fig. 4), where 100% represents the total number of vitroplantlets transferred into soil.

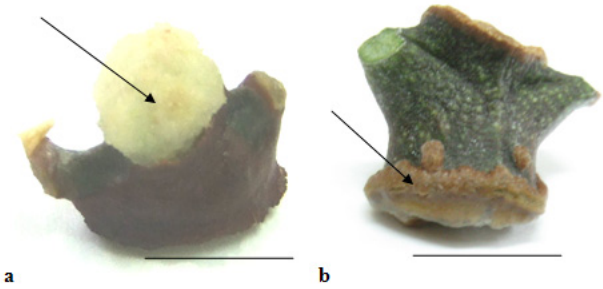

Figure 3. Callus formation (indicate by arrows) at apical level (a) and basal level (b) of *E. canariensis* inoculs, at 18 months of culture on MS-G medium prepared with distilled water (V_0 - control) (bars means 1 cm).

Table 3.

Average values of assimilating pigment quantities determined in extracts prepared from stem of *E. canariensis,* at 18 months of culture on MS-G medium prepared with distilled water (DW) (V_0 - control) or MS-G medium prepared with deuterium depleted water (DDW) (V_1).

Experimental type / Assimilating pigments	MS-G with DW (*control*)	MS-G with DDW	
	average (µg/g)	*average (µg/g)*	*% toward control*
Chl *a*	1.49±0.01	1.70±0.02 ns	114.1 %
Chl *b*	1.86±0.04	2.08±0.03 ns	111.8 %
Total Chl	3.35	3.78	112.8 %
Chl *a/b*	0,801	0,817	102,03%
Carotenoids	0.56±0.06	0.98±0.08*	174.6 %
Total assimilating pigments	3.91	4.76	121.7 %

Note: ns – no significant $p>0.05$; * = $p < 0.05$.

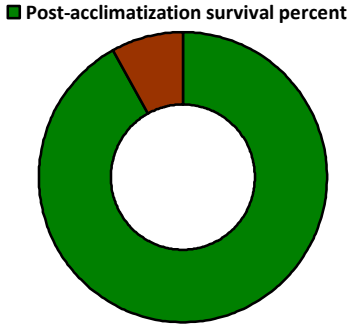

Figure 4. Post-acclimatization survival percent of *E. canariensis* plantlets from *in vitro* medium prepared with deuterium depleted water (V_1).

Thus, we have demonstrated that the initial hypothesis is true. In previous experiments we have increased the *ex vitro* survival rate of *Chrysanthemum* and *Saintpaulia ionantha* by watering their aerial parts, after transferring into soil, with DDW (Petrus - Vancea *et al.,* 2003; Petruş –Vancea *et al.,* 2013).

The results were positive in terms of the post-acclimatization survival rate, growth and ability to adapt to environmental conditions. All these results, obtained over the years, are in consensus with our presented in this study.

Conclusions

Deuterium depleted water (with 25 ppm D) stimulated the *in vitro* rhizogenesis of *E. canariensis*, because it eliminated callus formation at the basal level of plantlets and increase in stems the quantity of assimilating pigments, especially of carotenoids.

E. canariensis plantlets cultivated *in vitro* on medium prepared with deuterium depleted water (with 25 ppm D) had a good *ex vitro* survival capacity.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, 2, 2015 (p. 39-60)

The karstic lake Iezerul Ighiel (Transylvania, Romania): its first limnological study

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SUMMARY. The present study represents the first limnological survey made in Lake Iezerul Ighiel, considering the most important biotic communities in the water column (phyto- and zooplankton) and from the bottom (phyto- and zoobenthos). No data was collected on ichthyofauna, since the species existing in the lake were introduced. The lake was sampled in 2014 in three seasons, from three sampling sites located in different regions of the water body, having different characteristics. Physical and chemical parameters were also analysed. The biotic communities considered for the study depicted a generally good ecological status of the lake. However, moderate organic pollution, together with initial phases of eutrophication were indicated by the algal and invertebrate communities considered for the present study, both planktonic and benthic.

Keywords: algae, benthic invertebrates, diversity, ecological status, microcrustaceans

Introduction

Lake Iezerul Ighiel represents the largest karstic lake in Romania, located in the southern Trascău Mountains, on the upper course of the Ighiel River (Valea Iezerului); in the limestone plateau Ciumerna, at 940 m altitude (Gâştescu, 1971). Natural damming processes caused by landslides led to the formation of this oval shaped lake, 400 m long and 140 m wide, having a total volume of 225000 m^3, a maximum depth of about 9 m and an average one of 4 m (Decei, 1981; Mihăiescu *et al*., 2012). The lake has a few surface tributaries, usually temporary and short brooks; but numerous underground springs are located in the central and northern parts of the lake. The water level fluctuates with minimums in summer and winter periods, the lake being recharged during spring and precipitation events by torrents (Pop and Măhăra, 1965; Duma, 2009). Natural dominant vegetation from the catchment

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area consists in beech forests, mixed with hornbeam and fir (*Fagus* sp., *Carpinus* sp. and *Abies* sp.). Pines and spruces were later planted to consolidate the slopes. At present, natural grasslands and transitional regions are also characteristic to the region (Pop and Măhăra, 1965; Mihăiescu *et al*., 2012).

Lake Iezerul Ighiel is a Natural Reserve since 1969, now included in ROSCI0253: Trascău and ROSPA0087: the Trascău Mountains. The impacts that influence the lake are both natural (siltation) and human-induced (deforestation, tourism, poaching and domestic wastes). Since 1966 constant fish stocking is permitted in the lake, with various species like: *Salmo trutta fario, Oncorhynchus mykiss, Hucho hucho, Phoxinus phoxinus, Leuciscus cephalus, Cottus gobio,* even *Ctenopharyngodon idella* and *Aristichthys nobilis* (Decei, 1981), or *Cyprinus carpio* and *Carrassius auratus* at present.

The present limnological study focused on three important biotic communities characteristic to standing waters: phytoplankton, phytobenthos, planktonic microcrustaceans and benthic invertebrates.

Algae develop in all aquatic basins, each taxon having specific requirements and preferences to the environment (Brönmark and Hansson, 2005). Stenobiont forms represent accurate indicators of water quality in the ecosystems they inhabit (Bellinger and Sigee, 2010), that is why the Water Framework Directive 2000/60/EC states that planktonic and benthic algae should be used in assessing ecological status of water bodies.

Zooplankton organisms occupy a central position in aquatic food webs, responding quickly to any changes in the status of their environment, natural or human-induced (Suthers and Rissik, 2009). Microcrustaceans (cladocerans and copepods) represent the most important group of animal plankton in terms of biomass, but also due to the fact that they occupy different guilds: filter-feeders, herbivores or predators. Even if they are not included in the stadardized methods of the Water Framework Directive 2000/60/EC, planktonic microcrustacean communities can offer valuable information on water quality, based on their taxonomical composition and community characteristics (Moss *et al*., 2003; Haberman and Haldna, 2014).

Benthic invertebrates represent by far the most studied and diverse group of organisms in rivers, used to assess water quality (Giller and Malmqvist, 1998). In lakes, however, their importance decreases in favor of water column communities, due to the particularities of lentic habitats: greater depths, oxygen and light depletion near the bottom etc. Even though the Water Framework Directive requires assessments of macroinvertebrates in lakes, their use is extremely difficult (Moss *et al*., 2003).

Previous studies from the area focused on geographical and abiotic parameters from the Lake Iezerul Ighiel (Pop and Măhăra, 1965; Decei, 1981; Duma, 2009; Mihăiescu *et al*., 2012). Limnological studies considering biotic communities were not found, except for the inventory of stocked fish species from the lake (Decei, 1981). However, Negrea (1983) cited two cladoceran species from the karstic Lake

Ighiel - Trascău *(Daphnia rosea* and *Pleuroxus truncatus*), while Damian-Georgescu (1963, 1966, 1970) cited three copepod species *Eucyclops serrulatus, Acanthodiaptomus denticornis* and *Canthocamptus staphylinus*.

Thus, the aim of the present study was: (1) to characterize Lake Iezerul Ighiel from the point of view of three biotic communities inhabiting standing waters: algae, microcrustaceans and benthic invertebrates, considering their taxonomic composition, structure and diversity; (2) to assess the ecological status of Lake Iezerul Ighiel, reflected by the three biotic communities under study, since human impacts should be generally low in the area.

Materials and methods

The samples were collected in 2014 from three sampling sites, in 10th of May, 15th of August and 1st of November 2014 (Figs. 1 and 2; Table 1). The sampling locations were chosen to be different, site 2 was sampled by boat, while the other two by wading, from the lake banks. Only benthic invertebrates were collected at site 3, due to the shallow water depth. The main characteristics of the sampling sites were depicted in Table 1.

The following physical and chemical parameters were recorded in the field or were measured subsequently in the laboratory: water temperature, dissolved oxygen (measured *in situ* with the portable meter YSI 52), pH and water conductivity (measured in the laboratory).

Plankton was collected using a 20-40 μm mesh size net in case of algae and a 50-55 μm mesh size one for microcrustaceans. Benthic algae 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 in 4% formaldehyde. Identifications were made to the species level in case of algae (Krammer and Lange-Bertalot, 1986, 1988, 1991; Ettl, 1983) and microcrustaceans (Negrea, 1983; Damian-Georgescu, 1963; 1966; 1970; Einsle, 1993; Janetzky *et al*., 1996), and to different taxonomical levels in case of benthic invertebrates (Sansoni, 2001).

Relative abundance for planktonic and benthic invertebrates, expressed in percentages, represented a usefull tool in order to illustrate the structure of their communities. In order to compare diversities in different microcrustacean samples, diversity profiles were made, using a method that defines a family of diversity indices, dependent upon a single continuous parameter: the so-called Renyi index, which depends upon a parameter alpha (Tothmeresz, 1995). On the diversity ordering plot, the curve on top represents the most diverse community, while intersecting diversity profiles are non-comparable.

Algal communities have been long used to assess the ecological status of their environment, in terms of organic pollution, saprobity and trophicity. To assess the trophic state of the lake, the following indices were used in the present study: alpha-eutrophicity index, beta-eutrophicity index (Oltean, 1977); Nygaard compound index (1949), the Q index of eutrophy (Järnefelt, 1951) and the diatom index (Stockner, 1972). The saprobic indicator values of certain algal species were considered, following Rott (1997), Hindak (1978), Sládeček (1973). The organic pollution index (Palmer, 1969) was also used.

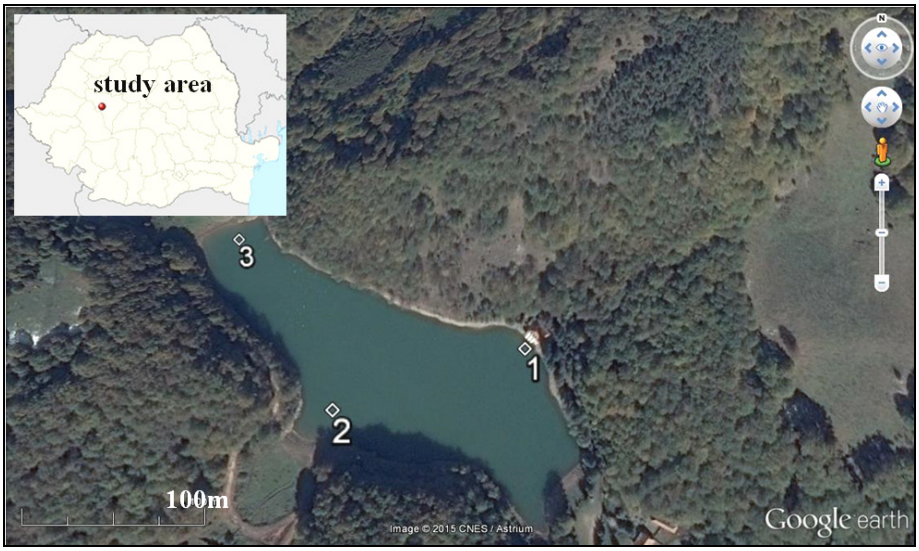

Figure 1. Location of Lake Iezerul Ighiel and the three sampling sites considered for the present study

Figure 2. View towards sampling site 1 in spring (left) and autumn (right), showing drastic drops in water level

The indication values of the most abundant microcrustaceans were considered. The species were sorted based on their frequency (the percentage of samples with the species) and on their average abundance in the samples. Cosmopolitan species were excluded, thus only species with indication values were selected, according to Sládeček (1973), Damian-Georgescu (1963, 1966, 1970), Negrea (1983).

Table 1.

Main characteristics of the three sampling sites considered for the present study (SP - spring; SU - summer; AU - autumn; al - algae; mc - microcrustaceans; bi - benthic invertebrates)

Site	Site code	Date of sampling	GPS	Samples	Location of sampling	Characteristics
1	SP1 SU1 AU1	10.05.2014 15.08.2014 01.11.2014	N46°10'51.1" E23°21'57.2"	al; mc	- north-eastern part of the lake, near the touristic cottage - samples taken from the cottage pier in spring and summer, and from the lake banks in autumn - maximum depth of sampling: 1 - 1.5 m	- highly variable water levels: extremely high in spring (when surplus water was channeled on a tributary) and extremely low level in autumn, due to severe drought - substratum near the banks: rocks, silt, no submerged macrophytes
2	SP2 SU2 AU2	10.05.2014 15.08.2014 01.11.2014	N46°10'47.2" E23°21'48.4"	al; mc; bi	- south-western part of the lake, opposite to site 1; a tributary enters the lake there - near the maximum depth region of the lake - maximum depth of sampling: 3m for boat samples; 1 m for wading samples	- variable water levels: spring samples collected from the lake banks included a flooded area, covered with herbaceous vegetation (usually above water levels) - substratum near the banks: rocks, silt, submerged macrophytes
3	SP3 SU3 AU3	10.05.2014 15.08.2014 01.11.2014	N46°10'57.9" E23°21'44.3"	bi	- north-western part of the lake - maximum depth of sampling: 1 m for wading samples	- variable water level: spring samples collected from the lake banks included a flooded area, covered with herbaceous vegetation (usually above water levels) - substratum near the banks: rocks, silt, submerged macrophytes

Biotic indices based on zooplankton species are rare. The one chosen for this paper represents the ratio between large cladocerans (C_l) and the density of all cladoceran species (C_t) (Moss *et al.,* 2003). The values of this index indicate five water quality classes, according to the Water Framework Directive: when the values are lower than 0.2, the water quality is bad or poor; when the values vary between 0.2 and 0.5, the water quality is moderate; if they exceed 0.5, the water quality is good or high. The explanation resides in the fact that there is a greater proportion of large cladocerans in lakes at high ecological status, finding refuges from fish predation among the plant communities (Moss *et al.,* 2003).

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.

Multivariate analyses were performed using XLSTAT Version 2015.3.01.19199, while diversity profiles were made using PAST version 2.17c.

Results and discussion

Physical and chemical parameters

Several physico-chemical parameters measured in the field or in the laboratory differentiated the three sampling locations according to their seasonal variation (Fig. 3). Thus, higher temperatures were recorded in summer 2014, a normal situation in the northern hemisphere, while lower dissolved oxygen saturations were recorded in autumn, probably due to the input of leaf material coming from the deciduous forest surrounding the lake, since high levels of organic carbon coincide with a lowering of dissolved oxygen concentrations.

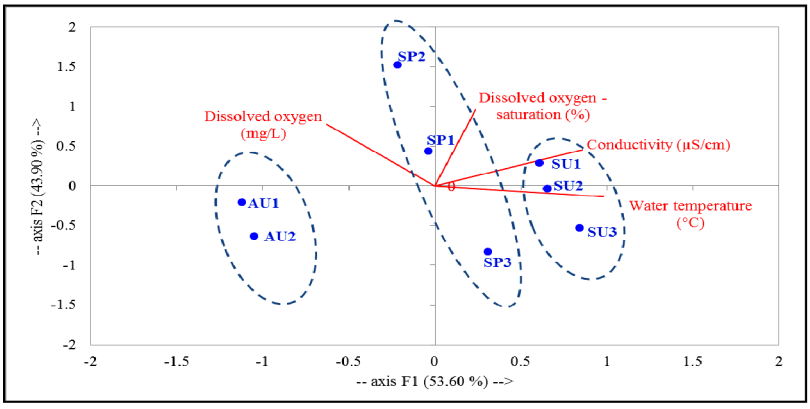

Figure 3. Principal Component Analysis (PCA) biplot (axes F1 and F2: 97.50 %) for the three sampling sites and three seasons considered for the present study, and their aggregation based on physical and chemical parameters (abbreviations as in Table 1).

Conductivity values varied around 300 µS/cm, except for autumn samples, where they decreased by half. pH was above 8, typical for karst areas.

According to Mihăiescu *et al*. (2012), the maximum concentration of Total Phosphorus (TP) found in a survey conducted in 2010 was 0.010 mg P /L, thus showing oligotrophic conditions from this point of view. However, Total Nitrogen (TN) values from the same study depicted a tendency towards eutrophication.

Algal taxonomical survey

A total number of 152 algal taxa was identified at sampling sites 1 and 2 in 2014, belonging to the following phyla: Cyanophyta, Chlorophyta, Euglenophyta, Chrysophyta, Bacillariophyta and Dinophyta. Bacillariophyta dominated the algal communities, reaching a percentage of 69%, followed by Chlorophyta (24%), Chrysophyta (3%), Cyanophyta (2%), Euglenophyta (1%) and Dinophyta (1%).

Only planktonic samples were taken in spring 2014, while epiphytic and epilithic samples were added in summer and autumn 2014. Hence, the total number of taxa from the three seasons differed, from 36 in spring to 100 and 105 in summer and autumn, respectively (Fig. 4). Table 2 depicts the list of algal taxa identified in the study area, in summer and autumn 2014 plankton and periphyton considered together.

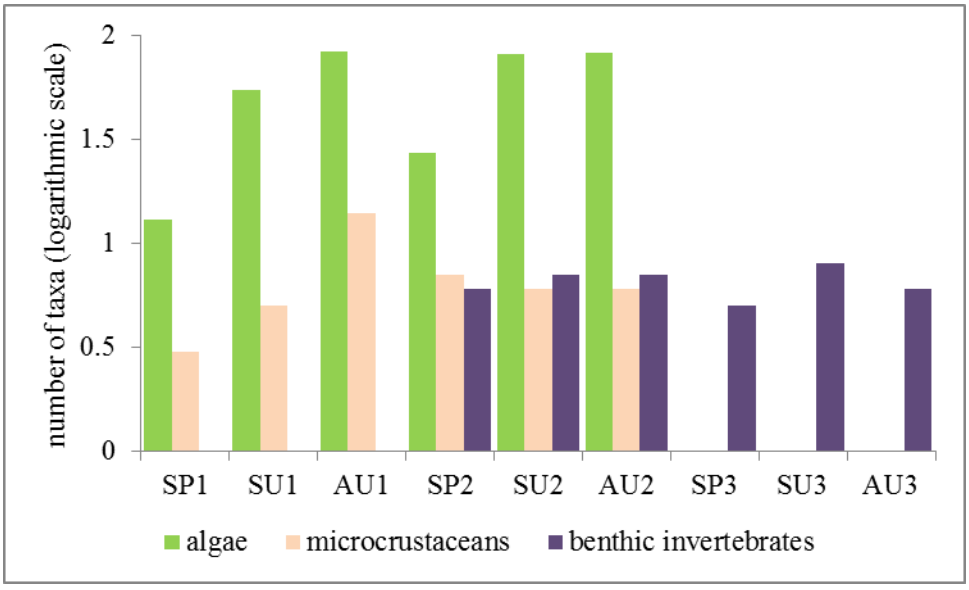

Figure 4. The number of algal, microcrustacean and benthic invertebrate taxa found in Lake Iezerul Ighiel (sampling sites as in table1; logarithmic transformation based on log10).

Table 2.

Algal taxa identified in two sampling sites from Lake Iezerul Ighiel
(sampling sites as in Table 1)

TAXA	SP1	SP2	SU1	SU2	AU1	AU2
Phylum Cyanophyta						
Merismopedia glauca (Ehrenberg) Kützing 1845			+		+	
Oscillatoria limosa C.Agardh ex Gomont 1892			+	+	+	+
Oscillatoria redekei Goor 1918						+
Phylum Dinophyta						
Ceratium hirundinella (O.F.Müller) Dujardin 1841			+	+		
Phylum Euglenophyta						
Trachelomonas pulcherrima (Playfair) Popova 1955						+
Trachelomonas volvocina (Ehrenberg) Ehrenberg 1834						+
Phylum Chrysophyta						
Dinobryon bavaricum var. *medium* (Lemmermann) Krieger			+	+		
Dinobryon divergens O.E.Imhof 1887			+	+		
Epipyxis natans (Ruttner) Hilliard et Asmund					+	
Mallomonas crassisquama (Asmund) Fott 1962					+	+
Uroglena americana G.N.Calkins 1892		+	+	+	+	+
Phylum Bacillariophyta						
Achnanthes biasolettiana Grunow in Cleve & Grunow 1880			+	+	+	+
Achnanthes flexella (Kützing) Brun 1880				+	+	+
Achnanthes lanceolata (Brébisson ex Kützing) Grunow in Van Heurck 1880		+	+	+		
Achnanthes minutissima Kützing 1833		+	+	+	+	+
Amphipleura pellucida (Kützing) Kützing 1844						+
Amphora lybica Ehrenberg 1840			+		+	+
Amphora ovalis (Kützing) Kützing 1844					+	+
Anomoeoneis vitrea (Grunow) R.Ross in Patrick & Reimer 1966			+	+	+	+
Asterionella formosa Hassall 1850	+	+	+	+	+	+
Aulacoseira ambigua (Grunow) Simonsen 1979					+	
Caloneis bacillum (Grunow) Cleve 1894					+	+
Caloneis silicula (Ehrenberg) Cleve 1894		+	+	+	+	+
Cocconeis placentula Ehrenberg 1838		+			+	+
Cyclotella antiqua W.Smith 1853			+	+	+	+
Cyclotella distinguenda Hustedt 1928		+	+		+	+
Cyclotella iris Brun & HéribaudJoseph	+	+	+	+	+	+
Cyclotella ocellata Pantocsek 1901	+		+	+	+	+
Cyclotella planctonica Brunnthaler 1901					+	+
Cymatopleura solea (Brébisson) W.Smith 1851				+	+	+
Cymbella affinis Kützing 1844				+		
Cymbella amphicephala Näegeli in Kützing 1849			+		+	+
Cymbella aspera (Ehrenberg) Cleve 1894					+	
Cymbella cistula (Ehrenberg) O.Kirchner 1878			+	+	+	+
Cymbella cymbiformis C.Agardh 1830			+			+
Cymbella ehrenbergii Kützing 1844		+		+	+	+

Table 2 continued

Cymbella lanceolata (C.Agardh) Kirchner 1878						+
Cymbella leptoceros (Ehrenberg) Kützing 1844				+		+
Cymbella minuta Hilse in Rabenhorst 1862		+	+	+	+	+
Cymbella silesiaca Bleisch in Rabenhorst 1864				+		
Cymbella simonsenii Krammer in Krammer & LangeBertalot 1985				+		
Cymbella tumida (Brébisson) van Heurck 1880				+		
Denticula tenuis Kützing 1844			+	+	+	+
Diploneis elliptica (Kutzing) Cleve 1894					+	
Diploneis oblongela (Nägeli ex Kützing) CleveEuler in CleveEuler & Osvald 1922				+		+
Diploneis petersenii Hustedt 1937			+	+	+	+
Eunotia arcus Ehrenberg 1837			+	+	+	+
Eunotia bilunaris (Ehrenberg) Schaarschmidt 1880					+	+
Fragilaria biceps Ehrenberg 1843					+	+
Fragilaria capucina var. *vaucheriae* (Kützing) LangeBertalot 1980					+	
Fragilaria crotonensis Kitton 1869	+	+	+	+	+	+
Fragilaria exigua (W.Smith) Lemmermann 1908			+	+		+
Fragilaria leptostauron var. *martyi* (HéribaudJoseph) LangeBertalot 1991		+				
Fragilaria parasitica (W.Smith) Grunow in van Heurck 1881					+	
Fragilaria pinnata Ehrenberg 1843			+	+	+	+
Fragilaria ulna (Nitzsch) LangeBertalot 1980	+	+		+	+	+
Fragilaria ulna forma *claviceps*					+	+
Fragilaria ulna var. *acus* (Kützing) LangeBertalot 1980	+		+	+	+	+
Fragilaria ulna var. *angustissima* (Grunow) Krammer & LangeBertalot 1991	+					
Fragilaria virescens Ralfs 1843				+	+	+
Frustulia rhomboides (Ehrenberg) De Toni 1891				+		
Frustulia vulgaris (Thwaites) De Toni 1891				+		
Gomphonema olivaceum (Hornemann) Brébisson 1838				+		
Gomphonema parvulum Kützing 1849				+	+	+
Gomphonema truncatum Ehrenberg 1832				+	+	+
Gyrosigma acuminatum (Kützing) Rabenhorst 1853					+	
Gyrosigma scalproides (Rabenhorst) Cleve 1894					+	+
Gyrosigma spenceri (Bailey ex Quekett) Griffith & Henfrey 1856		+	+	+	+	+
Hantzschia amphioxys (Ehrenberg) Grunow in Cleve & Grunow 1880				+	+	+
Melosira varians C.Agardh 1827					+	+
Meridion circulare (Greville) C.Agardh 1831	+					+
Navicula cincta (Ehrenberg) Ralfs in Pritchard 1861		+	+	+	+	+
Navicula concentrica Carter & BaileyWatts 1981				+		
Navicula cryptocephala Kützing 1844				+	+	+
Navicula cryptotenella LangeBertalot in Krammer & LangeBertalot 1985		+		+	+	
Navicula cuspidata (Kutzing) Kutzing 1844				+		+

Table 2 continued

Navicula cuspidata var. *ambigua* (Ehrenberg) Cleve 1894			+			+
Navicula elginensis (W.Gregory) Ralfs in Pritchard 1861				+		
Navicula gregaria Donkin 1861			+	+		
Navicula hasta Pantocsek 1892					+	+
Navicula laevissima Kützing 1844					+	
Navicula lanceolata Ehrenberg 1838				+		
Navicula menisculus Schumann 1867		+				
Navicula minuscula var. *muralis* (Grunow) LangeBertalot in LangeBertalot & Rumrich 1981			+			
Navicula nivalis Ehrenberg 1853			+	+		
Navicula pupula Kützing 1844			+	+	+	+
Navicula pygmaea Kützing 1849					+	+
Navicula radiosa Kützing 1844			+	+	+	+
Navicula trivialis LangeBertalot 1980				+	+	+
Navicula viridula (Kützing) Ehrenberg 1836				+		
Neidium affine (Ehrenberg) Pfizer 1871					+	
Neidium binodeforme Krammer in Krammer & LangeBertalot 1985			+	+		+
Neidium bisulcatum (Lagerstedt) Cleve 1894				+	+	+
Neidium dubium (Ehenberg) Cleve 1894		+		+		+
Neidium iridis (Ehrenberg) Cleve 1894				+		+
Nitzschia amphibia Grunow 1862		+		+		
Nitzschia dissipata (Kützing) Rabenhorst 1860					+	+
Nitzschia elegantula Grunow in van Heurck 1881					+	+
Nitzschia intermedia Hantzsch in Cleve & Grunow 1880		+	+	+	+	+
Nitzschia linearis W.Smith 1853					+	+
Nitzschia palea (Kützing) W.Smith 1856		+				
Nitzschia sinuata var. *delognei* (Kützing) W.Smith 1856				+		
Nitzschia subacicularis Hustedt 1922		+				
Pinnularia borealis Ehrenberg 1843			+			
Pinnularia maior (Kutzing) Cleve			+		+	+
Pinnularia microstauron (Ehrenberg) Cleve 1891				+		+
Rhoicosphenia abbreviata (C.Agardh) LangeBertalot 1980			+			
Stauroneis anceps Ehrenberg 1843				+	+	+
Stauroneis phoenicenteron (Nitzsch) Ehrenberg 1843						+
Stauroneis smithii Grunow 1860				+	+	
Surirella angusta Kützing 1844				+	+	+
Surirella brebissonii Krammer & LangeBertalot 1987						+
Surirella linearis W.Smith 1853				+		
Surirella spiralis Kützing 1844				+		
Tabellaria fenestrata (Lyngbye) Kützing 1844					+	
Tabellaria flocculosa (Roth) Kützing 1844	+					+
Phylum Cholorophyta						
Chloroccocum dissectum Korshikov 1953			+			
Chloromonas chlorogoniopsis (H.Ettl) Gerlof & H.Ettl in H.Ettl 1970		+				
Closteriopsis acicularis (Chodat) J.H.Belcher & Swale 1962	+					

Table 2 continued

Coelastrum astroideum De Notaris 1867				+	+	
Coelastrum microporum Nägeli in A.Braun 1855			+			
Coenochloris fottii (Hindák) Tsarenko 1990	+		+			
Coenococcus planctonicus Korshikov 1953	+			+		
Coenococcus polycoccus (Korshikov) Hindák 1977					+	+
Coleochaete divergens Pringsheim 1860			+	+		
Cosmarium contractum var. *minutum* (Delponte) Coesel 1989		+		+		
Cosmarium laeve Rabenhorst 1868				+		+
Cosmarium regnellii Wille 1884				+		
Cosmarium subgranatum (Nordstedt) Lütkemüller 1902				+		
Cosmarium tenue W.Archer 1868					+	
Crucigeniella apiculata (Lemmermann) Komárek 1974		+	+	+	+	+
Crucigeniella rectangularis (Nägeli) Komárek 1974		+				
Crucigenia triangularis Chodat 1902			+			
Didymocystis bicellularis (R.Chodat) Komárek 1973			+		+	+
Gonatozygon brebissonii De Bary 1858					+	
Monoraphidium griffithii (Berkeley) KomárkováLegnerová 1969					+	+
Pandorina morum (O.F.Müller) Bory in Lamouroux, Bory de SaintVincent & Deslongschamps 1824				+		
Pandorina smithii Chodat 1931		+				
Pediastrum boryanum (Turpin) Meneghini 1840			+	+	+	
Pediastrum boryanum var. *longicorne* Reinsch 1867			+			
Pediastrum integrum Nägeli 1849					+	
Pleurotaenium trabecula Nägeli 1849				+	+	
Scenedesmus acutus Meyen 1829			+	+	+	+
Scenedesmus bicaudatus Dedusenko 1925						
Scenedesmus dispar Brébisson 1868						+
Scenedesmus ecornis (Ehrenberg) Chodat 1926			+			
Scenedesmus linearis Komárek 1974			+			
Scenedesmus quadricauda (Turpin) Brébisson in Brébisson & Godey 1835					+	
Schroederia ecsediensis Lemmermann 1898					+	
Staurastrum paradoxum Meyen ex Ralfs 1848				+	+	
Tetraëdron muticum (A.Braun) Hansgirg 1888			+			
Tetrastrum triangulare (Chodat) Komárek 1974	+			+	+	+
TOTAL TAXA	**13**	**27**	**54**	**81**	**83**	**82**

A high number of taxa were cosmopolitan: *Fragilaria virescens, Achnanthes minutissima, Cymbella minuta, Navicula cryptocephala* etc. However, numerous species identified in Lake Iezerul Ighiel were known to have a subalpine or alpine distribution: *Achnanthes flexella, Anomoeoneis vitrea, Caloneis bacillum, Cyclotella antiqua, Cyclotella planctonica, Cymbella affinis, Cymbella leptoceros, Cymbella simonsenii, Diploneis elliptica, Diploneis petersenii, Gomphonema acuminatum, Gonatozygon brebissonii, Navicula concentrica, Navicula nivalis, Neidium binodeforme,*

Neidium bisulcatum. Moreover, several species had different preferences to physical and chemical parameters of the environment, like low water temperature: *Cyclotella planctonica, Diploneis elliptica, Gomphonema acuminatum*; higher values of pH: *Cosmarium regnellii, Fragilaria crotonensis* or high calcium content: *Achnanthes biasolettiana, Achnanthes flexella, Cymbella ehrenbergii, Cymbella leptoceros, Cymbella simonsenii, Eunotia arcus, Meridion circulare, Surirella spiralis*. True planktonic species were also found: *Asterionella formosa, Fragilaria crotonensis, Ceratium hirundinella* etc.

Three algal species are first cited for Romania, according to Cărăuş (2012): *Epipyxis natans, Schroederia ecsediensis, Uroglena americana*. Several other species can be considered rare in Romania, having only two previous citations: *Coleochaete divergens, Cosmarium contractum* var. *minutum, Dinobryon bavaricum* var. *medium, Fragilaria virescens* var. *exigua, Navicula concentrica, Nitzschia sinuata* var. *delognei, Pandorina smithii.*

Microcrustacean community structure

Sixteen taxa of microcrustaceans were identified in sites 1 and 2 in spring, summer and autumn 2014: 11 species of cladocerans and 5 species of cyclopoid, calanoid and harpacticoid copepods, together with immature copepod stages: copepodites and nauplii.

The number of taxa identified at site 1 ranged between 3 and 14 (Fig. 4, Table 3), probably due to the variable water level that changed drastically between the three sampling seasons. On the other hand, the number of taxa present at site 2 was more balanced throughout the year, probably due to the fact that the samples were taken by boat from a region where shore influences were decreased.

Seven taxa were only present at sample site 1 (Table 3), generally small-size cladocerans like *Alona guttata* or *Disparalona rostrata*, while *Daphnia* cf. *rosea* and immature copepod stages were the only microcrustaceans identified in all sampling occasions.

Several species were cosmopolitan, like the cladoceran *Chydorus sphaericus* or the harpacticoid copepod *Canthocamptus staphylinus*. Most of the species identified in the study area were planktonic, many preferring habitats with submerged macrophytes, while two were benthonic: *Ilyocryptus sordidus* and *Disparalona rostrata*.

The taxonomical identification of *Daphnia* sp. became especially problematic, since next to individuals belonging to *rosea* species, cited by Negrea (1983), several others, with different key taxonomical features were identified in all three seasons (Fig. 5). This particular genus is known to be one of the most difficult in terms of taxonomy, because of the co-existence of populations with intermediate morphologies, the presence of hybrids or different local races in large parts of its area (Petrusek *et al*., 2008). Thus, all individuals were considered to be *D.* cf. *rosea*, while subsequent genetic studies should solve the taxonomical incertitudes.

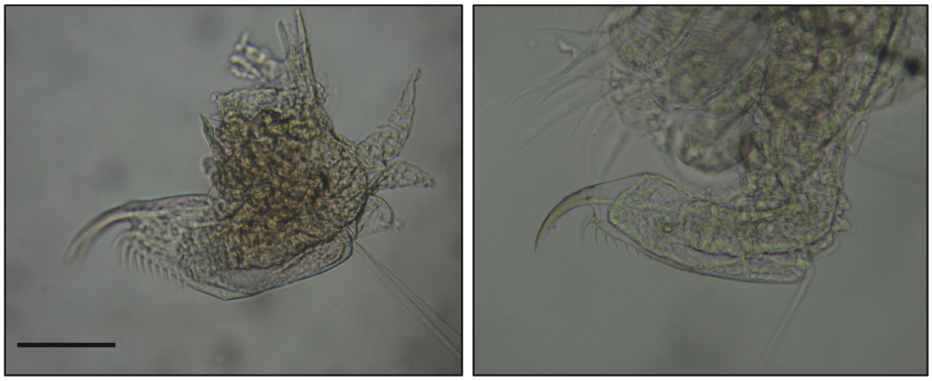

Figure 5. Two different postabdomen morphologies in *Daphnia* cf. *rosea* identified in Lake Iezerul Ighiel in 2014: left - postabdomen typical to *D. rosea*, right - postabdomen typical to *D. galeata* (scale bar - 100 µm)

Diveristy profiles of the microcrustacean communities from sampling sites 1 and 2 were constructed, based on the sums of all individuals from each taxon, in order to compare them (Fig. 6). Surprisingly, sampling site 1 supported a greater diversity compared to the more balanced, macrophyte-covered sampling site 2 (with significant differences, $t = 10.541$, $p < 0.01$).

This is due to the fact that large-size taxa like Da*phnia* cf. *rosea, Cyclops vicinus* and *Acanthodiaptomus denticornis* inhabited the sampling site 2, since they could use the macrophytes as hiding place from visual predators like fish.

On the other hand, numerous small-size species were identified at site 1: *Alonella nana, Alona guttata, Chydorus sphaericus,* even *Attheyella crassa*. These species had no need to hide from visual predators, and probably they lost the competition with the larger *Daphnia* at sampling site 2.

The correspondence analysis in Fig. 7 shows this aggregation of small-size taxa in site 1 and large-size ones in site 2.

Benthic invertebrates

A total number of 15 benthic invertebrate groups were identified, 12 at the sampling site 2 and 9 at the sampling site 3. The highest number of taxa, at both sampling sites was recorded in summer 2014, probably due to the life cycle of the benthic invertebrate populations (Table 4).

Three groups were present in all sampling occasions: Oligochaeta, Chironomidae and Acari-Hydrachnidia. On the other hand, other groups were present only at the sampling site 2: Hirudinea, Trichoptera, Coleoptera, Megaloptera, Stratiomydae, with low abundances, not exceeding 1.5%.They were probably carried into the lake by the tributary entering the water body near the location of the site.

Table 3.

Microcrustaceans identified in the two sampling sites from Lake Iezerul Ighiel (R - rare taxa; S - sporadic taxa; C - common taxa; ♀ - parthenogenetic cladoceran females and copepod females; † - gamogenetic cladoceran females; ♂ - males; sampling sites as in Table1)

TAXA (abbrevitations in brackets)	**SP1**	**SP2**	**SU1**	**SU2**	**AU1**	**AU2**
Cl. Branchiopoda, Subcl. Phyllopoda, Ord. Diplostraca, Subord. Cladocera						
Alona affinis (Leydig 1860) (*Alo.aff*)	-	R,♀	-	-	-	-
Alona guttata Sars 1862 (*Alo.gut*)	-	-	-	-	R,♀	-
Alona rectangula Sars 1862 (*Alo.rec*)	-	R,♀	-	-	R/S, ♀,†,♂	R,♂
Alonella nana (Baird 1843) (*Alo.nan*)	S,♀	-	S,♀	R/S,♀	S/C,♀	R/S,♀
Bosmina longirostris (O. F. Muller 1776) (*Bos.log*)	-	R,♀	-	S,♀	R,♀	R,♀
Ceriodaphnia pulchella (Sars 1862) (*Cer.pul*)	-	R,♀	-	R/S,♀	R,♀,†	S,♀,†,♂
Chydorus sphaericus (O. F. Muller 1776) (*Chy.sph*)	-	-	-	-	R/S,♀	-
Daphnia cf. *rosea* Sars 1862 (*Dap.ros*)	S,♀	C,♀,♂	R,♀	C,♀,♂	S,♀,†	S/C,♀,†
Disparalona rostrata (Koch 1841) (*Dis.ros*)	-	-	S,♀	-	S,♀,†,♂	-
Ilyocryptus sordidus (Lievin 1848) (*Ily.sor*)	-	S,♀	-	-	R/S,♀	-
Pleuroxus truncatus (O. F. Muller 1785) (*Ple.tru*)	-	-	S,♀	-	-	-
Cl. Maxillopoda, Subcl. Copepoda						
Acanthodiaptomus denticornis Kiefer 1932 (*Aca.den*)	-	-	-	S,♀, ♂	S/C,♀,♂	C,♀, ♂
Attheyella crassa (Sars 1863) (*Att.cra*)	-	-	R, ♂	-	S,♀, ♂	-
Canthocamptus staphylinus (Jurine 1820) (*Can.sta*)	-	-	-	-	R, ♀	-
Cyclops vicinus (Ulianine 1875) (*Cyc.vic*)	-	S/C,♀,♂	-	R, ♂	R,♀, ♂	-
Eucyclops serrulatus proximus (Lilljeborg 1901) (*Euc.ser*)	R,♀	-	-	-	S,♀, ♂	-
Cyclopoid copepodites	R/S	S/C	R	R	S	R/S
Calanoid copepodites	-	R	S/C	C	S	S/C
Harpacticoid copepodites	-	-	-	-	R	-
Nauplii	S	C	S/C	S/C	S	S/C
TOTAL TAXA	**3**	**7**	**5**	**6**	**14**	**6**

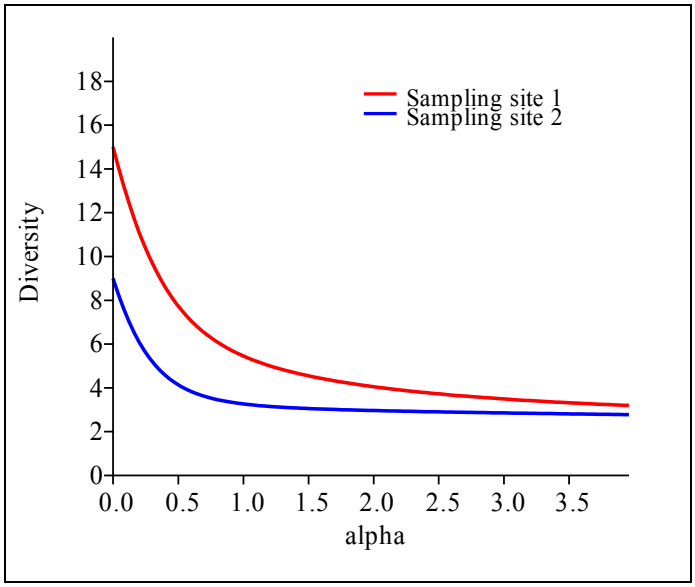

Figure 6. Diversity profiles for the microcrustacean communities from sampling sites 1 and 2, Lake Iezer Ighiel, in 2014

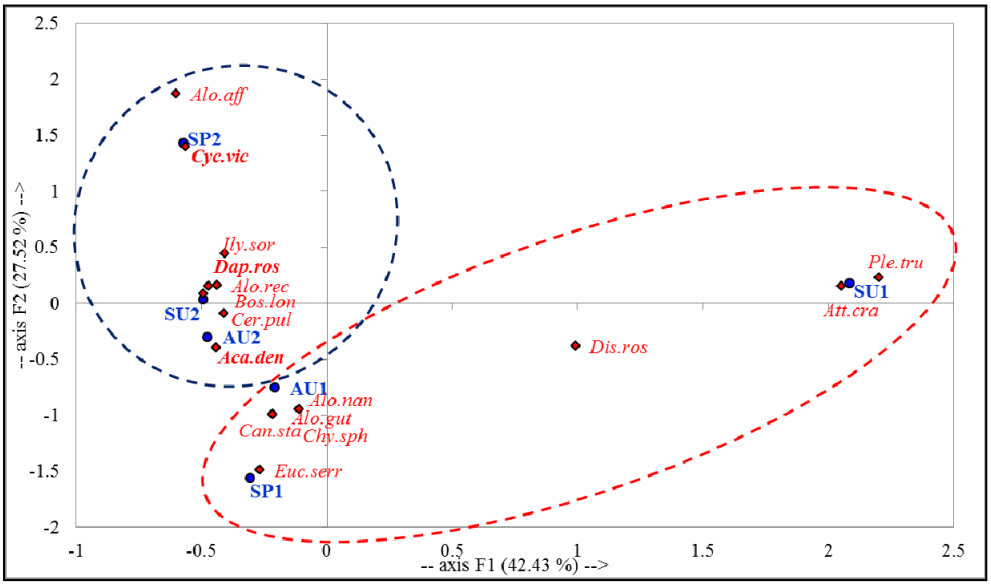

Figure 7. Correspondence Analysis (CA) plot (axes F1 and F2: 69.95 %) showing the aggregation of sampling sites with the number of individuals from each microcrustacean species (sampling sites as in Table 1; species as in Table 3)

Table 4.

Benthic invertebrate taxa identified in two sampling sites from Lake Iezerul Ighiel (sampling sites as in Table1)

Taxa	SP2	SP3	SU2	SU3	AU2	AU3
Nematoda						+
Annelida, Oligochaeta	+	+	+	+	+	+
Annelida, Hirudinea					+	
Arthropoda, Chelicerata, Acari, Hydrachnidia	+	+	+	+	+	+
Athropoda, Hexapoda, Insecta, Ephemeroptera		+	+	+	+	+
Athropoda, Hexapoda, Insecta, Trichoptera					+	
Athropoda, Hexapoda, Insecta, Odonata			+	+		
Athropoda, Hexapoda, Insecta, Megaloptera					+	
Athropoda, Hexapoda, Insecta, Coleoptera	+					
Athropoda, Hexapoda, Insecta, Heteroptera	+	+	+	+		+
Athropoda, Hexapoda, Insecta, Diptera, Chironomidae	+	+	+	+	+	+
Athropoda, Hexapoda, Insecta, Diptera, Chaoboridae			+			
Athropoda, Hexapoda, Insecta, Diptera, Culicidae				+		
Athropoda, Hexapoda, Insecta, Diptera, Psychodidae				+		
Athropoda, Hexapoda, Insecta, Diptera, Stratiomydae	+					
TOTAL TAXA	**6**	**5**	**7**	**8**	**7**	**6**

Benthic invertebrate communities from the two sampling sites had a heterogeneous and dissimilar structure. Mayflies (Ephemeroptera) recorded high abundances during spring 2014 at sampling site 3, and during summer and autumn at site 2. Water mites (Hydrachnidia) reached the highest abundances in spring at both sampling sites, while at site 2 they maintained high numbers during summer too. True bugs (Heteroptera) dominated the benthic communities in spring at both sampling sites. Chironomids recorded higher abundances in summer and autumn in both sampling locations. Oligochaetes prevailed in autumn (Figs. 8 and 9).

Four water mite taxa (Acari - Hydrachnidia) were identified in the present study. The most frequent species was *Limnesia konikei*, known to be tolerant to high nutrient loads - up to 10 mg/L Total Nitrogen (Haaren and Tempelman, 2009). Four individuals (1♂; 3♀) belonging to *Neumania deltoides* were also found. This species occurred in waters strongly influenced by man in Netherlands (Smit and Hammen, 2000) or in eutrophic lakes in Italy (Cicolani and Di Sabatino, 1985). Two taxa typical for rivers were identified at sampling site 2: *Hygrobates foreli* (1♀) and *Lebertia* sp. (deutonymph), probably from the tributary flowing into the lake in that area.

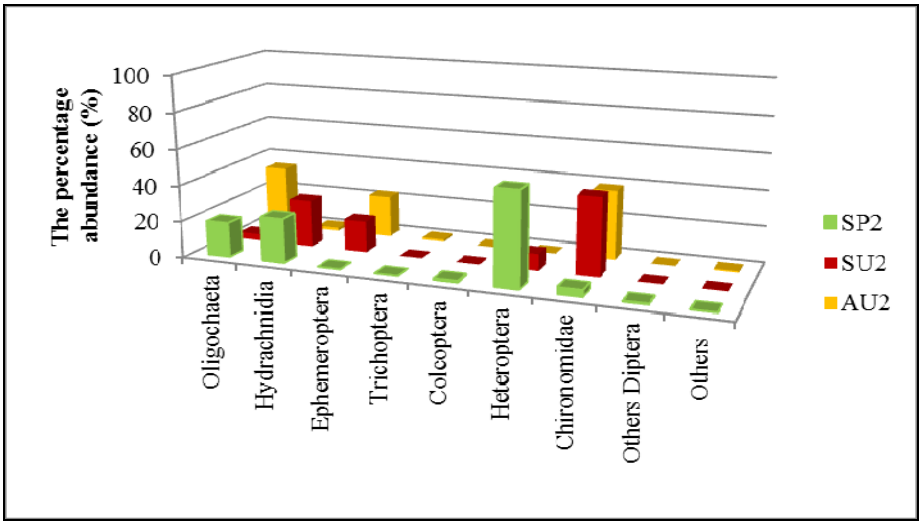

Figure 8. The percentage abundance (%) of benthic invertebrate groups from the second sampling site (sampling sites as in Table 1, Others - Diptera: Chaoboridae, Stratiomidae; Others: Hirudinea, Odonata, Megaloptera)

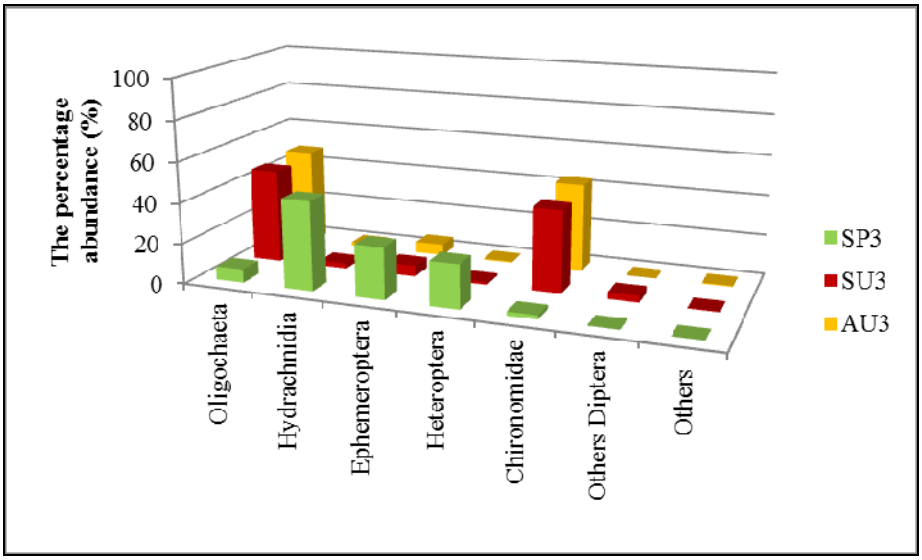

Figure 9. The percentage abundance (%) of benthic invertebrate groups from the third sampling site (sampling sites as in Table 1, Others - Diptera: Culicidae, Psychodidae; Others: Nematoda, Odonata)

Ecological status of the lake, as indicated by biotic communities

The structure of algal communities, both phytoplankton and periphyton, were used to assess the ecological status of Lake Iezerul Ighiel, considering the saprobity and organic pollution characteristic to the system, but also water trophicity.

In terms of organic pollution, eight out of the 20 indicator algal genera, included in the Palmer genus index (Palmer, 1969) were identified in Lake Iezerul Ighiel: *Cyclotella, Gomphonema, Melosira, Navicula, Nitzschia, Oscillatoria, Pandorina* and *Scenedesmus*. Similarly, 7 indicator species for organic pollution, from a total of 20, were found in the two sampling sites considered for the present study: *Gomphonema parvulum, Melosira varians, Navicula cryptocephala, Nitzschia palea, Oscillatoria limosa, Pandorina morum* and *Scenedesmus quadricauda*. The values of the organic pollution index, both at genus and at species levels (19 and 20, respectively) indicated moderate organic pollution in the water.

These findings were supported by the number of algal taxa with saprobic indicator values, identified at each sampling site (Fig. 10). Even if no trends were visible in terms of clear relationships between indicator taxa and one sampling site or sampling season in particular, most algal taxa indicated oligosaprobic - β mesosaprobic waters (Fig. 10), meaning clean to slightly polluted waters in terms of the quantiy of decomposing organic matter existing in the system.

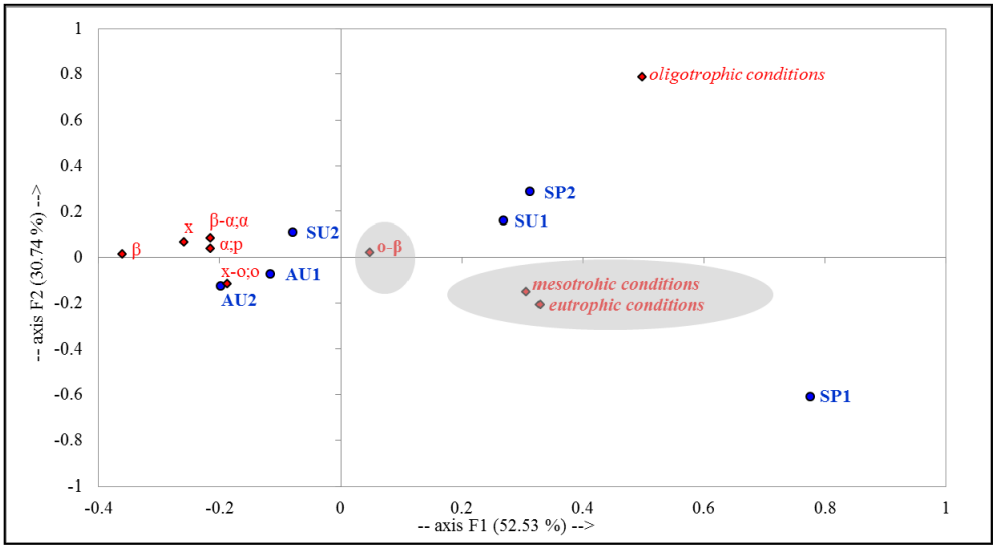

Figure 10. Correspondence Analysis (CA) plot (axes F1 and F2: 83.26 %) showing the aggregation of sampling sites with the number of algal taxa with indicator value (sampling sites as in Table 1; x - xenosaprobic; o - oligosaprobic; β: β - mesosaprobic taxa; α: α - mesosaprobic taxa; p - polysaprobic taxa; intermediate classes also depicted)

The trophic state of the ecosystem was assessed using several indices based on algal communities. According to Oltean (1977), alpha-eutrophic conditions were detected in spring 2014, because Bacillariophyceae led to "water blooms" in the system; while beta-eutrophic ones were depicted in summer 2014, due to the fact that Dinophyta was responsible for the "water blooms" that season. The values of the indices (0.4668 and 0.3644, respectively), together with the values of two other indices: 4.5 for Nygaard index and 2.28 for Stockner index showed an early phase of eutrophication in Lake Iezerul Ighiel. Similar results were depicted in Fig. 10, where mesotrophic-eutrophic conditions were placed at the center of the CA plot. Thus, the highest number of algal taxa with indicator value showed increasing nutrient loads in the lake, coming probably from the touristic and fish-stocking activities in the area.

Five microcrustacean species were considered for the assessment of water quality in Lake Iezerul Ighiel, based on their high frequency and abundances at the sampling occasions. Thus, the first two dominant taxa, *Daphnia* cf. *rosea* and *Acanthodiaptomus denticornis* indicated oligosaprobic conditions. However, the other three species, *Bosmina longirostris, Cyclops vicinus* and *Ceriodaphnia pulchella* indicated oligosaprobic to β-mesosaprobic conditions, and mesotrophic towards eutrophic waters.

The biotic index represented by the ratio between the number of large cladocerans (C_l) to all cladoceran species (C_t) (Moss *et al.,* 2003) revealed a general good condition (0.72 - high water quality).

The ratio between calanoid and cyclopoid copepods can give valuable information on the trophic state of the water body. Nicholls and Tudorancea (2001) correlated the disappearance of calanoid species to oxygen concentration depletion and significant nutrient loads in the system. In Lake Iezerul Ighiel however, the ratio between calanoids (*Acanthodiaptomus denticornis*) and cyclopoids (*Cyclops vicinus; Eucyclops serrulatus proximus*) had a general value of 5:1, thus showing good trophic conditions.

Benthic invertebrate communities were difficult to use in assessing the ecological status of the lake. However, generally speaking, high abundances of pollution intolerant groups like Ephemeroptera and Trichoptera indicated a good water quality in Lake Iezerul Ighiel at sampling sites 2 and 3.

Conclusions

The present study focused on the most important planktonic and benthic communities from Lake Iezerul Ighiel. Both phytoplankton and periphyton were well represented in the lake, comprising more than 150 taxa with different indicator values of water saprobity and/or trophic state. Three species were first cited for Romania.

Microcrustacean communities were diverse and relatively balanced, with smaller taxa inhabitting the shallower, macrophyte-free regions of sample site 1 and with larger taxa concentrated on the area covered in aquatic macrophytes from sample site 2, where they could escape visual predators like fish.

Benthic invertebrate communities were also well represented near the banks of the lake. They were heterogeneous, with different groups prevailing in different periods of time.

The ecological status of Lake Iezerul Ighiel was assessed based mainly on algal and microcrustacean communities, that provided the same information. Thus, the lake was characterized by a moderate organic pollution, with low quantities of decomposing organic matter in the system. Both algal and microcrustacean species depicted initial eutrophication conditions in the lake.

To conclude, the ecological status of Lake Iezerul Ighiel was not oligotrophic and oligosaprobic, as expected in a relatively isolated, mountainous lake, but with moderate organic pollution and increasing nutrient loads, probably due to tourism and fish-stocking.

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Distribution, population size and population dynamics of the White Stork (*Ciconia ciconia*) in Cluj County (Romania)

Ferenc Kósa[1,✉]

SUMMARY. During the VII[th] International White Stork Census the breeding population of the White Stork was censused in Cluj county. In 2014 at 90 localities 123 White Stork nests were identified. The population of the White Stork in Cluj county was estimated at 110-120 breeding pairs (HPa), and the total density amounted to 1.39 pairs/100 km^2. The mean distance between each nest and the nearest White Stork nest was 4320.54 m. The majority (55.28%) of the nests were found at altitudes between 300-500 m. Between 1996-2014 there was a moderate increase in the proportion of nests built in the 301-500 m altitudinal range, from 48.61% in 1996 to 55.28% in 2014. During the last 18 years there was a steep increase in the proportion of nests built on overhead electricity line poles, from 59.72% in 1996 to 91.86% in 2014. The average breeding success (JZa) and productivity (JZm) values for the county were 3.12 and 3.38, values which are higher than the estimated JZa and JZm values needed to keep the population stable. In comparison to the last survey in 1996, the 2014 census shows a moderate 5.35% increase in the number of the breeding pairs (HPa).

Keywords: breeding success, distribution, nest site selection, population trends, white stork

Introduction

In 2014 during the VII[th] International White Stork Census data of more than 5600 nests were obtained from more than 2500 romanian localities distributed in 39 counties (Kósa, unpublished data). The total romanian White Stork population can be estimated to 5000-6000 breeding pairs (Kósa, 2013). With the exception of high mountainous regions and forested area, the White Stork is distributed over the entire territory of Romania.

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The first regional White Stork census in Cluj county was conducted in 1956 by Miklós Béldi (Béldi, 1959). The first detailed census covering the whole area was made 40 years later, in 1996 (Kósa *et al.*, 1998). Some data on the numbers and population dynamics of the White Stork in Cluj county were published later by Kósa *et al.* (2002).

In 2014, the White Stork population from Cluj county was censused again. The main goal of this study was to assess the changes occurring after 18 years in the distribution, population size, breeding parameters and population dynamics of the White Stork in this area.

Materials and methods

The study area covers 6650 km^2 and is situated in the central-western part of Romania. The geographical range of Cluj county is from 22º36'E to 24º15'E and from 46º24'N to 47º22'N.

Between June 30th and July 21th 2014, 175 villages were surveyed for White Stork nests. The survey was conducted according to the standardized count methodology accepted in Romania (Kósa, 2014). In order to ensure the compatibility of our results with the data of the VIIth International White Stork Census, the following parameters were determined (Schulz 1999):

HPa – Number of breeding pairs (HPa=HPm+HPo+HPx);

HPm – Number of successfully breeding pairs;

HPo – Number of unsuccessfully breeding pairs;

HPx – Number of breeding pairs with unknown success;

JZG – Total number of fledged young.

The following numbers were calculated from the compiled data:

JZa – Productivity (breeding success) - the mean number of fledged young from all breeding pairs (JZG/HPa);

JZm – Mean fledged brood size – mean number of fledged young from successful nests only (JZG/HPm);

Std – "Stork density" or population density - number of breeding pairs (HPa) per 100 km^2.

Number of fledglings was recorded for each breeding pair by direct observation with binoculars.

Locations and altitude of White Stork nests were recorded with a Garmin Oregon 650t GPS receiver (GPS accuracy: 3–5 m). All nests were photographed.

Data analysis was made with the FileMaker Pro software and maps were produced with the QGIS 2.8.2 software.

Results and discussion

Distribution, abundence and population size

During the VIIth International White Stork Census the breeding population of the White Stork was censused in Cluj county. Compared to the last census (Kósa *et al.*, 1998) the number of surveyed localities increased from 78 to 175 (Fig. 1). In 2014 at 90 localities 123 White Stork nests were identified (Table 1). The mean number of nests/localities was 1.36 and the maximal number of 4 nests/localities were observed in Morlaca and Brăişoru.

Over the period of 1996 to 2014 the proportion of localities with one nest decreased from 86% to 40%, while the proportion of localities with 2 nests increased from 10.9% to 21.11%.

The mean distance between each nest and the nearest White Stork nest was 4320.54 m. The greatest distance recorded between neighbouring White Stork nests was 25399 m.

In Southeast Europe the presence of the White Stork nests is probably determined by a set of environmental variables with the greatest negative influence being topography and the amount of forest and the greatest positive influence beig presence of human settlements, the availability of open habitats such as grasslands or non-irrigated arable lands in proximity to White Stork nests (Radovic *et al.,* 2015).

The distribution of the White Stork throughout Cluj county is uneven. The highest densities of nests were recorded in the western part of the county in the upper valley of Crişul Repede (24 nests). About half of the total number of nests concentrated in the valleys of five larger watercourses: Someş (19 nests), Nadăş (19 nests), Borşa (11 nests), Luna (7 nests) and Hăşdate (7 nests). The lowest densities of White Stork nests were recorded in the eastern part of the county in an 1237 km^2 area situated between Apahida, Cămăraşu and Viişoara localities. In this area, characterised by more intensive agriculture, from the 29 surveyed localities only 3 had 3 storks nests.

No White Stork nests were identified at the following 85 localities: Agârbiciu, Apahida, Ardeova, Bădeni, Băişoara, Beliş, Berchieşu, Bogata, Boian, Bolduţ, Borşa-Cătun, Boteni, Buru, Buteni, Călăraşi, Cămăraşu, Căprioara, Ceanu Mare, Ciucea, Chesău, Ciumafaia, Coasta, Cojocna, Colonia, Coruşu, Corpadea, Crişeni, Cristorel, Dezmir, Dâmbu Mare, Dingău Mare, Dingău Mic, Domoşu, Dumbrava, Feleacu, Fânaţe, Frata, Gădălin, Ghirişu, Hodai-Boian, Horlacea, Huedin, Iacobeni, Ignita, Jucu Herghelia, Juriu de Câmpie, Lacu, Leghia, Liteni, Măcicaşiu, Mănăstirea, Mănăşturel, Mihaiu Viteazul, Moldoveneşti, Morău, Oşorhel, Păglişa, Petreştii de Mijloc, Plăeşti, Popeşti, Pustuţa, Prelucele, Răchiţele, Rediu, Râşca, Săcel, Săcuieu, Salatiu, Sălişte, Sănduleşti, Sânmartin, Săvădisla,

Scrind-Frăsinet, Soporu de Câmpie, Suceagu, Stoiana, Tiocu de Jos, Tiocu de Sus, Tritenii de Jos, Tritenii de Sus, Văleni, Vaida-Camaraş, Viişoara, Viştea, Zorenii de Vale (Fig. 1).

Population size was assessed based on the number of nests occupied by breeding pairs only (HPa). During the census 93 White Stork breeding pairs (HPa) were identified (Table 1): 86 successfully breeding pairs (HPm), 2 unsuccessfully breeding pairs (HPo) and 5 breeding pairs with unknown success (HPx). As about 20% of the county was not covered by the censuses, the total population is estimated to 110-120 breeding pairs (HPa).

The mean population density (StD) for Cluj county was 1,39 breeding pairs (HPa)/100 km^2.

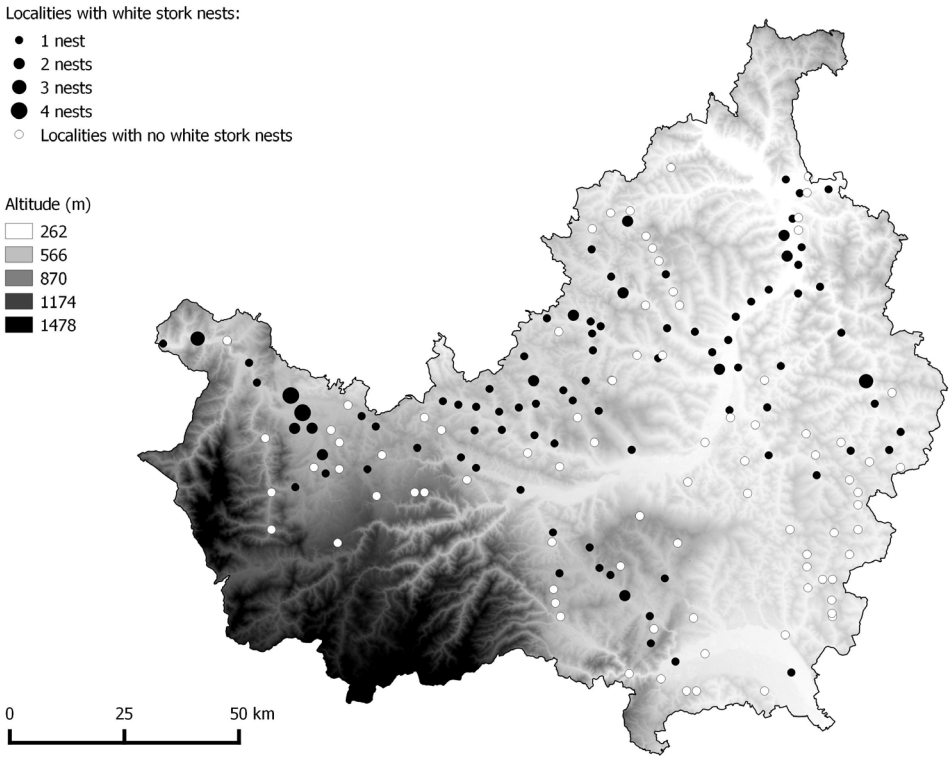

Figure 1. Distribution of localities with White Stork nests in Cluj county in 2014

Table 1.

List of White Storks nests in Cluj county in 2014 (breeding parameters: uH – unoccupied nest, HE – nest with one bird, HPm1-5 – stork pair with 1-5 fledglings; nest support: E0 – electric pylon without artificial support, E1 – electric pylon with artificial support, C – chimney, R – roof, B – barney, T – tree).

Localities	Latitude	Longitude	Altitude (m)	Breeding data	Nest support
Aghireşu	46.87192	23.23611	452	HPm2	E1
Aghireşu Fabrici	46.86732	23.26634	447	HPm2	E0
Alunişu	46.83562	22.94535	605	HPo	E0
Alunişu	46.83410	22.94386	605	HPm4	E0
Aşchileu Mare	46.98675	23.49200	367	HPm4	E1
Aşchileu Mare	46.98096	23.48039	365	HPm4	E0
Aşchileu Mic	46.98261	23.44014	379	HPm4	E1
Băbuţiu	46.93987	23.53026	377	HPm4	E0
Băgara	46.86444	23.30134	433	HPo	B
Băiţa	47.02085	23.87492	279	HPm3	E0
Bologa	46.89680	22.87153	498	HPm4	E0
Bonţida	46.91694	23.81497	269	HPm5	E1
Bonţida	46.90370	23.81256	273	uH	E0
Borşa	46.92938	23.65812	308	uH	E0
Brăişoru	46.85653	22.96099	552	HPm3	E0
Brăişoru	46.85641	22.96196	554	HPm2	E0
Brăişoru	46.85756	22.95527	550	HPm4	B
Brăişoru	46.85755	22.95532	550	HPm4	B
Bucea	46.94878	22.68750	377	HPx	E0
Bunești	47.06537	23.91097	255	HPm3	E0
Bunești	47.04284	23.90046	269	HPm4	E0
Căianu Vamă	46.79964	23.87478	323	HPm3	E0
Călata	46.80028	22.99993	614	HPm4	E0
Călata	46.80673	22.99837	611	HPm4	E1
Călăţele	46.77525	23.00621	658	HPm2	E0
Căpuşu Mare	46.78276	23.30118	467	HPm3	C
Căpuşu Mic	46.79684	23.27156	473	HPm5	E0
Chinteni	46.85905	23.54174	467	HE	E0
Chinteni	46.85901	23.53850	463	HPm3	E1
Ciurila	46.64851	23.54308	589	HPm3	E0
Cluj-Napoca	46.80690	23.60627	354	HPm3	E0
Comşeşti	46.63454	23.67126	533	uH	E0
Cornești	46.88839	23.32749	455	HE	E0
Cornești	47.04121	23.67317	373	uH	E0
Cornești	46.52275	23.69208	351	HPm4	E0
Crăeşti	46.61139	23.59279	502	HPm3	E0
Crăeşti	46.61872	23.58034	497	HPm2	E0
Cuzdrioara	47.16747	23.90849	278	HPm4	E0
Dăbâca	46.96941	23.67568	321	HPm4	E1
Dârja	47.01665	23.58933	357	HPm4	E0
Dârja	47.01838	23.58890	360	HPm3	E1

Table 1 continued

Localities	Latitude	Longitude	Altitude (m)	Breeding data	Nest support
Deuşu	46.89938	23.51613	493	HPm4	E0
Fizeşu Gherlii	47.02460	23.97556	274	HPm3	E0
Fodora	46.97831	23.52579	351	HPm4	E0
Fundătura	46.95365	23.79559	293	HPm4	E1
Gârbău	46.83354	23.35166	408	HPm5	E1
Geaca	46.86864	24.08285	300	uH	E0
Geaca	46.86380	24.08539	308	HPm4	E0
Gilău	46.75328	23.38832	417	HPm3	C
Hodaie	46.83078	24.13361	307	HPm2	E1
Iclod	46.98472	23.81029	290	HPm4	E0
Izvoru Crişului	46.83813	23.10455	583	HPm3	E0
Jucu de Sus	46.86000	23.79799	328	HPm5	E0
Lita	46.64142	23.46465	563	uH	E0
Livada	47.00487	23.84053	291	HPm4	E1
Lujerdiu	46.96462	23.73017	326	uH	E0
Luna	46.50803	23.91923	279	HPx	E0
Luna de Jos	46.93731	23.76418	293	HPm3	E1
Macău	46.83280	23.29824	454	uH	E0
Mănăstirea	47.11533	23.92180	270	HPm3	E1
Mănăstireni	46.78090	23.08815	718	HPm2	E0
Mărgău	46.75686	22.94674	755	HPm4	E0
Mera	46.81540	23.45489	405	uH	B
Mera	46.82101	23.44932	409	HPm3	E0
Mica	47.14938	23.93580	261	uH	E1
Mihăieşti	46.89939	23.41402	406	HPm5	E1
Mihăieşti	46.90147	23.41587	411	HPm2	E0
Mihăieşti	46.90832	23.41695	412	uH	E1
Mintiu Gherlii	47.05382	23.93292	272	HPm4	E0
Mintiu Gherlii	47.05457	23.93866	275	HE	E1
Mociu	46.80567	24.03563	318	uH	E0
Morlaca	46.87952	22.93789	519	HPm4	E0
Morlaca	46.88164	22.91732	519	HPm3	E0
Morlaca	46.86155	22.92934	540	HPm4	R
Morlaca	46.86056	22.91697	538	HPm3	E0
Nădăşelu	46.82635	23.41571	382	HPm4	O
Negreni	46.95560	22.75526	414	HPx	E0
Negreni	46.95977	22.73954	408	HPx	E0
Negreni	46.96153	22.72987	402	HPx	E0
Nicula	47.01576	23.93250	270	HPm3	E0
Nima	47.09327	23.90494	251	HPm3	E0
Nima	47.07842	23.90952	253	HPm5	E1
Păniceni	46.80958	23.18588	648	HPm1	E0
Panticeu	47.03810	23.56598	377	HPm4	E0
Panticeu	47.04199	23.55652	372	uH	C
Petreşti	47.07730	23.93946	278	uH	E0
Petreşti	47.08054	23.93583	273	uH	E0
Petreştii de Jos	46.58392	23.64178	472	HPm3	E0
Petreştii de Sus	46.54738	23.64383	552	uH	E0

Table 1 continued

Localities	Latitude	Longitude	Altitude (m)	Breeding data	Nest support
Poieni	46.92320	22.85613	480	uH	E0
Pruniş	46.63905	23.56461	569	HE	E0
Răscruci	46.91472	23.77838	276	HPm1	E1
Răscruci	46.89934	23.77538	276	HPm5	E1
Recea Cristur	47.07453	23.52779	399	uH	E1
Recea Cristur	47.08582	23.52182	404	HPm5	E1
Sălicea	46.67620	23.52385	631	HPm4	E0
Săliştea Nouă	46.87288	23.49073	465	HPm5	E0
Săliştea Veche	46.88649	23.47220	432	uH	E0
Săliştea Veche	46.89245	23.46420	421	HPm3	E0
Sâmboleni	46.80676	24.11115	330	HPm3	E0
Sâncraiu	46.83586	22.97960	570	HPm2	E0
Sâncraiu	46.83001	22.98531	581	HPm3	E1
Sânmărghita	47.15438	23.99221	280	HPm4	E0
Sânpaul	46.86863	23.41848	402	HPm4	E1
Sântioana	46.96343	24.01726	286	uH	E0
Şardu	46.86337	23.38504	409	HPm3	E0
Şaula	46.85200	23.07676	567	HPm3	E0
Sic	46.91901	23.89866	317	uH	E0
Sic	46.92581	23.89670	343	uH	E1
Şoimeni	46.96239	23.52871	353	uH	E0
Suatu	46.77280	23.96894	362	HPm2	T
Sucutard	46.89879	24.06582	295	HPm4	E1
Sucutard	46.89349	24.07005	308	HPm4	E1
Sucutard	46.89298	24.07270	310	HPm3	E1
Topa Mică	46.93191	23.39554	439	HPm2	E0
Turea	46.85792	23.34642	424	HPm2	E0
Vâlcele	47.11188	23.59831	350	HPm3	E0
Vâlcele	46.67794	23.65018	534	HPm4	E0
Vişea	46.86361	23.87197	330	HPm2	E0
Vlaha	46.69631	23.45192	448	HPm2	E1
Vultureni	46.97209	23.54549	347	uH	E0
Vultureni	46.97243	23.53946	346	uH	E0
Vultureni	46.97023	23.54756	350	uH	E0

Altitudinal distribution

In Cluj county, the majority (55.28%) of the nests were found at altitudes between 300-500 m (Fig. 2). The mean altitude of all localities with stork nests was 409.6 m. The highest occupied stork nest was at Mărgău, 755 m above sea level. Between 1996-2014 an uphill shift took place in the altitudinal distribution of White Stork nests. There was a moderate increase in the proportion of nests built in the 301-500 m altitudinal range, from 48.61% in 1996 to 55.28% in 2014. The opposite was true for nests built in the 100-300 m altitudinal range; their share decreased from 30.55% in 1996 to 21.95% in 2014. Similar tendency was observed in other countries (Tryjanowski et al., 2005) and in other regions of Romania (Kósa *et al.,* 2002).

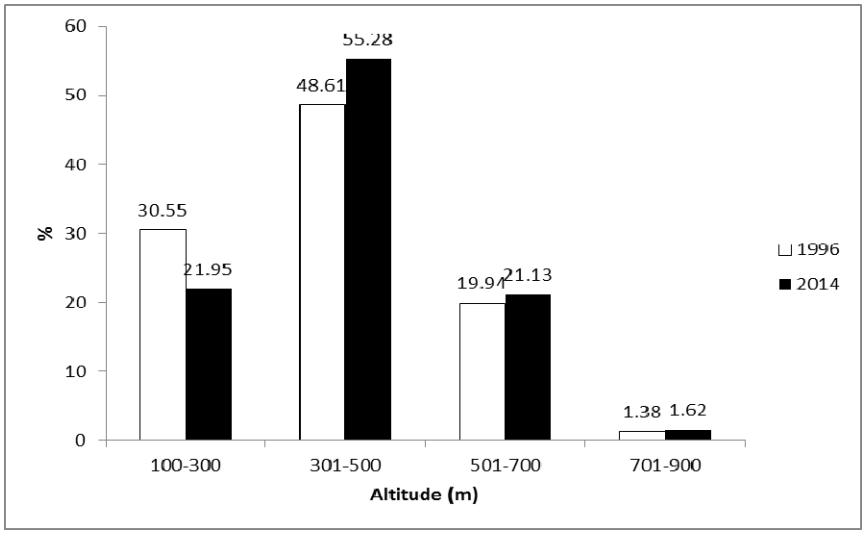

Figure 2. Changes in the altitudinal distribution of White Stork nests in Cluj county (Kósa *et al.,* 1998)

Uphill shift in distribution of the White Stork is a new phenomenon, started in the first half of the last century. It is believed to be the consequence of anthropogenic changes of habitats resulting in the improvement of food supply (Wuczyński 2006), and possibly also an effect of climate warming (Tryjanowski *et al.,* 2005).

Nest placement

The majority (91.86%) of all recorded White Stork nests were located on electricity line poles (Fig. 3). The absolute majority of White Stork nests on power lines are located on poles of low-voltage overhead electrical lines that have horizontal placement of the wires, which is particularly suitable for supporting nests. Nests on overhead electricity line poles with artificial nesting platforms accounted for 24.39% of the total. In 2000, the first artificial nest platforms began to be installed on electricity poles in Cluj county in cooperation with the national electricity company, and by 2014 about 30 poles had been equipped with such platforms.

Nests on various buildings accounted for 6.5% of all nests, and only 1.62% were in various other locations (trees etc.).

Particularly prominent and significant changes over the 18-year period took place in the location of White Stork nests (Fig. 3). There was a steep increase in the proportion of nests built on overhead electricity line poles, from 59.72% in 1996 to 91.86% in 2014. The opposite was true for nests built on buildings; their share decreased from 31.94% in 1996 to 6.5% in 2014.

This same tendency has also been observed in Romania, both in the entire country, where the proportion of nests on electricity line poles increased from 51.75% in 1994/1995 to 83.9% in 2004/2005 (Kósa, 2013), and in different regions of the country (e.g. Kósa *et al.,* 2002, Kósa and Papp, 2007).

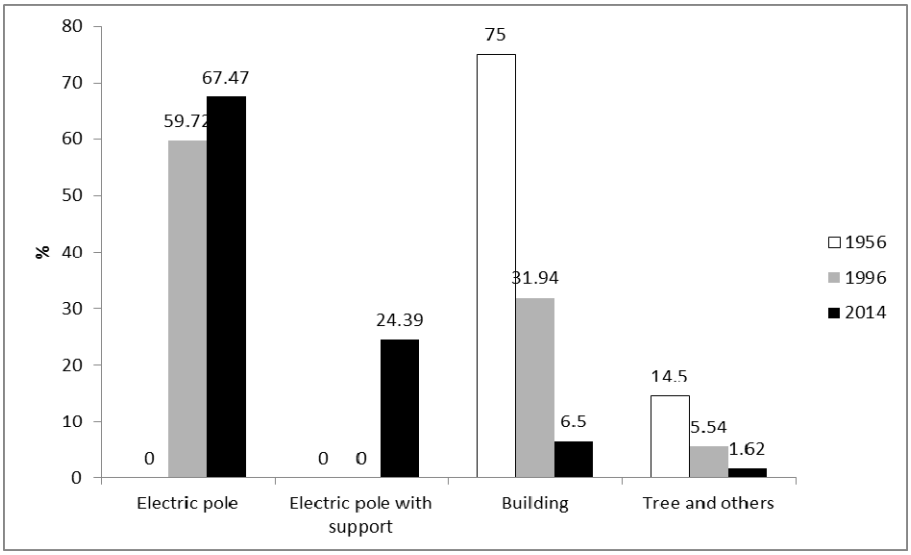

Figure 3. Changes in proportion of White Stork nest localisation in Cluj county (Béldi, 1959, Kósa *et al.,* 1998)

Breeding parameters

Breeding success was recorded in 86 successful nests (HPm). In total, 291 young (JZG) were raised in these nests, with average breeding success of 3.38 young per successful pair (JZm). This was similar to the value recorded in 1996: 3.14 young per number of successfully breeding pairs (HPm) (Kósa *et al.* 1998).

The number of young per successful pair ranged between 1 (2.32%) and 5 (10.46%), with 3 (32.56%) and 4 (38.37%) being the most common, accounting for 70.93% of all successful nests, while 5 young were registered in 9 nests only.

In 2014 the percentage of breeding failure (%HPo) was low, only 6.66%.

The productivity (JZa), the mean number of fledged young from all breeding pairs (HPa) for Cluj county was 3.12.

Thus the average breeding success (JZm) and productivity (JZa) values for the region were above 2.5 and 2.0, values which are higher than the estimated JZm and JZa values needed to keep the population stable (Burnhauser, 1983; Lakeberg, 1995).

Population dynamics

The Romanian breeding White Stork population underwent a large decline between 1958 and 1978 (Klemm, 1983). Among the causes of the decline Klemm listed the disappearance of wetlands due to drainage and river regulation following a systematic government plan and structural changes of the human settlements.

A large population decline could be observed also in Cluj county for the 1956-1996/1997 time interval: the number of occupied White Stork nests decreased by -47.36% (Kósa *et al.,* 2002).

After decades of decline of the White Stork population, the 2014 census revealed a positive development in Cluj county. Comparing the breeding pairs for the 56 localities surveyed in both years (1996 and 2014) we can see (Table 2), that in comparison to the last census in 1996, the 2014 survey shows a moderate 5.35% increase in the number of the breeding pairs (HPa).

Table 2.

Changes in the number of the breeding pairs (HPa) of the White Stork in the localities of Cluj county between 1996 (Kósa *et al.*, 1998) and 2014

Localities	1996	2014
Aghireşu	1	1
Aghireşu Fabrici	0	1
Apahida	1	0
Aşchileu Mare	1	2
Aşchileu Mic	0	1
Băbuţiu	1	1
Băgara	1	1
Băiţa	0	1
Bologa	1	1
Brăişoru	3	4
Bucea	0	0
Bunești	1	2
Căianu Vamă	0	1
Călăraşi-Gară	1	0
Călata	1	2
Cămăraşu	1	0
Câmpia Turzii	1	0
Căpuşu Mare	1	1
Căpuşu Mic	1	1
Chinteni	1	1
Ciurila	0	1
Cluj-Napoca	0	1
Cojocna	1	0
Crăeşti	1	2
Cuzdrioara	0	1

Table 2 continued

Localities	1996	2014
Dârja	1	2
Fodora	2	1
Fundătura	1	1
Geaca	1	1
Gheorgheni	1	0
Gilău	1	1
Hodaie	1	1
Iclod	1	1
Livada	1	1
Luna	1	1
Mănăstirea	1	1
Mihăieşti	1	2
Mintiu Gherlii	1	1
Morlaca	2	4
Nădăşelu	1	1
Negreni	0	0
Nima	1	2
Păniceni	1	1
Panticeu	1	1
Răscruci	2	2
Recea Cristur	2	1
Sălicea	0	1
Sâncraiu	1	2
Sânpaul	1	1
Şoimeni	1	0
Suatu	1	1
Sucutard	3	2
Şutu	1	0
Ţaga	1	0
Turda	1	0
Viişoara	3	0
Total	56	59

In the last 18 years the White Stork disappeared from Apahida, Cămăraşu, Câmpia Turzii, Cojocna, Gheorgheni, Şoimeni, Şutu, Ţaga, Turda and Viişoara, but appeared as nesting bird in the following localities: Aghireşu Fabrici, Aşchileu Mic, Băiţa, Căianu Vamă, Ciurila, Cluj-Napoca, Cuzdrioara, Sălicea.

Conclusions

In 2014 at 90 localities 123 White Stork nests were identified. The population of the White Stork in Cluj county in 2014 was estimated at 110-120 breeding pairs (HPa), and the total density amounted to 1.39 pairs/100 km^2.

The majority (55.28%) of the nests were found at altitudes between 300-500 m. Between 1996-2014 there was a moderate increase in the proportion of nests built in the 301-500 m altitudinal range, from 48.61% in 1996 to 55.28% in 2014.

During the last 18 years there was a steep increase in the proportion of nests built on overhead electricity line poles, from 59.72% in 1996 to 91.86% in 2014.

The average breeding success (JZa) and productivity (JZm) values for the county were 3.12 and 3.38, values which are higher than the estimated JZa and JZm values needed to keep the population stable. In comparison to the last survey in 1996, the 2014 census shows a moderate 5.35% increase in the number of the breeding pairs (HPa).

From a conservational point of view it is necessary to continue the monitoring of the White Stork populations in Cluj county and to continue the installation of artificial nest platforms on electricity poles.

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I would like to thank Lucian Barbu for helping during the fieldwork.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, 2, 2015 (p. 75-84)

Acute administration of Red Bull affects blood parameters in untrained and trained young males

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SUMMARY. The worldwide consumption of energy drinks has grown globally during the last few decades among athletes and youth, because of their stimulant properties. Although these beverages are consumed in order to improve concentration, physical and cognitive performance, there is insufficient evidence or studies to confirm these claims. The aim of our study was to investigate if acute administration of Red Bull energy drink influences certain biochemical parameters of the blood as well as blood pressure and heart rate, under physical activity in trained and untrained healthy males.

Thirteen healthy voluntary males between the ages 20-25 years participated in this study. They were organized in two groups: trained and untrained. Blood samples were drawn and certain hemodynamic parameters were measured before and after physical activity/Red Bull administration. Anthropometrical measurements were also determined.

A significant increase of blood pressure and heart rate were noticed after Red Bull administration in all subjects, while the glucose concentration decreased. The concentration of total protein increased significantly after Red Bull administration, as did the activities of serum LDH, AST and ALT. These results indicate that acute consumption of energy drinks can affect biochemical blood parameters as well as the hemodynamic parameters.

Keywords: blood, caffeine, energy drinks, Red Bull

Introduction

The worldwide consumption of energy drinks has grown globally during the last few decades amoung athletes and youth, because of their stimulant properties (Kumar *et al.,* 2015). The investigations conducted among athletes showed that energy drinks are frequently consumed by athletes prior to competitions in order to improve their performance (Astorino *et al.,* 2011; Desbrow and Leveritt, 2007).

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Alford *et al.* (2001) reported that consumption of energy drinks delay the onset of exhaustion. The stimulating properties of Red Bull stem from the combined action of its ingredients: caffeine, taurine, carbohydrates (glucose and sucrose), B3 vitamin, glucuronolactone etc. Usually, energy drinks contain high amounts of carbohydrates along with nutrients purposed to improve self-perceptions of attention and/or mental alertness. Low calorie energy drinks are also marketed to increase mental alertness, energy metabolism, and performance (Cambell *et al*., 2014). These ingredients taken separately should increase athletic performance.

Caffeine is a potentially useful ergogenic resource for the short loading period after weigh in (Lopez-Silva *et al*., 2014). It has been found that athletes use caffeine before competitions, a discovery made via urine testing during doping checks (Del Coso *et al*., 2012). The ingestion of caffeine increases mental performance (Da Silva *et al*., 2015) and in combination with carbohydrates before and during a badminton match can maintain serve accuracy, anticipation timing and sprinting actions around the court (Clarke *et al*., 2015). It also produces mild central nervous system stimulation, similar to that of amphetamines, reducing fatigue and increasing concentration and alertness. Physiological effects include increased heart rate and output, metabolic rate and urine production (Avois *et al*., 2006). Reisenhuber *et al.* (2006) showed that caffeine in energy drinks has diuretic and natriuretic effects. High doses can cause anxiety, insomnia, and nervousness. In 2004, caffeine was removed from the list of prohibited substances and is now part of the monitoring programme (Avois *et al*., 2006).

There is clear evidence in literature that a normal taurine level is important for the normal functioning of skeletal muscle. Its main roles are to facilitate Ca^{2+}-dependent excitation-contraction coupling, to contribute to the regulation of cellular volume, and to help in antioxidant defense from stress responses (Spriet *et al*., 2015). Taurine, which is an aminosulphonic acid, reduces oxidative stress during exercise and also acts as an antihypertensive agent (Luckose *et al*., 2015). El Idrissi *et al.* (2014) mentioned that acute administration of taurine may be beneficial to lowering blood pressure.

Niacin is important for maintenance of cellular integrity and energy production and is involved in a lot of intracellular reactions. It has been shown that niacin has positive effects on all cardiovascular events on atherosclerosis evolution (Julius, 2010). Ringseis *et al.* (2013) showed that the niacin-induced changes in skeletal muscle phenotype are indicative of an increased capacity of skeletal muscle for oxidative utilization of fatty acids.

The long term effects of energy drinks consumption as well as short term effects are controversial. Shearer *et al.* (2014), reported two opposing effects of caffeine and caffeine-containing energy drinks, their positive effects on athletic performance and their negative impacts on glucose tolerance in the sedentary state. Negative effects of energy drinks affect especially physiological and behavioral moods (Jackson

et al., 2013). An increasing number of problems with behavior modification and cognitive capabilities in adolescents who use energy drinks have been reported (Van Battenburg-Eddes *et al*., 2013). Positive effects of energy drinks were mentioned in a few studies. According to Duncan and Hankey (2013), beneficial effects of energy drinks were observed on perception of extertion, leg muscle pain perception and readiness to invest effort during submaximal cycling in active adults. Peacock *et al*. (2013) demonstrated that long term consumption has positive effect on performance, reducing the reaction time.

Short term negative effects of energy drinks have also been proven. Acute ingestion of Red Bull increases the heart rate and both diastolic and systolic blood pressure, but it does not cause alteration in ventricular repolarization (Elitok *et al.,* 2015; Marczinski *et al.,* 2014; Grasser *et al.,* 2014; Grasser *et al.,* 2015). On the other hand, there are researches that showed positive effects such as soft improvements in physical endurance and mental performance, including concentration and memory (Alford *et al*., 2001; Scholey and Kennedy, 2004). Moreover, Menci *et al.* (2013) showed that the acute consumption of energy drinks causes a significant increase of right and left ventricular myocardial function.

Further knowledge about the effects of energy drink consumption on health is very important, especially given its prevalence among young people and athletes. Therefore, the aim of our study was to investigate whether acute administration of an energy drink, Red Bull, affects certain biochemical parameters of the blood as well as blood pressure and heart rate, while performing intense physical activity (sport exercises) in physically fit and unfit young males, henceforth referred to trained (T) and untrained (U).

Materials and methods

Participants. Thirteen healthy voluntary males between the ages of 20-25 years were selected to participate in this study. They were organized in two groups: trained (T) and untrained (U). The trained volunteers were rugby players of Rugby Team of the Babes-Bolyai University of Cluj-Napoca. The untrained volunteers were young college students who self-reportedly do not engage in regular demanding physical activity. All participants were informed about the purpose and demands of the study before giving their written consent to participate. All volunteers were self-declared as healthy, with no history of cardiovascular, urinary, digestive or metabolic diseases (determined by questionnaire). The protocol was in accordance with the *Declaration of Helsinki* (http://sites.jamanetwork.com/declaration-of-helsinki/index.html, 1964) for research on human subjects. Table 1 shows the main characteristics of participants in this study.

Experimental design. All subjects were instructed not to consume food, energy drinks, coffee and alcohol 12 h prior to the onset of the experiment. In the first day anthropometrical measurements were recorded. Blood samples were drawn and blood preassure and heart rate were measured twice, once before and once after performing physical exercises. Following a warm-up the participants were asked to undergo Astrand Cycle Ergometer Test for five minutes. One day later, the protocol was repeated, however after the initial recordings and blood sampling, the subjects ingested one dose of Red Bull. A period of 45 minutes of rest followed, after which the warm-up and Astrand test were perfomed.

Assays. Blood was collected from the antecubital forearm vein and processed for biochemical examinations at Medstar Laboratory from Cluj-Napoca, Romania. A Conelab.30i determined glycemia, proteinemia, LDH, AST and ALT activities.

Data analysis. The results are presented as mean ± standard error (SE). The data were analyzed for statistical significance using unpaired Student's *t* test. A value of $p < 0.05$ was considered significant.

Table 1.

Comparison of anthropometric data between trained (n=6) and untrained (n=7) subjects

	TRAINED	UNTRAINED
WEIGHT (kg)	102.4±2.7	77.74±4.85
HEIGHT (m)	1.83±0.03	1.83±0.03
BMI (kg/m^2)	30.66±1.06	23.15±1.30
BF %	19.2±1.6	42.54±1.43
Bm %	41.16±1.02	13.32±1.78

BMI-body mass index; Bm%-percent of body muscle; BF%-percent of body fat. Data are mean±standard error.

Results and discussion

The aim of our study was to investigate if acute administration of Red Bull energy drink influences some biochemical parameters of the blood as well as blood pressure and heart rate, under intense physical activity in trained and untrained males.

As expected, physical activity increased the systolic pressure in both groups (Table 2). The results of this study confirm the literature data, according to which physical activity increases sistolic pressure (Marczinski *et al.,* 2014). However, physical activity decreased diastolic pressure, contrary to previous studies in the literature which reported that effort in fact increases diastolic pressure (Elitok *et al.,* 2015; Grasser *et al.,* 2014; Grasser *et al.,* 2015). Furthermore, Red Bull has been shown to increase even more the systolic pressure in both groups and to restore

diastolic pressure to both subject categories. Restoration of diastolic pressure may be due to caffeine, which has diuretic and natriuretic effects (Reisenhuber *et al.,* 2006) and causes peripheral vasoconstriction (Knight *et al.,* 2015), which is followed by tachycardia (heart rate increase) (Higuchi *et al.,* 2015).

The results of this study indicate that the heart rate increases during physical activity (Table 2), an increase which is intensified by the energy drink consumption, more specifically by the caffeine it contains (Steinke *et al.,* 2009; Marczinsky *et al.,* 2014; Elitok *et al.,* 2015).

Table 2.

Effects of exercise and Red Bull on hemodynamic and biochemical blood parameters

	TRAINED			UNTRAINED		
Parameter	RT	T	TRB	RU	U	URB
HR (beats/min)	74.71±4.6	167±7.98	172.28±6.1	68.83±4.49	163±4.37	171.66±6.4
SBP (mmHg)	129±6.37	153.33±8.13	176.66±9.88	117.14±4.34	142.25±8.85	135±5.45
DBP (mmHg)	77.33±3.07	74.16±2.38	78.33±2.78	72.14±2.14	67.14±1.84	72.14±2.14
Glycaemia (mg/dl)	94.5±1.89	91.5±1.78	73.66±7.45*	85.28±3.12	90.28±5.45	85.57±5.09
Proteinemia (g/dl)	7.19±0.13	7.85±0.09**	7.64±0.15	7.11±0.08	8.01±0.15***	8.01±0.10***
LDH activity (U/l)	165±7.58	187.8±4.39	194.8±7.85	136.28±7.85	157.57±11.50	174.14±5.87
ALT activity (U/l)	24.8±1.39	26.6±1.50	27.25±1.28	17±2.98	18.85±3.58	20.14±2.91
AST activity (U/l)	21.2±3.30	21.8±3.59	24.2±1.31	18.71±1.56	19.28±2.21	25±5.77

RB-Red Bull; HR-heart rate; SBP-sistolic blood pressure; DBP-diastolic blood pressure; RT-resting trained group; T-trained group after exercises; TRB-trained group after exercises and Red Bull administration; RU-resting untrained group; U-untrained group after exercises; URB-untrained group after exercises and Red Bull administration. The results are expressed as mean±SE.
Glycaemia *$p < 0.05$ *vs* exercises
Proteinemia **$p < 0.01$ and ***$p < 0.001$ *vs* resting

Physical activity did not produce significant changes in glucose blood concentration (Table 2, Fig. 1 a). However, Red Bull was observed to have determined a significant decrease of glycaemia in trained males. These results are in accordance with the results of Phillips *et al.* (2014). It is possible that the results in this study were influenced by the niacin in Red Bull, which intensified the use of glucose as a cofactor for the enzymes that are involved in the glycolytic pathway. The amount of niacin found in one dose of Red Bull is slightly higher than daily recommended dose.

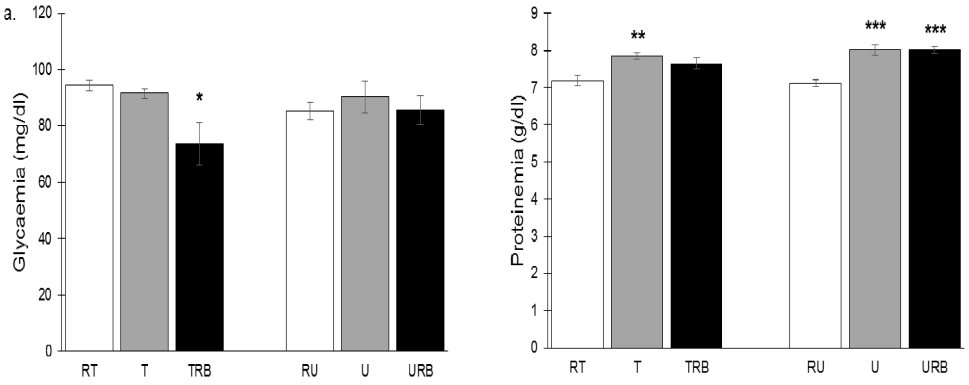

Figure 1. Changes in glycaemia (*p < 0.05 *vs* exercises) (a) and proteinemia (**p < 0.01 and ***p < 0.001 *vs* resting) (b) after exercises and Red Bull administration in the two experimental groups. n=6 in trained group and n=7 in untrained group. RT-resting trained group; T-trained group after exercises; TRB-trained group after exercises and Red Bull administration; RU-resting untrained group; U-untrained group after exercises; URB-untrained group after exercises and Red Bull administration. The results are expressed as mean±SE.

The question is why the same effect was not observed in untrained subjects. It is well known that physical activity increases insulin sensitivity by activating the AMP-activated protein kinase (AMPK) in the skeletal muscle and adipose tissue, which translocates the GLUT-4 to the membrane and facilitates the glucose uptake (O'Neill, 2013). In theory, the consequence would be that the glycaemia would drop following physical activity and Red Bull consumption in both groups, trained and untrained. In this study, we can only assume that the trained muscle is more sensitive to insulin and more responsive to glycaemia increase.

The proteinemia increased significantly in both groups after physical activity coupled with Red Bull administration (Table 2, Fig. 2 b). The increase of protein concentration may be due to muscle damage that occurs during intense effort and this subject matter requires further investigations.

The activities of LDH and ALT increased after physical activity. Red Bull intensified this increase in both groups (Table 2, Fig. 2 a and b). In the case of LDH activity, the increase may be due to the high amount of caffeine which stimulates LDH activity to sustain the lactate production (Dias *et al*., 2015).

ALT and AST activities increase due to muscle injury (Hazar *et al*. 2014). In trained subjects the increase of ALT activity may be due to the release of enzymes from both the liver and skeletal muscles following physical activity (Hazar *et al*., 2014). Red Bull consumption is believed to have increased the serum ALT activity even further (Table 2, Fig. 2 b). At this moment, the mechanism behind this reaction is unclear.

As depicted in Table 2 and Fig. 2 c, AST activity was not affected by physical activity, but rather increased after Red Bull administration. Similarly, Hazar *et al.* (2011) did not find any difference in the AST value after exercise in their study, which was carried out with professional sportsmen. As with ALT activity, the mechanism behind AST activity increase following Red Bull consumption is unclear.

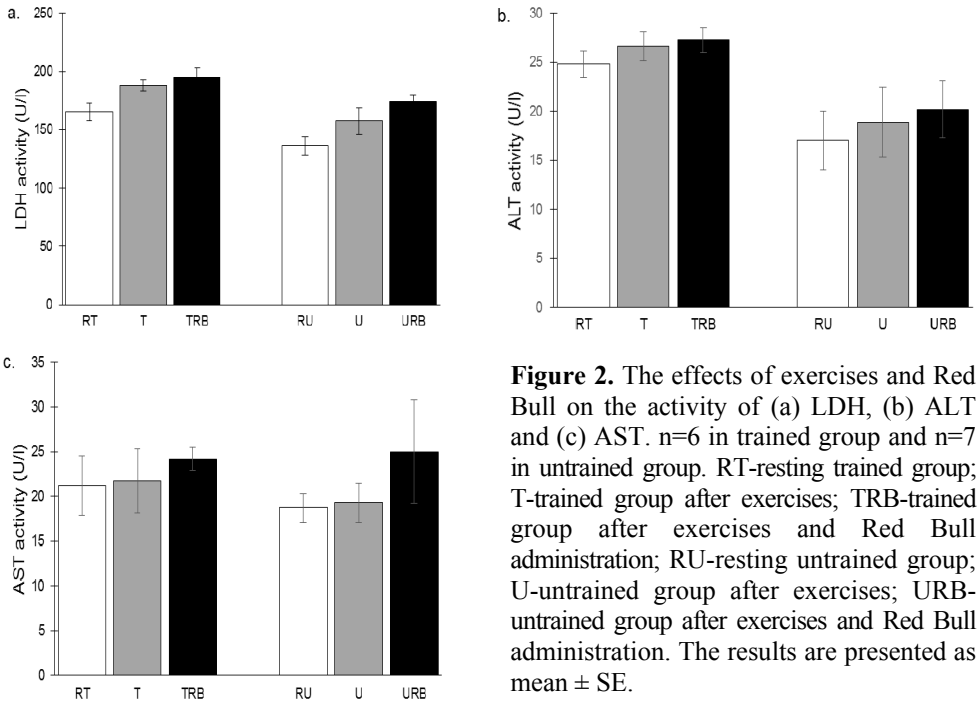

Figure 2. The effects of exercises and Red Bull on the activity of (a) LDH, (b) ALT and (c) AST. n=6 in trained group and n=7 in untrained group. RT-resting trained group; T-trained group after exercises; TRB-trained group after exercises and Red Bull administration; RU-resting untrained group; U-untrained group after exercises; URB-untrained group after exercises and Red Bull administration. The results are presented as mean ± SE.

Conclusions

The results of the present study indicate that energy drinks such as Red Bull can affect biochemical blood parameters, as well as blood pressure and heart rate. Furthermore, energy drinks may be a risk factor for the development of cardiovascular diseases. Further research is required, while raising awareness among young people and athletes about the potential acute effects of energy drink consumption is important.

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*** "Declaration of Helsinki History Website". Ethical Principles For Medical Research. The JAMA Network

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, 2, 2015 (p. 85-88)

Computer sound card used as analog-to-digital converter in a teaching physiology laboratory

Ştefan-Claudiu Mirescu[1,✉]

SUMMARY. Teaching electrophysiology concepts can be a challenging task when confronted with the lack of educational electronic devices. Currently, there are a number of such devices available on the market, but our physiology laboratory does not own an integrated electrophysiology system. So, in order to provide a more practical approach to teaching electrophysiology, we were motivated to seek available alternatives. The sound card microphone port of a computer converts analog signal (sound) into digital signal, also being able to display its waveform in real time. Considering this, we developed a simple device for displaying and recording the photopletismographic peripheral pulse wave and, also, converting it in the second derivative photopletismogram wave, based on which students were able to estimate arterial stiffness. Besides this topic, students were able to learn basic concepts concerning analog-to-digital conversion and data acquisition in biological systems using our device.

Keywords: analog-to-digital converter, computer sound card, electrophysiology laboratory

Introduction

An analog-to-digital converter (ADC) is an electronic device that converts an analog value (usually a voltage) to a digital number. This is very useful especially because computers can acquire and process data only in digital format and all the natural phenomena occur in analog format. Therefore, in order to create an interface between computer and any natural phenomenon, the presence of an ADC is mandatory (Trifa, 1992).

A computer soundcard is such an ADC, converting the analog sound signal into digital, computer accessible digital signal. Actually, a soundcard is a two-way converter, converting analog sound into digital signal through the microphone input port, and also converting digital signal into analog sound, through the headphones/ speakers output port (Liu, 2015).

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Considering these characteristics, the sound card can successfully be used as a data acquisition device, with two conditions: (1) to manufacture the appropriate transducer for interfacing the biological system and (2) to comply to the voltage range tolerated by the soundcard (0 – 5 V).

A sound card-based photopletismography (PPG) sensor was easy to build, required non-expensive electronic components and the PPG trace provided accurate information about pulse wave and arterial stiffness (Qawqzeh, 2010; Simek, 2005; Bortolotto, 2000). Also, constructing laboratory-made (*do-it-yourself*) didactic equipment is not unusual in the field of physiology (see also Sircar, 2015).

Materials and methods

We built a LED-based photoelectric system that collects infrared light emitted by the blood that is pulsed rhythmically through peripheral capillaries (finger pulse meter). The two infrared optic components (one LED and one photodiode) were mounted on the opposite side of a clothespin (the spring was removed in order not to compress the finger too tightly and stop the blood flow). The circuit was powered by a 9V battery, and the output voltage was around 1.5V, which is within the voltage range supported by the sound card input. No external amplifier or filter was used (Fig. 1).

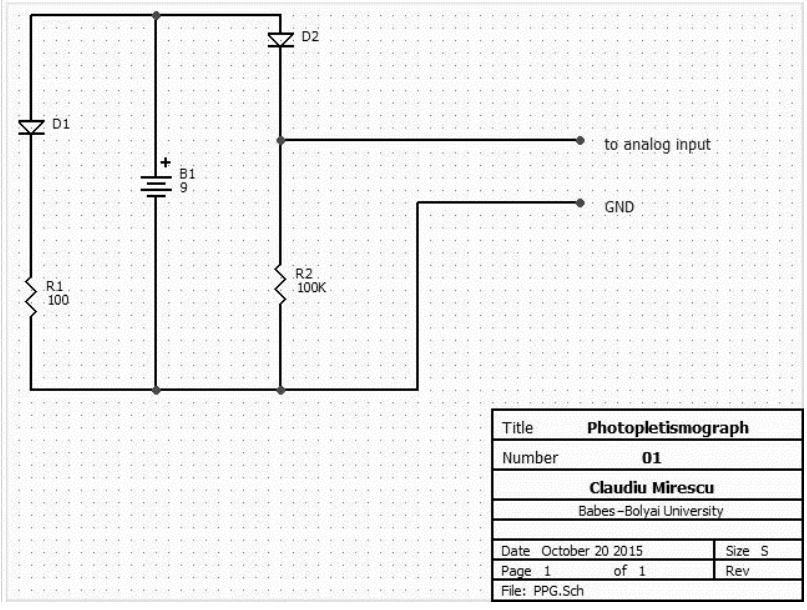

Figure 1. The circuit diagram of the infrared interface, designed using 5Spice Analysis, version 2.31.0. D1 – light emitting diode; D2 – photodiode; R1, R2 – resistors.

For interfacing the computer, we used a sound card oscilloscope software (Zeitnitz SoundScope V1.40, available online for non-commercial purposes at *www.zeitnitz.eu*). This software instrument interfaces the 2 channel computer soundcard, plots the soundwave and, also, has the feature of saving the data in Microsoft Office Excel format, for further processing. Also, the *Cursors* option enables the user to manually measure intervals and amplitudes, directly on the plot of the waveform. The signal can be amplified or filtered using built-in features of the software.

Results and discussion

The device was introduced to the students during two laboratory classes: (1) *Data acquisition principles in biological systems* and (2) *Photoplethysmography and the second derivative photoplethysmogram*. The students observed the components of the device and were explained the concepts of data acquisition, analog-to-digital conversion and transducer, using the device and its components as an example. After this introductory lesson, the students were instructed to perform a PPG trace, using a finger of their choice. The PPG trace was transferred to a Microsoft Office Excel datasheet, were students were able to calculate (as instructed) the second derivative of the photopletismogram (2D-PPG), which studies confirm that is relevant for estimating the arterial age (Figs. 2 and 3).

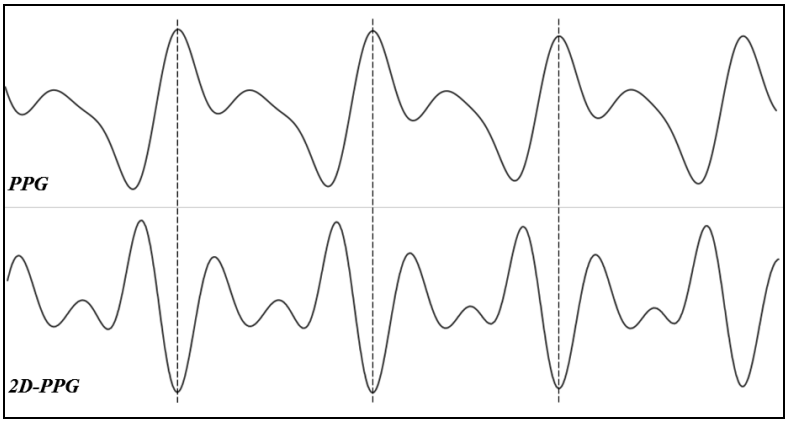

Figure 2. The photopletismographic (PPG) signal, compared with the second derivative photopletismogram (2D-PPG)

According to student feedback forms and personal testimonials, they appreciated that the device offered an explicit approach in teaching the principles of data acquisition, the components were at sight and the experiment also had a purpose: evaluating arterial stiffness.

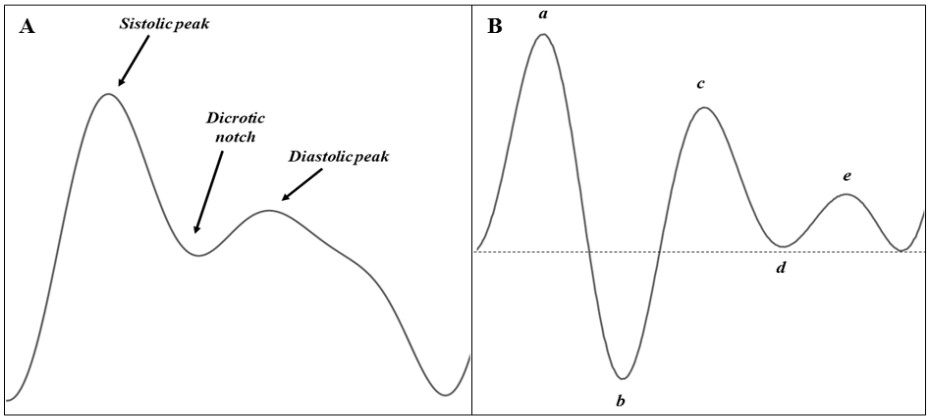

Figure 3. A complete cardiac cycle: A – photopletismogram; B – second derivative photopletismogram (normal curve, corresponding to a decreased arterial index)

Conclusions

Using devices with „at sight“ components for teaching electrophysiology proved to have a number of advantages, compared with standard, commercially available „all-in-a-box“ devices: (1) the ease of concept understanding, because all components can be seen; (2) low cost of manufacturing; (3) greater attractiveness for students. On the other hand, one major disadvantage is the lack of aesthetics.

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=== REVIEW ===

Overview on nanoparticulate formulations for 5-fluorouracil delivery in colorectal cancer treatment

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SUMMARY. 5-Fluorouracil (5-FU) is an antimetabolite drug used to treat various cancer types, especially colon cancer. Its mechanism of action includes the inhibition of the biosynthetic processes in proliferating cells by inhibiting the normal function of DNA and RNA. 5-FU has a short half-life since 80% of the administered drug is catabolized in the liver. Moreover, the therapies based on the 5-FU administration are accompanied by severe side effects such as immunosuppression, cardiotoxicity, and neurotoxicity. Therefore, the use of the nanoparticle-encapsulated 5-FU can target efficiently colorectal cancer, could reduce the major drawbacks of conventional administration of 5-FU and also increase the drug lifetime and its tumour accumulation, and finally 5-FU therapeutic index will be enhanced. To this end, this article aims to review the recent research regarding nanoparticles encapsulating 5-FU for colorectal cancer targeted therapy.

Keywords: 5-fluorouracil, colorectal cancer, nanoparticles

Introduction

Colorectal cancer (CRC) is the third most common cause of cancer, with over 1.2 million new cases being diagnosed every year and the second cause of death by cancer worldwide (Jemal *et al.*, 2011). Moreover, CRC has a high incidence in developed countries and affects both genders equally (Jemal *et al.*, 2011). The main therapies applied in the colon cancer include surgery, biological therapy (hormone

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therapy, immunotherapy), radiation therapy, and chemotherapy. Depending on the cancer stage, various combinations of these types of treatments are used to achieve a better response (Krishnaiah *et al.*, 2002). Nevertheless, systemic chemotherapy is the only available treatment in colorectal cancer with advanced, nonresectable tumours (Arias, 2008). Among most active cytostatic drugs used in the management of this malignancy is 5-fluorouracil (5-FU). This cytotoxic agent was first synthetized over 40 years ago (Wigmore *et al.*, 2010) and is a widely used antimetabolite drug for cancer treatment (Longley *et al.*, 2003) including colorectal cancer but also respiratory, breast, head, and neck cancers (Cheng *et al.*, 2012b). The low efficiency of the clinical applicability of 5-FU is caused by its high toxicity associated with the lack of tumour tissue specificity, low bioavailability, and the acquisition of drug resistance in the CRC cells. Therefore, 5-FU targeting to colorectal carcinoma offers the possibility to increase its antitumour efficacy and to reduce strongly many serious side effects associated with tumour therapy. Therefore, the main goal of this review is to offer a brief overview on the 5-FU mechanisms of action and principal causes of its low antitumour efficiency as well as on the current status of the nanoparticulate formulations encapsulating 5-FU, especially for the cytotoxic drug delivery to colorectal tumours.

Limitations of conventional anticancer therapies based on 5-FU administration

Being a fluorinated analogue of uracil, 5-FU can rapidly enter the cells and further be converted intracellularly to its three active metabolites: fluorouridine triphosphate (FUTP), fluorodeoxyuridine monophosphate (FdUMP) or fluorodeoxyuridine triphosphate (FdUTP). These compounds can either disrupt RNA and DNA synthesis by incorporating themselves within these macromolecules or block the action of thymidylate synthase (TS) (Longley *et al.*, 2003). FUTP and FdUTP cause apoptosis as a result of cell cycle arrest (Anitha *et al.*, 2014). Moreover, FUTP is extensively incorporated into RNA, causing high toxicity on these molecules at different levels. FUTP can inhibit pre-mRNA processing and splicing, disrupt post-transcriptional modifications of tRNAs. In conclusion, 5-FU disrupts normal function and processing of RNA, leading to major effects on cellular viability.In addition, FdUMP-induced inhibition of TS decreases deoxythymidyne triphosphate (dTTP) synthesis. As a result uracil-DNA-glycosylase, the nucleotide excision repair enzyme, will preferentially incorporate FdUTP in DNA leading to irreversible DNA damage (Longley *et al.*, 2003).

Besides the multiple possibilities of action shown above 5-FU is rapidly metabolized in the liver. Dihydropyrimidine dehydrogenase is abundantly expressed in the liver and is the rate limiting enzyme that is responsible for the rapid metabolism of 5-FU, with a half-life of about 5-10 minutes (Zhang *et al.*, 2008). Due to the activity of this enzyme, more than 80% of the administered dose of 5-FU is metabolized (Longley *et al.*, 2003). Therefore several strategies were developed to increase 5-FU anticancer efficiency. Thus, it has been noted that long-term administration of 5-FU via

continuous infusion exerts better response than its bolus injection (van Kuilenburg *et al.*, 2000). Moreover, 5-FU antitumour activity can be improved if the cytotoxic drug is administered in combination with folinic acid (leucovorin) (Dhawale *et al.*, 2010). Consequently, the modulation strategies developed over the last 20 years have increased 5-FU efficacy with up to 40-50% (Longley *et al.*, 2003). However, the limited anticancer efficiency and the low bioavailability of this chemotherapeutic agent require the usage of high dosages that is accompanied by severe adverse effects such as hematopoietic bone marrow suppression, small bowel ulceration, vascular toxicity, cardiotoxicity, and neurotoxicity (Fata *et al.*, 1999; Arias, 2008; Wigmore *et al.*, 2010; Cheng *et al.*, 2012b). Another major disadvantage of the conventional therapy with 5-FU is the development of cancer cell resistance to the treatment (Ortiz *et al.*, 2012). Therefore, tumour-targeted therapies based on 5-FU delivery systems would be capable to overcome the major drawbacks of the conventional administration of this drug (Nair *et al.*, 2011).

Nanoparticles for 5-FU delivery to tumors

Nanoparticles are nanosized drug delivery systems that exhibit the so-called "nanosize effect" (Yassin *et al.,* 2010). Nanosized particles possess different properties than their bulk counterparts, which are caused by their reduction to the nanoscale. This in turn makes the matter to follow quantum mechanics as opposed to Newtonian physics (Leopold, 2009). Due to this effect they have the capacity to make complexes with variety of drugs including 5-FU (Yassin *et al.*, 2010b). Moreover "sterically stabilzed" nanoparticles can extravasate through the leaky pathological vasculature and thereby accumulate in malignant tissue. This effect is referred to as the "enhanced permeability and retention (EPR) effect" and is enabled by the leaky vasculature and poor lymphatic drainage of tumours, which allows nanoparticles to efficiently accumulate in the tumours as a result of their small size (Banciu, 2007; Nair *et al.*, 2011). While in most normal tissues the aperture of vascular endothelial cells is 2 nm, the non-continuous blood vessels in tumours have a much greater aperture, ranging between 100 nm and 780 nm (Cheng *et al.*, 2012b). Therefore, modulation of nanoparticle size allows the drug delivery systems to agglomerate 5-FU passively in the desired area (Nair *et al.*, 2011; Cheng *et al.*, 2012b). Noteworthy, since previous data demonstrated that continuous infusion of the drug induces cell death more effectively than a single injection of 5-FU, nanoparticle-based sustained release of 5-FU would furthermore improve its therapeutic efficacy (Nair *et al.*, 2011). Therefore, several studies described efficient encapsulation of 5-FU in a huge variety of nanoparticles such as those based on polymers (alginate, Poly(ε-caprolactone), chitosan, eudragit, guar gum, Poly(alkylcyanoacrylates), Poly(glutaraldehyde), methacrylic acid, Poly(α -malic acid), Poly(methilidenemalonate 2.1.2), Polyacrylamide, Poly(ortho-ester)s, Poly(D,L-lactide) (PLA) and poly(D,L-lactide-co-glycolide), hydrogels), lipids (liposomes,

niosomes), lipoproteins, inorganic biomaterials (magnetic drug delivery systems, nanoparticles based on clay minerals and anionic clays, metals) and ion exchange resins (Arias, 2008; Zhang *et al.*, 2008; Yan *et al.*, 2010; Yassin *et al.*, 2010b; Nair *et al.*, 2011; Cheng *et al.*, 2012a; Cheng *et al.*, 2012b; Ortiz *et al.*, 2012; Clares *et al.*, 2013; Mishra *et al.*, 2014; Subudhi *et al.*, 2015).

Colorectal specific targeting with nanoformulations containing 5-FU

Nanoparticles based on different drug delivery mechanisms for CRC-specific drug targeting have been developed (Subudhi *et al.*, 2015). Although, most of them had pH-dependent mechanisms, several systems delivered cytotoxic drugs via time-dependent or microflora activated mechanisms (Subudhi *et al.*, 2015).

The pH-sensitive drug delivery systems provide a high controlled release of the cytostatic agent in the colon by taking advantage of the pH variation along the gastrointestinal tract (Dhawale *et al.*, 2010; Subudhi *et al.*, 2015). However, these drug delivery systems did not ensure a specificity for colon region as the pH of the colon is very similar to the pH of the small intestine (Subudhi *et al.*, 2015). Nevertheless, some pH-dependent colon targeted nanoencapsulated formulations containing 5-FU are described in the literature (Yassin *et al.*, 2010a; Mishra *et al.*, 2014; Subudhi *et al.*, 2015). One of them is based on stimuli-responsive hydrogels, such as poly[2-(methacryloyloxyethyl)trimethylammonium chloride-co-methacrylic acid] (Mishra *et al.*, 2014) hydrogels (PMAAc). These hydrogel-based nanoparticles that encaspulated 5-FU were shown to induce apoptosis of HCT116 cells (Mishra *et al.*, 2014). An advantage of PMAAc nanoparticles is given by pKa value (5.6-7) that was close to the pH of the tumour extracellular environment, which led to a maximum swelling ratio at pH 7.4 with a release efficacy of 5-FU by 93.2% (Mishra *et al.*, 2014).

Another example for pH-sensitive drug systems are solid lipid nanoparticles that present important advantages for 5-FU administration such as: a higher incorporation of hydrophilic drug, improved physical stability, good biocompatibility and low toxicity (Kumar, 2000). Thus, Yassin *et al.* obtained 5-FU incorporated in spherical solid lipid nanoparticles. These nanoparticles presented a biphasic drug release and an accumulation in the tumours due to EPR effect (Yassin *et al.*, 2010b). Subudhi *et al*. described a pH-dependent system for colon cancer treatment using curcumin and 5-FU co-encapsulated in thiolated chitosan nanoparticles. These spherical nanoparticles exhibited high release in acidic pH of the tumour microenvironment (Subudhi *et al.*, 2015). Moreover, antitumour efficacy studies demonstrated a notable cytotoxicity on HT-29 human colon carcinoma cells *in vitro* as well as an improved drug bioavailability and tumour growth inhibition *in vivo* (Anitha *et al.*, 2014; Subudhi *et al.*, 2015). These important antitumour effects of this formulation were related to cyclooxygenase-2 inhibition as a result of the synergic effect of curcumin and 5-FU (Subudhi et al., 2015).

Chitosan-based nanoparticles have also been studied for colon targeting, as these systems are capable of protecting the drugs through the upper gastrointestinal tract, and can release the encapsulated agents in the colon via degradation by colonic microflora (Park *et al.*, 2010). Thus, Li *et al.* developed chitosan nanoparticles for the administration of 5-FU in combination with leucovorin (Li *et al.*, 2011). A biphasic and simultaneous release phase of these drugs from the chitosan nanoparticles has been observed. Moreover, efficient drug encapsulation and increased loading capacity for both drugs were reported (Li *et al.*, 2011). To actively target colon cancer cells, Anekant Jain and Sanjay Jain developed 5-FU-loaded chitosan nanoparticles coupled with hyaluronic acid, as receptors of this ligand are overexpressed in HT-29 colon cancer cells (Jain and Jain, 2008). These nanoparticles showed a cell internalization 7.9 fold higher than in the case of nanoparticles without ligand after 4h incubation (Jain and Jain, 2008).

It is known that microflora-activated systems take the advantage of the enzymes produced by colonic bacteria that enhance drug release from the biodegradable polymeric nanoparticles (Yassin *et al.*, 2010b). An example is the Citrus pectin nanoparticles for active targeting of colorectal cancer cells since Citrus pectin is a ligand for galectin-3 receptors overexpressed in these cancer cells (Subudhi *et al.*, 2015). Moreover, Citrus pectin has also been shown to have anti-proliferative activity on the same cell line (Subudhi *et al.*, 2015). These nanoparticles were also coated with Eudragit S100 that provided an intestinal fluid pH-sensitive release system. Apart from this, drug release can also be facilitated through degradation of pectin which is mediated by the intestinal microflora (Subudhi *et al.*, 2015).

Another promising anticancer therapy that combined chemotherapy with hyperthermia was described by Clares *et al.* who used FU-loaded magnetoliposomes (Clares *et al.*, 2013). Multilamellar liposomes were used to incorporate supermagnetic magnetite nuclei and 5-FU (Clares *et al.*, 2013). The sustained release pattern of the chemotherapeutic drug from these liposomes was improved and accelerated by electromagnetic field (Clares *et al.*, 2013).

To reduce side effects of 5-FU, a formulation of chitosan poly(ε-caprolactone) nanoparticles encapsulated the hydrophobic prodrug of 5-FU, doxifluridine (Wang and Peng, 2011). This system presented a slow release profile of doxifluridine, which was only intracellularly converted to 5-FU by thymidine phosphorylase, leading to increased anticancer effects in HT-29 colon cancer cells (Wang and Peng, 2011).

Despite the multitude of the nanoparticulate formulations developed for 5-FU delivery to CRC, serious adverse effects could not be overcome. For instance, these nanoparticles could accumulate easily in different organs such as liver, kidneys and spleen (Yan *et al.*, 2010). Moreover, small quantities of nanoparticles have been detected even in the heart, brain and lung (Tsai *et al.*, 2011; Anitha *et al.*, 2014). Therefore, future studies for the optimization of the tumour targeted therapies based on nanoparticles incorporating 5-FU are needed.

Conclusions

CRC-targeted 5-FU delivery discussed within this review by using nanoparticulate delivery systems lead to achievement of intratumour high concentrations of the cytotoxic drug and a local drug release in a controlled manner, which could not be reached by conventional administration strategies of 5-FU. Nevertheless, future research is needed to improve specificity of these nanoparticulate carriers for the tumour targets.

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=== REVIEW ===

Strategies to improve the efficacy of curcumin in colorectal cancer treatment

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SUMMARY. Colorectal cancer is a severe type of disease, in which surgical therapy complemented by radio- or chemotherapy, is hindered by the chemoresistance or secondary effects. Due to the complex and dynamic interactions in tumor microenvironment, there is constant need in designing new anti-cancer strategies that simultaneously target directly cancer cells development and indirectly the pro-tumor processes mediated by the crosstalk of cells in tumor milieu. Curcumin, is a natural, biological safe polyphenol, with anti-tumor, pro-apoptotic and immunomodulatory actions in a wide spectrum of neoplasia including colorectal cancer. Specifically, its ability to orchestrate the processes associated with tumorigenesis such as cancer cell proliferation, metabolism, angiogenesis, inflammation, oxidative stress and immunosuppression, has been largely documented, but insufficiently exploited. However its use in preclinical and clinical studies is hindered due to low solubility in aqueous environments, poor absorption, instability and high rate of degradation. In this article we review the existing data on the anti-tumor actions of curcumin in colorectal cancer and potential strategies aiming at enhancing its efficacy in the treatment of this disease. Due to its ability to both prevent and treat colorectal cancer, by modulating multiple targets, active delivery of curcumin or curcumin analogues combined with other chemotherapeutic agents, is a promising therapeutic approach for this type of cancer, with minimal toxicity to healthy tissues.

Keywords: CRC, curcumin, cytotoxic actions, nanoformulations.

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Introduction

Colorectal cancer (CRC) is a severe chronic disease of the digestive tract affecting more than 1.2 billion people worldwide. The mechanisms responsible for CRC pathogenesis, specifically the neoplastic transformation of normal cells, proliferation, new blood vessel formation, invasion and metastasis have been attributed to genetic and epigenetic factors, and equally to oxidative stress, inflammatory and metabolic processes (Haggar and Boushey, 2009). The therapeutic approach for this type of cancer consists in tumor surgical resection complemented by radiotherapy and/or systemic administration of cytotoxic agents (5-fluorouracil, oxaliplatin, capecitabine), which, unfortunately, due to the lack of specificity for cancer cells, have significantly secondary effects. Additionally, some patients develop resistance to chemotherapeutic agents among which 5-fluorouracil (5-FU) (Chibaudel *et al.*, 2012). Co-administration of chemotherapeutic agents with naturally occurring drugs, which are pharmacologically safe, may overcome conventional chemotherapy side effects (Fantini *et al.*, 2015). In this sense, efficient adjuvant strategies for chemotherapy have been ascribed for flavonoids, polyphenols, stilbenes and other natural compounds (Aggarwal *et al.*, 2013). Among these, **curcumin**, an active polyphenol isolated from *Curcuma longa* possesses anti-tumor and immunomodulatory actions in gastric, cervical, melanoma, genitourinary, breast, esophagus, lung, neurological, hematological and intestinal cancers (Tuorkey, 2014). Curcumin pharmacological activities translate in inhibition of processes entertaining cancer cells development such as cell proliferation, epigenetic or metabolic processes, inflammation, angiogenesis, oxidative stress, invasion and metastasis.

In this article we review the anti-tumor actions of curcumin in CRC and strategies aiming at improving its efficacy in the treatment of this disease.

Thus, in the first part of this work we describe the main effects of curcumin on modulating tumor associated processes such as cell growth, apoptosis, metabolic and epigenetic alterations, inflammation, angiogenesis, and oxidative stress, followed up by a presentation of therapeutic strategies exploiting these effects.

1. Anti-tumor effects of curcumin that modulate tumor growth- associated processes

Modulation of cancer cell growth and apoptosis

Through its remarkable ability to modulate NF-kB expression and activation curcumin attenuates CRC cells (HCT116, HT29, Caco-2) growth by interfering with cell cycle regulatory proteins, arresting the cells in the G1, S/G2 or G2/M phase (Aggarwal *et al.*, 2003). Subsequentially, curcumin induces apoptosis by inhibiting

the expression of anti-apoptotic factors Bcl2, Bcl-xl, stimulating the expression of pro-apoptotic proteins: Bax, Bal, Bok, p21, p27 and (but not necessary) tumor suppressor p53 (Moos *et al.*, 2004; Shehzad *et al.*, 2013), activating caspase 3 and finally inducing the release of cytochrome c from mitochondria (Kunnumakkara *et al.*, 2008). Additionally, modulation of signaling pathways controlled by EGFR (Chen *et al.*, 2006), COX2 (Goel *et al.*, 2001), MAPKs, AMPKs and Wnt/β catenin (Collett and Campbell, 2004; Jaiswal *et al.*, 2002) or interfering with the ubiquitin-mediated degradation of proteins in the proteasome machinery, are effects described for curcumin induced-apoptosis (Hasima and Aggarwal, 2014).

Modulation of epigenetic events

Targeting epigenetic events in cancer cells is nowadays a strategy to prevent aberrant DNA methylation, histone acetylation/deacetylation, or miRNA expression (Lao and Grady, 2011; Vaiopoulos *et al.*, 2014). Curcumin is a recognized inhibitor of these epigenetic alterations exerting its anti-cancer effect at least in part through epigenetic modulation of global DNA hypomethylation or local DNA-hypermethylation in human HCT116 and HT29 CRC cells (Guo *et al.*, 2015b; Link *et al.*, 2013). Aberrant acetylation/deacetylation affecting histones or non-histones proteins, occur post-translationally and have been associated with CRC (Sadoul *et al.*, 2008). Curcumin has been found to be a potent inhibitor of the activity of both histone acetyltransferases and histone deacetylases (Reuter *et al.*, 2011). miRNAs are short, non-coding RNAs regulating post-transcriptionally the gene expression. Their altered expression has been associated with cell proliferation, growth, angiogenesis, migration and apoptosis of cancer cells. They have both oncogenic or tumor suppressor activities, which can be regulated by various agents, offering great anti-tumor therapeutic perspectives. While, in some studies, curcumin or its analogues have been demonstrated to downregulate oncogenic miRNA21 involved in migration, invasion and proliferation of HCT116 CRC cells, in other studies curcumin upregulated the tumor suppressive miRNA34a, miRNA27a, thus inhibiting cancer growth *in vitro* and *in vivo* (Gandhy *et al.*, 2012; Toden *et al.*, 2015a; Toden *et al.*, 2015b).

Modulation of inflammatory pathways and angiogenesis

It has long been recognized that targeting tumor-associated inflammation and angiogenesis, with different compounds (statins, glucocorticoids, non-steroidal anti-inflammatory drugs etc.) is both an attractive and efficient therapeutic anti-cancer strategy (Banciu, 2007; Rayburn *et al.*, 2009). In CRC tumor inflammation is driven mainly by the overexpression of the ubiquitous transcription factor NF-kB

(Voboril and Weberova-Voborilova, 2006). Curcumin inhibits the production of soluble mediators produced by tumor cells or the tumor-associated cells, entertaining a pro-inflammatory milieu (Casey *et al.*, 2015), by inhibiting, *in vitro* and *in vivo*, the activation by phosphorylation of NF-kB and its downstream inflammatory regulated effectors: COX-2, TNF-α, IL-6, PGE_2, MMP3, MMP9, ROS, iNOS and most importantly VEGF (Aggarwal *et al.*, 2006). VEGF, the major pro-angiogenic factor, is synthetized and activated by hypoxia, inflammation, oxidative stress, and other growth factors (bFGF, EGF, TGF-B, PDGF-BB). Curcumin has been shown to inhibit the activation of HIF-1α, which is constitutively express in hypoxic area of solid tumors, therefore inhibiting its main targets- VEGF or bFGF, MMP-1,2,3,9 and TIMP (Yadav and Aggarwal, 2011). In melanoma or colon tumors, macrophages have been shown to dominate the inflammatory infiltrate, manifesting a dual phenotype, depending on the localization and stage (Sica and Mantovani, 2012). They are equally responsible for sustaining tumor development by producing IL-1, IL-6, IL-10, TNF-α, IL-21, VEGF, TGF-b, MMPs, ROS, NO, or impairing it by phagocytosis of cancer cells or production of anti-inflammatory/anti-angiogenic factors (Erreni *et al.*, 2011). Curcumin has the ability to reeducate tumor associated macrophages as shown in animal models for breast cancer, activating their intrinsic anti-tumor functions (Shiri *et al.*, 2015; Zhang *et al.*, 2013). This offers great therapeutic perspectives for CRC treatment.

Modulation of invasion and metastasis

Cancer cells migration process at secondary sites is tightly controlled by growth factors, cytokines and cell adhesion molecules, as well as intracellular signaling systems (Chambers *et al.*, 2002). Among these up- regulation of NF-kB expression and deregulation of the Wnt/β-catenin signaling pathway, which is a major regulator of the cell proliferation, motility and migration, has been suggested to be a major cause of malignant dissemination (Chen *et al.*, 2013). Similarly, loss of E-cadherin and enhanced activity of matrix metalloproteinases contribute to tumor cells ability to invade and metastasize. Curcumin was shown to affect both Wnt signalling and cell-cell adhesion pathways (Jaiswal et al., 2002; Narayan, 2004) in human CRC cell lines (HCT-116, HT-29, HCT-15, HCC-2998, Colo205) or in animal models, inhibiting NF-kB, PKC, RhoA, MMP-2, MMP-9, COX2 gene expressions and enhancing E-cadherin expression, thereby preventing cancer cell invasion and metastatic potential (Shen *et al.*, 2014). Recently, it has been shown, that curcumin exerts anti-metastatic effects by modulating the TGF- β-mediated crosstalk between human HCT116 CRC cells and human fibroblasts (MRC-5) in co-culture. TGF-β is a major metastatic promoter of cancer cells (Buhrmann *et al.*, 2014).

Modulation of reactive oxygen species production

In cancer cells, low level of ROS foster the survival and maintenance of cellular viability and phenotype, whereas aberrant ROS production induces a cellular redox imbalance, which causes macromolecular damage and finally, cell death (Martindale and Holbrook, 2002). Curcumin functions, at low concentrations as an antioxidant (upregulating the antioxidants levels) whereas at higher concentrations curcumin manifests prooxidant cytotoxic activity (Das and Vinayak, 2014). This is due to curcumin ability to interfere with the expression and activation of different transcription factors (NF-kB, AP-1, Nrf-2), their downstream effectors (COX-2, MMPs, iNOS, VEGF, PPAR-γ) or signaling pathways (ERK, PI3K/Akt, JNK) (Lin, 2007; Surh, 2003). ROS-mediated cytotoxicity of curcumin has been demonstrated in human CRC cell lines (Colo205, HCT116, HCT115, HT29) or animal models (Su *et al.*, 2006).

Modulation of tumor energy metabolism

A hallmark of neoplastic transformed cells, is the deregulated energy metabolism. Cancer cells consume much higher level of glucose, fatty acids and glutamine to ensure their anabolic growth (DeBerardinis *et al.*, 2008). Glycolysis, the main energy furnisher in cancer cells, results also in generation of high levels of lactate and H^+ which in turn acidifies the tumor microenvironment promoting tumor invasion, as well as precursors for the synthesis of nucleotides or fatty acid synthesis (Zhao *et al.*, 2013). In addition to glucose, cancer cells rely also on glutaminolysis to support their growth, as glutamine is an important aminoacid fueling the TCA cycle (Phan *et al.*, 2014). Almost all of the glycolysis and TCA cycle participants, have been linked with cancer cell growth, invasion, metastasis (Kim and Dang, 2005). In several cancer models, the glucose transporters (Glut1, Glut3, Glut4) (Macheda *et al.*, 2005) and/or glycolytic enzymes (HKII, PFK, GAPDH, PK, LDH) (Zhang and Yang, 2013) are upregulated by cooperation of c-MYC and HIF-1α. MYC-enhanced glutamine catabolism is also observed (Kim *et al.*, 2007). Inhibiting key metabolic enzymes in CRC cells, would offer great therapeutic perspectives, as a recently published study from Wang *et al.*, 2015, describes pro-apoptotic action of curcumin on human HCCT116 and HT29 CRC cells, mediated by direct inhibition of the rate limiting glycolytic enzyme Hexokinase II in an Akt-dependent manner (Wang *et al.*, 2015). Moreover, administration 5-FU and dicholoracetate has already been shown to inhibit pyruvate dehydrogenase kinase *in vitro*, in human CRC cell lines LS174T, LoVo, SW620, and HT29 (Tong *et al.*, 2011). It is though tempting to speculate, that a powerful anti-cancer effect would result from the co-treatment of cancer cells with curcumin and 5-FU, in an attempt to blunt their energy metabolism. However, there should be great concern regarding the specificity of such treatments, as glycolysis occurs in both neoplastic and healthy cells.

2. Strategies aiming at improving the efficacy of curcumin in CRC treatment

Curcumin administration in combination with cytotoxic agents

It was demonstrated that the administration of therapeutic agents in combination usually engenders a greater anti-tumor effect, over monotherapy. Chemotherapeutics in CRC include oxaliplatin, irinotecan, capecitabine and 5-FU. Experimental studies show that curcumin is able to synergize with some of these agents, in the anti-tumor actions (Patel and Majumdar, 2009). Particularly, enhanced apoptotic effects were observed for 5-FU who remains the main chemotherapeutic agent for the treatment of both colorectal and breast cancer. Its anti-tumor effect in mammalian cells results from its ability to block the activity of thymidylate synthase, an enzyme involved in DNA replication and repair, leading to inhibition of cellular growth and apoptosis (Longley *et al.*, 2003). 5-FU clinical efficiency is limited by its low specificity for the target tumor tissue, the low biodisponibility, and especially the accelerated degradation by the liver. Additionally, the fact that tumor cells often develop resistance to this chemotherapeutic agent, by activating other salvaging signaling pathways, impedes the successful treatment of CRC in suffering patients (Malet-Martino and Martino, 2002). Curcumin or its analogues administrated as an adjunct to the chemotherapeutic drug 5-FU, offers a promising strategy for the treatment of CRC. *In vitro* studies on human CRC cell lines HCT116 envisage the ability of curcumin co-administrated to 5-FU, to reduce the proliferation and viability of cancer cells and to induce apoptosis by blocking the expression and activity of the constitutively activated NF-kB (Shakibaei *et al.*, 2013). When combined with oxaliplatin, curcumin is able to inhibit colon carcinoma *in vivo*, in nude mice xenografted with human CRC cells (LoVO) in an apoptotic manner (Guo *et al.*, 2015a). Multiple targeting of human CRC cells HCT116 or HT29, with 5-FU, oxaliplatin and curcumin, resulted in higher cytotoxic effects than each of the individual or dual treatments. Altered EGF-R, IGF-1R, Akt signaling pathway or COX-2 expression were associated with this cytotoxic action (James *et al.*, 2015; Shakibaei *et al.*, 2015). Additionally, inclusion of curcumin in conventional therapeutic regimens is an effective strategy to reverse the chemoresistance of cancer cells (Goel and Aggarwal, 2010). Curcumin reverses the multidrug resistance of CRC cells to vincristine, cisplatin, and hydroxycamptothecin *in vitro* and *in vivo* (Lu *et al.*, 2013) and enhances the chemosensitivity to 5-FU in human CRC cells (HCT116 or HT29) (Shakibaei *et al.*, 2014; Shakibaei *et al*., 2015; Toden *et al*., 2015b). Moreover, when applied on CRC cells and MRC-5 fibroblasts co-culture in a monolayer or high density tumor microenvironment model *in vitro*, curcumin and 5-FU were able to suppress cell synergism in tumor microenvironment and sensitize the cells to 5-FU (Buhrmann *et al*., 2014).

Administration of synthetic curcumin analogues

Another approach to overcome pharmacological problems for curcumin and enhance its efficacy is the design of new compounds which are superior to curcumin in the anti-tumor actions in various *in vitro* and *in vivo* models for colorectal tumorigenesis,

while retaining their non-toxicity to healthy tissues. Dimethoxy-curcumin, diphenyl-difluoroketone-EF24, hexahydroxycurcumin, difluorinated-curcumin are some examples of analogues of curcumin, chemically modified in relation to the parent compound (by methylation, reduction, condensation etc), found to affect cancer cells development with higher potency than curcumin. In HCT116 CRC cells dimethoxy-curcumin inhibited proliferation and induced apoptosis (Tamvakopoulos *et al.*, 2007). Diphenyl-difluoroketone tested on HCT116 and HT29 CRC cells, manifested anti-tumor actions by arresting the cells in G2/M phase of the cell cycle, reducing VEGF and COX2 expression, and inducing caspases- mediated apoptosis (*in vitro* and *in vivo*) (Subramaniam *et al.*, 2008). Another derivative of curcumin-hexahydroxycurcumin combined with 5-FU exhibited potent anti-tumor activity on HT29 human CRC cells by inhibiting COX2 mRNA and protein expression (Srimuangwong *et al.*, 2012). Moreover, difluorinated curcumin (CDF), was shown to inhibit the growth of CRC cells resistant to 5-FU and oxaliplatin, due to reduced expression of miR-21, therefore sensitizing the cells to these chemotherapeutics (Roy *et al.*, 2013).

Encapsulation of curcumin in nanoparticles

Although curcumin has theoretically, a huge therapeutic potential, its low solubility in aqueous environments, poor absorption, the high degree of instability and hepatic and intestinal rate of degradation limit its uses in preclinical and clinical studies (Shehzad *et al.*, 2010). To overcome these issues and to substantially improve curcumins biological activity different nanoformulations have been developed with aim of passively or actively targeting cancer cells as presented in detail in Table 1. For a detailed overview of these formulation please consult (Yallapu *et al.*, 2013). For example polymeric nanoparticles, liposomes, cyclodextrines, etc, have been ascribed to efficiently incorporate curcumin, stabilize the compound, enhance its cellular uptake, and cytotoxicity (Yallapu *et al*., 2013).

Moreover, to actively target the tumor cells with curcumin, functionalized bioconjugates have been also developed. One such hibrid formulation contains a hydrophobic core (poly(D,L-lactide-co-glycolide)(PLGA), a lipid based monolayer surrounding the PLGA and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-carboxy (polyethylene glycol) 2000 (DSPE-PEG_{2000}-COOH)-shell. This shell enhances the half-time of curcumin and binds a small RNA fragment (Aptamer) directed agains adhesion molecules overexpressed on CRC cells. Enhanced cellular uptake and cytotoxicity for this nanoformulation has been reported on HT29 human CRC cells (Li *et al*., 2014a). In colorectal cancer, administration of 5-FU as a free drug has a poor therapeutic effect due to the lack of the tumor site specificity, rapid metabolization/ degradation and associated side effects. Subsequently, many nano-formulations of the drug were developed with the aim to shorten the main drawbacks we mentioned as shown in Table 1 and described in detail elsewhere (Arias, 2008).

Table 1.

Curcumin and 5-FU nanoformulations used in CRC treatment

Nano-formulations for delivering curcumin	*Pre-clinical study*	*References*
Liposomes	*In vitro*, *in vivo*	(Lin *et al.*, 2012; Rahman *et al.*, 2012)
Pluronic/Polycaprolactone micelles	*In vitro*	(Raveendran *et al.*, 2013)
Polymeric micelles in thermosensitive hydrogel system	*In vitro*, *in vivo*	(Zhang *et al.*, 2015)
Polymeric nanoparticles	*In vitro*, *in vivo*	(Tan *et al.*, 2014)
Albumin nanoparticles	*In vitro*	(Kim *et al.*, 2011)
Thiolated chitosan nanoparticles	*In vitro*, *in vivo*	(Anitha *et al.*, 2014)
Cyclodextrin complexes	*In vitro*	(Yadav *et al.*, 2010)
Glycerol monooleate and pluronic F-127 based nanoparticles	*In vitro*	(Mohanty and Sahoo, 2010)
Silica nanoparticles	*In vitro*	(Singh *et al.*, 2015)
Eudragit S100 nanoparticles	*In vitro*	(Prajakta *et al.*, 2009)
Chitosan and gum arabic nanoparticles	*In vitro*	(Udompornmongkol and Chiang, 2015)
PLGA-lecithin-PEG–Apt-nanoparticles		(Li *et al.*, 2014a)

Nano-formulations for delivering 5-FU	*Pre-clinical study*	*References*
Enteric-coated chitosan polymeric nanoparticles	*In vitro* drug release studies	(Tummala *et al.*, 2015)
Polymeric hydrogels	*In vitro*	(Mishra *et al.*, 2014)
Poly(ε-caprolactone) nanoparticles	*In vitro*	(Ortiz *et al.*, 2012)
Solid lipid nanoparticles	*In vitro*	(Yassin *et al.*, 2010)
Thiolated Chitosan nanoparticles	*In vitro*, *in vivo*	(Anitha et al., 2014)
Hyaluronic acid coupled chitosan nanoparticles	*In vitro*	(Jain and Jain, 2008)
Chitosan nanoparticles	*In vitro*	(Li *et al.*, 2014b)
Magnetoliposomes	*In vitro*	(Clares *et al.*, 2013)
Layered double hydroxide nanoparticles	*In vitro*	(Chen *et al.*, 2014)

Conclusions

Curcumin is a promising natural compound able to target multiple processes associated with CRC. To remarkably improve its efficacy, co-administration with chemotherapeutic agents or the use of non-toxic analogues of curcumin, which, as described, seem to be superior in action to curcumin, might be a reasonable approach for CRC treatment. However, an active targeting strategy, by using functionalized nanoformulations, remains until now, the best alternative, to take advantage of curcumin cytotoxic effects on cancer cells. To demonstrate the clinical potential of such formulation, remains to be properly assessed.

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