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THE EPIGEAN FRESHWATER MALACOSTRACANS (CRUSTACEA: MALACOSTRACA) OF THE RIVERS IN THE ANINA MOUNTAINS (SW ROMANIA)

LUCIAN PÂRVULESCU[1]

SUMMARY. This paper represents a biogeographic study on the distribution of freshwater crustacean species of the Astacidae (Decapoda), Gammaridae (Amphipoda) and Asellidae (Isopoda) Malacostracans in the rivers of the Anina Mountains, in Southwestern Romania. Two species of crayfish, three species of Amphipods and one species of aquatic Isopods have been identified. The specimens were captured by hand from the river bad (crayfish) or using Surber collector (for small malacostracans). The data come to complete the existent data as to the distribution and the relative size of the population in the two national parks located in the investigated area - the Semenic-Cheile Caraşului (Semenic - Caras Gorges) National Park and the Cheile Nerei-Beuşniţa (Nera Gorges - Beuşniţa) National Park. The georeferenced data acquired can be used in the policymaking process for the protected areas located within this mountain range.

Keywords: Anina Mountains, *Asellus*, *Astacus*, *Austropotamobius*, distribution, *Gammarus*

Introduction

Freshwater malacostracans residing in the Romanian aquatic ecosystems belong to the Decapoda, Amphipoda and Isopoda orders (Băcescu, 1967), and they also represent water quality indicators (Chapman and Jackson, 1996). Of all the decapods, the stone crayfish *Austropotamobius torrentium* is the most relevant indicator of the habitat's health (Streissl and Hödl, 2002) due to the fact that it is very sensitive to water pollutants (Machino and Füreder, 2005). In the European Council's Directive 92/43, they are rated as "prioritary species" and thus a series of management measures are necessary within the protected areas, amongst which the periodic measurement of population density is of great importance (Pârvulescu, 2007). Moreover, both *A. torrentium* and *Astacus astacus* are considered to be "vulnerable" (IUCN, 2008). Thus, crayfish remain vulnerable to various threats: overexploitation, habitat modification and loss, pollution, the spread of non-indigenous crayfish species, crayfish plague (Holdich and Pöckl, 2005).

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The majority of amphipod species reside in marine or brackish environments, but they also inhabit a wide range of freshwater habitats, in close relation to the environmental conditions (Ciubuc, 1985). Species diversity is higher in flowing waters, either cold, or subterranean, while the number of epigeal species is lower (Väinölä *et al.,* 2008). As to isopods, the majority of species are marine, some species are adapted to terrestrial environments and very few of them prefer freshwater environments exclusively (Müller and Tomescu, in Godeanu, 2002).

The data published in regard to freshwater crayfish species in the Anina Mts. area are few and obsolete, and many of the locations mentioned are no longer in accordance with present reality. First records of the *Austropotamobius torrentium* in the Anina Mts. place this species in the Minis River, in Buhui and around Anina (Entz, 1912). In the Decapoda volume of the "Romanian Fauna", the species is mentioned in the Minis River, in the Righidia stream (affluent of the Minis, in the area around the village of Bozovici), as well as in the Ponicova stream – an affluent of the Caras that crosses the Comarnic Cave (Băcescu, 1967). Bănărescu and Oprescu (1971) mention the species in the affluent of the Nera River in the mountainous area, but without specifying the exact locations. The distribution map presented by Machino and Füreder (2005) for *A. torrentium*, refers only to a broad European context. Machino and Holdich (2006) mention the species in Southwestern Transylvania and in Western Walachia. The *Astacus astacus* species is mentioned in the literature as present in the Anina Mts. in the Buhui Lake (cohabitating with *A. torrentium*), in the Minis river (Băcescu, 1967) and in the Nera river (Bănărescu and Oprescu, 1971). There is no mention of the presence of *A. leptodactylus* or any other decapods in the Anina Mts. In the work of Machino and Holdich (2006), the only reference is that related to the distribution of *A. astacus* and *A. leptodactylus*. The most recent monograph on crayfish species of Europe, "Atlas of crayfish in Europe" (Souty-Grosset *et al.*, 2006) reflects the deficiency in data on the distribution of the three native crayfish species of Romania.

An important aspect of biodiversity conservation is the biocontamination of benthic macro invertebrates with alien species (Arbačiuskas *et al.*, 2008). Currently, nine species of freshwater crayfish are known to reside in Europe. These species were intentionally introduced here during the XIXth and XXth century. Four of these species belong to the *Orconectes* genus, one to *Procambarus*, three to *Cherax* and also one to *Pacifastacus* (Holdich and Pöckl, 2007; Souty-Grosset *et al.*, 2006; Henttonen and Huner, 1999). In many cases, these species have their own way of spreading from one area to another, through canals and rivers or during floods (Holdich and Pöckl, 2007). Among the countries that are close to Romania and are found upstream of the Danube, Hungary, Croatia and Slovakia have already been infested with 2 species: *Pacifastacus leniusculus* and *Orconectes limosus* (Puky *et al.*, 2005; Janský and Kautman, 2007; Petrusek and Petruscová, 2007; Maguire *et al.*, 2008; Maguire and Klobučar, 2008; Puky, 2009); in Serbia, *O. limous* is present (Pavlović

et al., 2006; Simić *et al.*, 2008). These species are potential competitors of the native species (Holdich, 2003) as well as potential carriers of the deadly crayfish plague *Aphanomyces astaci* (Kozubiková *et al., in press*). On the other hand, the Danube river acts like a passage for the upstream spreading of several amphipod species. During the last few years, these species have largely expanded from the Balkan and the Ponto-Caspian regions towards northwestern Europe (Grabowski *et al.*, 2007a), Thus, some species native to Romania have managed to colonize western Europe through the Rhine-Danube system, connected for the first time by the Ludwigskanal in 1845: *Echinogammarus ischnus, Pontogammarus robustoides, Obesogammarus crassus, Dikerogammarus haemobaphes, D. villosus* (Grabowski *et al.*, 2007c); ***D. villosus*** has even reached the alpine lake Lac du Bourget in France (Grabowski *et al.*, 2007b).

The investigated area, i.e. the Anina Mountains, is located in southwestern Romania; it has a surface of 770 km^2 (Sencu, 1978) mostly included in two National Parks: Semenic-Cheile Caraşului (Semenic – Caras Gorges) National Park and Cheile Nerei-Beuşniţa (Nera Gorges - Beusnita) National Park. The relief is mainly calcareous (Bleahu and Rusu, 1965). This geographical unit is drained by three main water courses: the Bârzava River, the Caraş River and the Nera River. The Bârzava River collects its waters from the northern and northeastern part of the Anina Mountains and from the western part of the Semenic Mountains, crossing a substratum of crystalline schists. The upstream stretch of the Caraş River drains the central-western Anina Mountains, of calcareous composition. The Nera River crosses the southern sector of these mountains, draining the waters from the southern and central-eastern sector; its main tributary, the Miniş River, crosses a calcareous substratum. The Caraş and the Nera rivers are the direct tributaries of the Danube River, and the Bârzava River flows into the Danube River, after the confluence with the Timiş River (Ujvari, 1972).

Since the existing data related to the distribution of freshwater crustacean species is rather obsolete, it is necessary to update this data in view of a better management of this region.

Materials and methods

Sampling sites and collection of specimens. Qualitative and quantitative biologic samples were collected in August 2007 and in August 2008 respectively, in a total of 52 sampling stations, on all the permanent waters in the upper sector of the Bârzava, Caraş and Nera rivers (Figure 1). Most of these sampling stations are located within two National Parks (Semenic-Cheile Caraşului National Park and Cheile Nerei-Beuşniţa National Park).

Each sampling station was comprised of at least 100 m of river under investigation. On this occasion, observations were carried out with regard to the riverbed morphology and the surrounding habitats. The crayfish were collected using

active methods (Dorn *et al.*, 2005), i.e. direct hand sampling from the water bed, by checking the spaces between rocks, roots and galleries within banks, and by sampling over 100 meters along each of the rivers investigated. If specimen collection was rendered impossible by the morphological features of the substratum, we declared the species as being absent when, after sampling over 200-300 meters, we found no specimens.

For the quantitative collection of small freshwater crustaceans (Amphipods and Isopods), we used a Surber sampler with a catchment area of 300 cm^2 and a mesh size of 350 μm, followed by further research among solid objects and plants on the bottom of the river. The collecting surface was established at five randomly chosen different squares per sampling station, on the 100-metre river sector, for covering more microhabitats.

Statistical analysis. Once the number of individuals captured on the river sector under investigation was established, the approximate dimension of the freshwater crayfish population was determined for each sampling station and employed in statistical analyses. To perform the statistical analysis and create the diagrams, we used the PAST software (version 1.88). Similarity between the preferences of the 6 malacostracans species, from the 52 sampling stations, were represented by the cluster correlation analyzes (Ceapoiu, 1968).

Identification of specimens. The crayfish were identified *in situ* according to their morphological features, sexed and photographed. Tissue for subsequent molecular analysis was sampled, by detaching the last pereiopod on the right and conserving it in alcohol (80%). Moreover, an inspection was carried out, to determine the specimens' health status and potential parasites (Holdich, 2003). Subsequently, the specimens were set free in the same location where they had been captured. To identify the specimens, we used the keys in Băcescu (1967) and Ingle (1997).

The amphipods and the isopods were brought to the laboratory, processed and analyzed using the optic stereomicroscope; for details, micro dissections and digital photography were carried out. The identification of amphipods was carried out on the species level, using the Karaman and Pinkster (1977, 1987) and the Cărăuşu *et al.* (1955) determination keys, and the Racovitza (1919) and Negoescu (1987, 1989) publications, respectively. Subsequently, we updated the data systematically, in accordance with the data in the Fauna Europaea project (Geoffrey, 2007; Geoffrey and Ronald, 2007). The collected specimens have been conserved in alcohol (70%), in our Faculty collection.

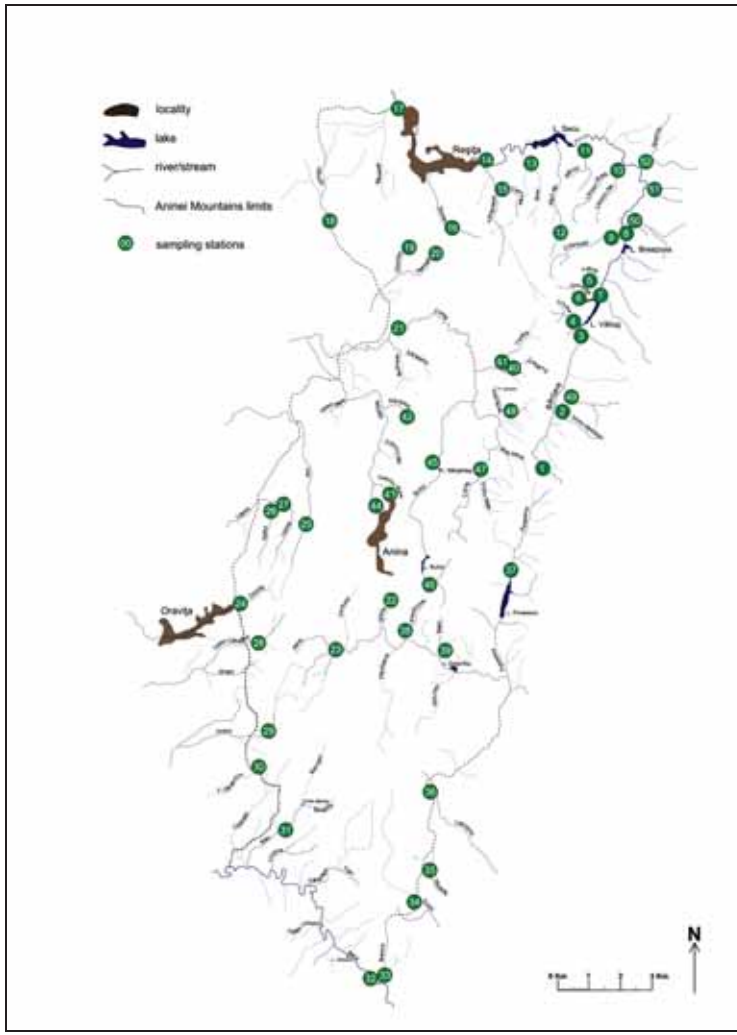

Fig. 1. Distribution of the 52 sampling stations in the lotic aquatic habitats of the Anina Mountains (see Table 1 for geographical coordinates and toponyms)

Results

Based on the findings of the 52 sampling stations dispersed over all the permanent rivers in the Anina Mountains, we were able to create a high-resolution image of the epigean species distribution and the relative dimension of the populations belonging to the Astacidae, Gammaridae and Asellidae groups. For a more detailed image, we will go over each hydrographical basin and its tributaries. To be noted that the rivers are presented according to their geographical position in Table 1.

The Bârzava hydrographical basin was investigated starting from the upper part (near the spring) up to the exit from the Anina Mountains (downstream from the town of Reşiţa) representing a total of 21 sampling stations, 6 on the main course of the river and 15 on its tributaries. As a result to the investigation of the main course, the *Astacus astacus* (Linnaeus 1758) decapod was identified in one location, upstream from the Văliug Lake. The amphipods were present in all the sampling stations (except downstream from the town of Reşiţa and downstream from the Văliug Lake) by means of *Gammarus balcanicus* Schäferna 1922 and *Gammarus fossarum* Koch, in Panzer 1835 species, while the aquatic isopods, by means of *Asellus aquaticus* (Linnaeus 1758), were present only downstream from the town of Reşiţa, in a oozy habitat. The 15 investigated tributaries of the Bârzava River revealed the presence of *A. astacus* and *Austropotamobius torrentium* (Schrank 1803) decapods. *A. astacus* is also present in the river's tributaries which flow directly into the Văliug Lake (Crivaia and Grindeşti) and also in the Doman River, while *A. torrentium* is present in the majority of the tributaries (Dignacea, Crainicului, Radomir, Bogatu, Liscov, Stârnic, Râul Alb, Cuptoare creeks). The *G. balcanicus* amphipod was found in Molidului, Dignacea, Crivaia, Grindeşti, Văliug, Crainicului, Radomir, Bogatu, Râul Alb, Groposu, Liscov, Cuptoare, Stârnic, Secu, while *G. fossarum* was found only in the Doman river.

The Caraş hydrographical basin was investigated in the upper part up to the exit from the mountain range, downstream from the Caraş Gorges, as well as its tributaries with a permanent water course, representing a total of 20 sampling stations. The *A. astacus* decapods were present in 13 sampling stations (Clocotici, Oraviţa, Jitin, Natra, Lişava, Călugăra, Ponicova, Comarnic, Topliţa, Răviştea, Celnicu Mare, Buhui downstream from Mărghitaş Lake and also upstream from Buhui Lake), while *A. torrentium* was present in only 2 locations at Cândeni and Buhui creek, upstream from Buhui Lake; in the last sampling station both species were captured, the specimens of *A. torrentium* being numerically predominant. The amphipods were identified in most of the locations: *G. balcanicus* (Buhui, Ponicova, Caraş creek, Răviştea, Comarnic, Topliţa, Celnicu Mare, Jitin, Oraviţa, Lişava, Călugăra, Cândeni, Vicinic and Caraş downstream from the Gorges) and *G. fossarum* (Oraviţa, Lişava, Natra, Nermed and Clocotici), while *Gammarus roeseli* Gervais 1835 was identified in two stations (Caraş downstream from the Caraş Gorges and Gelugu creek). The captured aquatic isopods (*A. aquaticus* species) were present in one of the sampling station, Gelugu River, where the habitat was represented by a dim water course with a muddy substratum.

Table 1.

Distribution of freshwater crustaceans, relative dimension of the populations and frequency in Anina Mountains Rivers (2007-2008)

Col. pts.	Location	Geographical coordinates N/E	Relative altitude	*Ast.ast*	*Aus.tor*	*Gam.bal*	*Gam.fos*	*Gam.roe*	*Ase.aqu*
			Bârzava basin						
A1	Bârzava creek	45°07’15’’/ 21°59’23’’	900 m			○			
A2	Molidului	45°10’06’’/ 21°59’59’’	700 m			●			
A49	Dignacea	45°10’55’’/ 22°00’19’’	700 m		○	●			
A3	Bârzava (ups. Văliug Lake)	45°12’03’’/ 22°00’42’’	655 m	○		○			
A4	Crivaia	45°12’10’’/ 22°00’38’’	658 m	○		●			
A6	Grindeşti	45°13’14’’/ 22°00’33’’	650 m	●		■			
A5	Văliug	45°13’50’’/ 22°00’49’’	600 m			■			
A7	Bârzava (dws. Văliug Lake)	45°13’11’’/ 22°01’28’’	560 m						
A8	Bârzava (dws. Breazova Lake)	45°15’10’’/ 22°02’58’’	480 m			●			
A9	Crainicului	45°15’23’’/ 22°02’37’’	460 m		○	●			
A50	Radomir	45°15’52’’/ 22°02’54’’	475 m		○	●			
A51	Bogatu	45°16’45’’/ 22°03’45’’	450 m		●	●			
A12	Râul Alb	45°17’32’’/ 21°59’41’’	430 m		○	●			
A52	Groposu	45°17’28’’/ 22°03’43’’	415 m			●			
A10	Liscov	45°17’32’’/ 22°02’33’’	380 m		○	●			
A15	Cuptoare	45°16’36’’/ 21°57’35’’	360 m		○	●			
A11	Stârnic	45°18’13’’/ 22°02’43’’	330 m		○	■			
A16	Doman	45°15’38’’/ 21°54’18’’	320 m	●			●		
A13	Secu	45°17’48’’/ 21°58’14’’	300 m			■			

Table 1. (continued)

Col. pts.	Location	Geographical coordinates N/E	Relative altitude	*Ast.ast*	*Aus.tor*	*Gam.bal*	*Gam.fos*	*Gam.roe*	*Ase.aqu*
A14	Bârzava m.c. (ups. Reşiţa)	45°17'39''/ 21°56'15''	275 m				●		
A17	Bârzava m.c. (dws. Reşiţa)	45°20'36''/ 21°50'08''	190 m						■
	Species frequency in the Bârzava basin (%)			19	38.1	80.9	9.5	0.0	4.7
			Caraş basin						
A46	Buhui creek	45°03'51''/ 21°53'20''	660 m	○	●				
A45	Buhui (dws. Mărghitaş Lake)	45°07'31''/ 21°54'00''	530 m	○		●			
A48	Ponicova	45°09'26''/ 21°57'03''	515 m	●		●			
			Caraş basin						
A47	Caraş creek	45°07'23''/ 21°56'00''	500 m			○			
A42	Răviştea	45°03'10''/ 21°52'35''	475 m	■		●			
A40	Comarnic	45°10'46''/ 21°57'10''	470 m	●		●			
A41	Toplita	45°10'56''/ 21°56'56''	465 m	●		●			
A43	Celnicu Mare	45°06'48''/ 21°51'46''	435 m	■		●			
A44	Gârliste	45°06'23''/ 21°50'53''	420 m						
A25	Jitin	45°07'13''/ 21°48'02''	395 m	●		●			
A24	Oravita	45°03'07''/ 21°45'02''	390 m	●		●	■		
A27	Lişava	45°06'22''/ 21°46'39''	300 m	●		●	●		
A28	Călugăra	45°01'45''/ 21°45'15''	300 m	○		●			
A30	Cândeni	44°56'49''/ 21°44'08''	295 m		■	■			
A26	Natra	45°06'21''/ 21°46'09''	295 m	■			■		
A29	Vicinic	44°58'28''/ 21°44'37''	292 m			■			
A20	Nermed	45°13'59''/ 21°52'26''	275 m				■		
A19	Clocotici	45°14'43''/ 21°50'28''	270 m	■			●		

Table 1. (continued)

Col. pts.	Location	Geographical coordinates N/E	Relative altitude	*Ast.ast*	*Aus.tor*	*Gam.bal*	*Gam.fos*	*Gam.roe*	*Ase.aqu*
A21	Caraş (dws. gorges)	45°12'06''/ 21°52'27''	240 m			●		●	
A18	Gelugu	45°16'01''/ 21°48'31''	230 m			○		■	●
Species frequency in the Caraş basin (%)				65	10	75	25	10	5
Nera basin									
A23	Miniş creek	45°01'29''/ 21°49'24''	595 m		●	●			
A22	Şteier	45°02'38''/ 21°51'34''	540 m			■			■
A38	Predilcova	45°01'57''/ 21°52'39''	505 m		●	■			
A37	Poneasca	45°03'29''/ 21°57'36''	465 m		○				
A39	Babii	45°01'18''/ 21°54'24''	350 m		○	●			
A36	Lăpuşnic	44°55'03''/ 21°55'37''	298 m		■	●			
A35	Moceriş	44°53'36''/ 21°53'53''	298 m		●	●			
A34	Ducin	44°52'31''/ 21°53'47''	280 m		●	●			
A31	Beiu	44°55'22''/ 21°46'28''	240 m		●	■			
A33	Bresnic	44°50'27''/ 21°51'21''	205 m			●			
A32	Nera m.c. (dws gorges)	44°50'19''/ 21°51'18''	200 m						
Species frequency in the Nera basin (%)				0.0	72.7	81.8	0.0	0.0	5
Species frequency in the rivers of the Anina Mountains (%)				**32.7**	**34.6**	**78.8**	**13.5**	**3.8**	**5.8**
Total specimens				**69**	**113**	**1567**	**1779**	**139**	**228**
Uncertain juveniles				**3**			**155**		**0**

Observations: the symbols in the cells have the following meanings for crayfish: ○ = rare (<0.04 ind/m^2); ● = present (0.05-1 ind/m^2); ■ = common (>1 ind/m^2); for amphipods and isopods: ○ = rare (<10 ind/m^2); ● = present (11-100 ind/m^2); ■ = common (>100 ind/m^2), empty cell = absent

Abbreviations: *Ast.ast = Astacus astacus*; *Aus.tor = Austropotamobius torrentium; Gam.bal = Gammarus balcanicus; Gam.fos = G. fossarum; Gam.roe = G. roeseli; Ase.aqu = Asellus aquaticus*; m.c. = main course; ups = upstream; dws = downstream

As far as the similarity in the preferences for a specific habitat (Figure 2) is concerned, no significant links were noticed among the six aquatic Malacostraca species in the Anina Mountains. However, affinities are noticed between the *Gammarus roeseli* amphipod and the *Asellus aquaticus* isopod, and between the *Gammarus fosarum* amphipod and the *Astacus astacus* decapod. A weak but positive-evidence association is determined for the *Gammarus balcanicus* amphipod and the *Austropotamobius torrentium* decapod, which were observed at the same sampling stations.

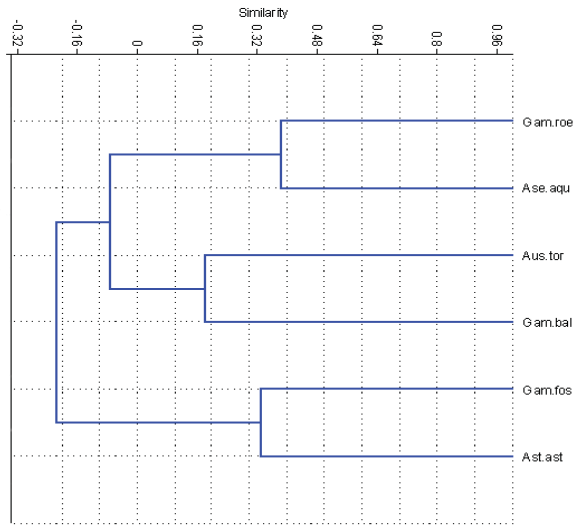

Fig. 2. The association of Malacostraca species in the rivers of Anina Mountains (coph. corr. 0.8434)

Discussion and conclusions

The researches made between summer 2007 and 2008 in the freshwaters of the Anina Mountains offer a good view regarding the distribution, frequency and relative dimension of the populations of the freshwater crustacean species in this region and the possibility of data interpretation.

By comparing our data to the data provided by previous research, one will notice that *A. astacus* can no longer be found in the Miniş River, any of its tributaries or the main course of the Nera River. *A. torrentium* was completely replaced by

A. astacus in the Ponicova stream. However, the co-existence of the two species upstream from the lake formed on the Buhui River is still confirmed. Taking into account that *Orconectes limosus* is a species which can live in small streams (Pöckl *et al.*, 2006), that it has already been attested in the area where the Nera river flows into the Danube, (Pârvulescu *et al.,* 2009), and that it is a potential carrier of the crayfish plague, which is deadly for the native crayfish species (Kozubiková *et al.*, *in press*), we consider that this species is a potential danger for the aquatic fauna of the Anina Mountains.

Since the diversity of the amphipods is low in the surface waters of Romanian mountains (Cărăuşu *et al.,* 1955), the presence of the three species from the Gammaridae family shows a good diversity of the aquatic habitats in the Anina Mountains. The most frequent species are *Gammarus balcanicus* (with a frequency of 78.8 %) and *Gammarus fossarum* (with a frequency of 13.5 %); the two species are common throughout the Balkan Peninsula. (Živić and Marcović, 2007) In Western Europe it is considered that *Gammarus roeseli* has extended its habitat in southeastern Europe. (Grabowski *et al.,* 2007c) Cărăuşu *et al*. (1955) mentions that *Rivulogammarus triacanthus* (sin. *Gammarus roeseli*) lives in the warmer waters of the Episcopia Bihor area (North-Western Romania).

A. aquaticus is a species living in slow lowland waters which display a higher degree of saprobity. Even if high numbers of specimens were collected at the Steier sampling station situated at an altitude higher than 500 meters, this was caused by the eutrophication of the stream as a result of pollutants released in the village of Steierdorf (Anina); the absence of the common but more pollution-sensitive crayfish (Machino and Füreder, 2005) in the Nera basin confirms this atypical situation. In the other two sampling stations the species was actually collected only in low-altitude streams polluted by various human activities specific to the villages and towns which the streams cross.

As far as the sensitivity of the three groups of freshwater crustaceans is concerned, according to the Biological Monitoring Working Party Score System (Chapman *et al.,* 1996), one notices that the Astacidae (score 8) and the aquatic Asellidae (score 3) do not cohabit. The Gammaridae's score 6 is not relevant for this comparison. We also notice that in the main course of the rivers, downstream from towns or reservoirs the diversity of freshwater crustaceans is very low. In the Bârzava River, downstream from the Văliug Reservoir (in the area right downstream of the dam), the freshwater crustacean are missing; the sample actually contains no insect larvae characteristic of that type of region. The absence of aquatic invertebrates at this sampling station is highly likely to be caused by the water from the deeper layers of the reservoir, which is rich in suspensions and low in oxygen (Pârvulescu and Hamchevici, *submitted manuscript*). *G. balcanicus* was reported at approximately 200 meters downstream from Breazova Lake. In the main course of Bârzava River, downstream from the town of Reşiţa, the aquatic crustacean fauna is represented by only one species and a high number of individuals: *A. aquaticus,* a species which

usually lives in high-nutrient waters. The results of the investigations carried out in the main course of Caraş River showed a normal diversity for that region, probably because there is no village or town upstream from the sampling station (Pârvulescu and Hamchevici, *submitted manuscript*). The absence of freshwater crustaceans in Nera River is most probably caused by the released pollutants upstream in the river, from the towns and villages (Pârvulescu, 2009; Pârvulescu and Hamchevici, *submitted manuscript*).

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NESTING AND REPRODUCTIVE CHARACTERISTICS OF THE GREAT CRESTED GREBE (*PODICEPS CRISTATUS*) BREEDING IN CÂMPENEŞTI PONDS FROM NW ROMANIA

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SUMMARY. The paper presents the results of nest and reproductive characteristics of the Great Crested Grebe (*Podiceps cristatus*) population that breeds solitary in the Câmpeneşti ponds (Cluj County, NW Romania), investigated between 2006 and 2007. We have studied the dimensional parameters of nest (external and internal diameter), water depth at the nesting site, clutch and egg size. The goal of this paper is to provide basic data on the breeding ecology of the Great Crested Grebe in the South-Eastern area of the species' geographic range. Average clutch size was of 3.31 ± 0.48 in 2006 (n=17) and 3.83 ± 1.04 in 2007 (n=28). Water depth at the place of nesting varied between 60-147 cm in 2006 and 79-120 cm in 2007. Repeatability between dimensions of all eggs measured in this study was not very high (0.408 - 0.457). However, we noticed a low within clutch variation of egg traits.

Keywords: Clutch size, egg size, nest characteristic**s,** repeatability

Introduction

The Great Crested Grebe (*Podiceps cristatus*) is distributed throughout Europe. However, as a breeding bird, it is more common in the Northern parts of Europe (Cramp and Simmons, 1977; Fjeldsa and Lammi, 1997; Vogrin, 1999). Studies about breeding, nest-site selection, breeding biology and ecology from those regions are numerous (Cramp and Simmons, 1997; Glutz von Blotzheim *et al.,* 1987; Vogrin, 1999; Stanevićius, 2002). On the other hand, the data from other regions are scarce, especially from the Southern locations (Sarrocco, 1986; Vogrin, 1999).

The main aims of this paper were to document the variations in some basic characteristics of the nesting and reproduction behavior of Great Crested Grebe breeding in the South-Eastern part of Europe; especially in Romania where, for a proper conservation of this species, we need studies in this direction.

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Materials and methods

The *study area* is on the Feiurdenilor Valley between two geographical units: the Transylvanian Plains and the Someşan Tableland. The Câmpeneşti wetlands include six ponds, on a 120 ha surface. Those ponds are originally from 1984 and they are used for pisciculture.

The research was carried out on pond number 5, where the entire Great Crested Grebe population of the area is nesting solitarily: on this pond fishing is forbidden by law and it is covered for 70% by emergent vegetation, mainly *Typha angustifolia* and *Phragmites australis*.

Data were collected between 2006 and 2007. During this period all places of the pond suitable for the nesting of Great Crested Grebe were checked in the breeding season (May-July) at intervals of 3 to 15 days (Stanevičius, 2001). Attempts were made to find all nests, by making a systematic search of the vegetation throughout the pond. In the searching of the nests we have used the transect method (Bibby *et al.,* 2000).

The nest of this species is like a floating platform. We measured the external and the internal diameter of the nest. The external diameter was measured at water level. The water depth was also measured at the nest site. Only the nests with eggs, egg shells or nestlings were considered (Goc, 1986).

The length (L) and breadth (B) of eggs in all complete clutches were measured by the same person with calipers to the nearest 0.1 mm. A clutch was considered complete if between two visits no eggs were added. Egg volume (V, in cm^3) was estimated following Hoyt's (1979) formula: $V = 0.000507*L*B^2$. Egg shape index expressed egg breadth as a percentage of egg length following the formula: ES = B/L *100 (Rizi *et al.,* 1999).

In order to characterize the differences between the water depths at the nest site in the two years of the study, the one-way ANOVA test was applied; to verify the homogeneity of the data, the LEVENE test was applied.

Repeatability between dimensions of all eggs was calculated from the components of variance as intraclass correlation coefficients (Sokal and Rohlf, 1981) and their standard errors were estimated following Rizi *et al.* (1999) and Becker (1984).

Results

Descriptive characteristics of nest and clutch size of the Great Crested Grebe breeding in the Câmpeneşti ponds in 2006 and 2007 are presented in Table 1.

The depth of the water at the nest sites varied largely among nests during one year. All the nests on pond no. 5 had been built at sites with water depths ranging from 60-147 cm in 2006 and from 79-120 cm in 2007.

Analyzing the data with reference to the water depth, there were however no statistically significant differences between the two years (Table 1; F=0.013, p=0.97). Also, no significant differences between the external and internal diameter of the nest were found (external diameter F=1.156, p=0.289; internal diameter F=1.161, p=0.287). There was no correlation between water depths and external diameter of the nest (p=0.044).

The external diameter of the nests varied in accordance with the different types of plant species used to build the nests (Rizi *et al.*, 1999). Most of the nests that were situated at a greater distance from the shore were made of roots and leaves of *Typha angustifolia* and those that were situated closer to the shore were made of roots and leaves of *Phragmites australis.* These two plant species were the dominant ones in the nesting area.

Table 1.

Nest characteristics and clutch size of Great Crested Grebe breeding on Câmpeneşti pond No. 5 in 2006 and 2007

Variable	Câmpeneşti Pond (No. 5)			
	2006 (n=17)		2007 (n=28)	
	Mean ± SD	Min-Max	Mean ± SD	Min-Max
Water depth (cm)	95.941±19.168	60-147	96.636±16.596	79-120
Ext. diameter (cm)	73±17.208	35-95	77.32±8.678	46-87
Int. diameter (cm)	28.352±3.296	22-37	29.88±5.158	20-40
Clutch size	3.307±0.48	3-4	3.833±1.043	2-5

We have counted a total of 164 eggs laid in 45 full clutches of Great Crested Grebes. Clutch size ranged from 3 to 4 in 2006 and from 2 to 5 in 2007 (Table 1). Average clutch size was 3.31±0.48 in 2006 and 3.83±1.04 in 2007 (Table 1). 13.33% from all complete clutches contained 2 eggs, 31.11% 3 eggs, 35.55% 4 eggs and 17.77% 5 eggs (Fig. 1). In this study, the optimal clutch size was 4 eggs.

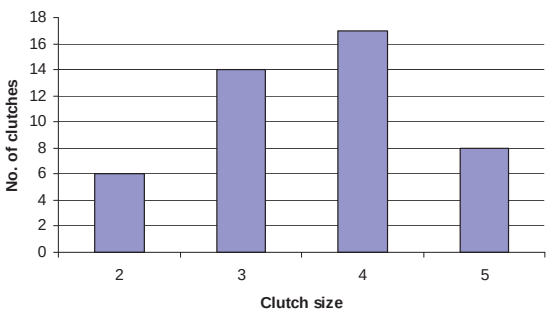

Fig. 1. Frequency distribution of clutch size measured in Great Crested Grebe breeding solitary on Câmpeneşti pond No. 5 between 2006 and 2007.

A total of 112 eggs were measured in length and breadth. The length of the eggs varied from 34.82 mm to 42.24 mm in 2006 and from 34.12 mm to 43.77 mm in 2007. Also the egg breadth varied from 18.91 mm to 23.42 mm in 2006 and from 18.01 mm to 23.08 mm in 2007 (Table 2). We found a positive correlation within clutch between values of egg length and egg breadth (r=0.648, p=0.000), (Fig. 2). Egg volume varied from 6.395 cm^3 to 11.647 cm^3 in 2006 and from 5.716 cm^3 to 12.635 cm^3 in 2007, and the egg shape index from 48.488 to 61.403 in 2006 and from 43.614 to 67.926 in 2007 (Table 2). No significant differences in egg dimensions were found between clutch size classes (Table 3).

Still, there were statistical differences in length and shape variations of all the eggs in these two years (length F=14.78, p=0.002; shape F=10.321, p=0.001) but no differences in eggs' breadth and eggs' volume variation (breadth: F=0.51, p=0.476; volume: F=3.91, p=0.001).

The measures of repeatability of egg length (L) between and within the clutches (3.798 and 4.505, respectively) and of the repeatability of shape index between and within the clutches (5.614 and 7.447, respectively) showed significant variations of these parameters. On the opposite, the repeatability of egg breadth (B) and volume between and within the clutches (1.306 and 1.894; 1.688 and 2.258, respectively) indicated a relative uniformity of the observed parameters.

Table 2.

Variation in egg traits of Great Crested Grebe breeding on Câmpeneşti pond No. 5 in 2006 and 2007

Egg traits	2006		2007	
	Mean ± SD	Min-Max	Mean ± SD	Min-Max
Length (L) (mm)	38.820±2.173	34.82-42.24	40.131±3.004	34.12-43.77
Breadth (B) (mm)	20.822±1.372	18.91-23.42	21.067±1.991	18.01-23.98
Volume ($0.000507*L*B^2$) (cm^3)	8.452±1.47	6.395-11.647	9.196±2.196	5.716-12.635
Shape index (B/L*100)	54.677±2.99	48.488-61.403	52.538±3.712	43.614-67.926

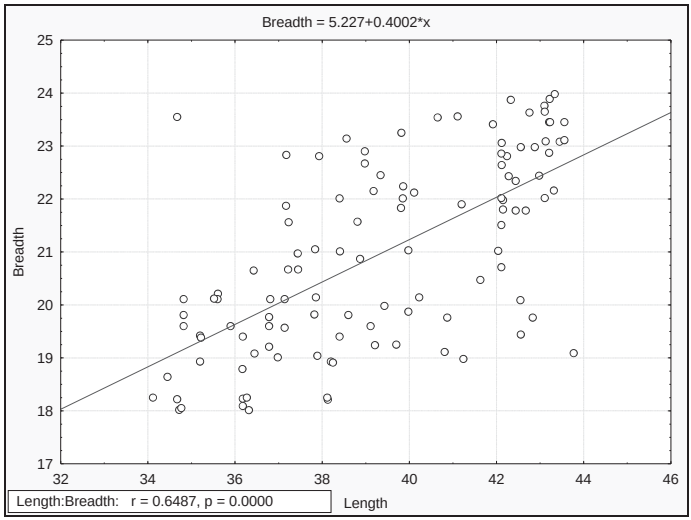

Fig. 2. Correlation between within-clutch values of eggs length and eggs breadth in the Great Crested Grebe

Table 3.

Repeatability (r) of eggs length, breadth, volume and shape index in the Great Crested Grebe

Egg traits	r	SE	F-ratio	Df	p	95% CI	r	
							Between clutches	Within clutches
Length (L) (mm)	0.457	0.339	1.224	3	0.036	0.263-0.651	3.798	4.505
Breadth (B) (mm)	0.408	0.168	0.703	3	0.552	0.213-0.611	1.306	1.894
Volume (0.000507*L*B^2) (cm^3)	0.427	0.270	0.860	3	0.465	0.233-0.627	1.688	2.258
Shape index (B/L*100)	0.429	0.186	0.835	3	0.478	0.235-0.628	5.614	7.447

Discussion and conclusions

The significant intra-year variation of the water depth at the nest sites points at a lower specificity of the Great Crested Grebe for this parameter. This was demonstrated in other studies, too, where the depth of the water varied between 0-150 cm (Stanevičius, 2002).

The low variation in the egg's breadth measured in 2006 and 2007 may have resulted from the fact that this trait is constrained by the structure of the female oviduct (Van Noordwijk *et al.*, 1981).

Even though Hor's experimental studies (1984) suggested that a poor amount of food could lead to serious variations of egg dimensions within clutches, the low intra-clutch variation found here insinuates that females were in good physical conditions during laying and had access to stable energy resources throughout. Indeed, fish is abundant at pond no. 5 as fishing is forbidden at this reproductive pond. As between clutch size class comparisons showed no meaningful differences, variations in egg volume were related to inter-clutch differences independent of clutch size. Possibly differences existed in the inherent qualities of different females (related to age, body size or other).

In different bird species, differences in metrical characteristics between females have been proved to be the major source of variation in egg traits (Lessells *et al.*, 1989; Oró *et al.*, 1996). The values of repeatability between dimensions of all eggs measured reported in the present study and ranging from 0.408 to 0.457 are low as compared to other studies on other bird species (Piotr and Marcin, 2003; Bańdura and Zieliński, 1990). This fact could a priori support egg differences originating from metrical differences between females.

There should be future studies in other similar habitats or with different environmental conditions to assess in how far the findings of this study are more generally valid.

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A CONTINUOUS MODEL FOR THE AGE GROUP POPULATION DYNAMICS

MOHAMMAD REZA MOLAEI[1]

SUMMARY. In this paper the population dynamics of a society with immigration to the society and from the society is considered. A continuous model for this dynamics is presented. This model has this ability to determine the rate function of those who immigrate to the society, to keep constant the number of population in a special age group.

Keywords: continuous Model, age group, immigration, observer

Introduction

The population age group dynamics (Leslie, 1945, 1948) as a discrete model has been considered from different viewpoints (Farkas, 2001, Dordea and Coman, 2006). This model has been extended to a discrete model for a population with respect to the immigration (Molaei and Mohammadhasani, 2008). We would like to use this model to present a continuous model for population. This model will be determined by five observers (Molaei, 2009) which are: per capita birth, mortality rate, rate of those who immigrate to the society, rate of those who immigrate from the society, and per capita birth of those who immigrate to the society.

The continuous model

We divide the population into n age groups, and we denote the number of individuals in the k^{th} group at time t by $x_k(t)$, where $k \in \{1,2,...,n\}$. We also denote the number of offspring and mortal people at time t by $B(t)$ and $D(t)$ respectively. If we choose a discrete time τ as a unit time, then the per capita birth and the mortality rate of the k^{th} age group at time t are defined by $b_k(t) = \frac{B(t+\tau) - B(t)}{B(t)}$ and $d_k(t) = \frac{D(t+\tau) - D(t)}{D(t)}$, respectively. By the graphs of $b_k(t)$ and $d_k(t)$ deduced from the discrete model we can approximate them by two suitable continuously differentiable functions. So we assume that both of them are continuously

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differentiable functions. We ask the author to pay attention to this point that if $\dot{b}_k(t) = 0$ for all t in an open interval then $b_k(t) = 0$ on this interval. In other words, if $b_k(t)$ is a constant function on an open interval, then $b_k(t)$ is zero on that interval. Because $\frac{B(t+\tau)-B(t)}{B(t)} = \frac{B(t+u)-B(t)}{B(t)}$ for a small τ and for all u with $0<u<\tau$. So $B(t+\tau) = B(t+u)$ for all $0<u<\tau$, and the continuity of $B(t)$ implies $B(t+\tau) = B(t+u) = B(t)$. Thus $b_k(t) = \frac{B(t+\tau)-B(t)}{B(t)} = 0$.

By the same method we can define the function $e_k(t)$, as the rate of those immigrate from society and also leave k^{th} age group at time t.

If $s_k(t) = 1-d_k(t)$ then $x_k(t)s_k(t)$ is the number of those survive in k^{th} age group at time t and $x_k(t)s_k(t)e_k(t)$ is the number of those who survive and also immigrate from the society, from k^{th} age group at time t, and $x_k(t)s_k(t)e_k(t)b_k(t)$ is the number of those who are born at time t by the people who immigrate from the society with this assumption which the people who immigrate, take their offspring with themselves. $c_k(t)$ is the rate of those who immigrate to the society and increase the number of that and enter into k^{th} age group at time t. Let $f_k(t)$ be the per capita birth of those immigrate to the society and are placed in k^{th} age group at time t. Then $x_k(t)c_k(t)f_k(t)$ is the number of those who are born by the immigrants and enter to the society.

So for arbitrary $t \in R$, and small positive number τ we have the following discrete model for the number of population in the age groups.

$$x_1(t+\tau) = \sum_{k=1}^{n} (x_k(t)\frac{\int_t^{t+\tau} b_k(u)du}{\tau} - x_k(t)\frac{\int_t^{t+\tau} s_k(u)e_k(u)b_k(u)du}{\tau} + x_k(t)\frac{\int_t^{t+\tau} c_k(u)f_k(u)du}{\tau})$$

and for $1 \le k \le n-1$

$$x_{k+1}(t+\tau) = x_k(t)\frac{\int_t^{t+\tau} s_k(u)du}{\tau} - x_k(t)\frac{\int_t^{t+\tau} e_k(u)du}{\tau} + x_k(t)\frac{\int_t^{t+\tau} c_k(u)du}{\tau}$$
$$= x_k(t)\frac{\int_t^{t+\tau} (s_k(u) - e_k(u) + c_k(u))du}{\tau}.$$

The conditions $x_1(t) = \lim_{\tau\to 0} x_1(t+\tau)$ and $x_k(t) = \lim_{\tau\to 0} x_k(t+\tau)$ imply

$$x_1(t) = \sum_{k=1}^{n} (x_k(t)b_k(t) - x_k(t)s_k(t)e_k(t)b_k(t) + x_k(t)c_k(t)f_k(t))$$

and

$$x_{k+1}(t) = x_k(t)(s_k(t) - e_k(t) + c_k(t)).$$

Thus,

$$\frac{x_1(t+\tau) - x_1(t)}{\tau} = \sum_{k=1}^{n} (x_k(t) \frac{\frac{\int_t^{t+\tau} b_k(u)du}{\tau} - b_k(t)}{\tau}$$
$$- x_k(t) \frac{\frac{\int_t^{t+\tau} s_k(u)e_k(u)b_k(u)du}{\tau} - s_k(t)e_k(t)b_k(t)}{\tau}$$
$$+ x_k(t) \frac{\frac{\int_t^{t+\tau} c_k(u)\, f_k(u)du}{\tau} - c_k(t)\, f_k(t)}{\tau})$$

and

$$\frac{x_{k+1}(t+\tau) - x_{k+1}(t)}{\tau} = x_k(t) \frac{\frac{\int (s_k(u) - e_k(u) + c_k(u))du}{\tau} - (s_k(t) - e_k(t) + c_k(t))}{\tau}.$$

If we tend τ to zero in the above two equalities then we deduce the following model for the age group population dynamics.

$$\begin{cases} \dot{x}_1(t) = \frac{1}{2} \sum_{k=1}^{n} \alpha_k(t) x_k(t) \\ \dot{x}_{k+1}(t) = \frac{1}{2} [\dot{s}_k(t) - \dot{e}_k(t) + \dot{c}_k(t)] x_k(t), \quad for\ k \in \{1,2,3,...,n-1\} \end{cases}$$

where

$$\alpha_k(t) = \dot{b}_k(t) - \dot{s}_k(t)e_k(t)b_k(t) + s_k(t)\dot{e}_k(t)b_k(t) + s_k(t)e_k(t)\dot{b}_k(t)$$
$$+ \dot{c}_k(t)\, f_k(t) + c_k(t)\, \dot{f}_k(t).$$

Another approach to the model

In this section we also use of the same symbols of the previous one.

For $t \in R$ and small τ there exists a function $u(\tau)$, with $0 \le u(\tau) \le \tau$ such that we can approximate $x_1(t+\tau)$ and $x_k(t+\tau)$ by:

$$x_1(t+\tau) = \sum_{k=1}^{n} (x_k(t)b_k(u) - x_k(t)s_k(u)e_k(u)b_k(u) + x_k(t)c_k(u)\, f_k(u))$$

and

$$x_{k+1}(t+\tau) = x_k(t)(s_k(u) - e_k(u) + c_k(u)).$$

So

$$x_1(t) = \sum_{k=1}^{n} (x_k(t)b_k(t) - x_k(t)s_k(t)e_k(t)b_k(t) + x_k(t)c_k(t)f_k(t))$$

and

$$x_{k+1}(t) = x_k(t)(s_k(t) - e_k(t) + c_k(t)).$$

So

$$\frac{x_1(t+\tau) - x_1(t)}{\tau} = \sum_{k=1}^{n} (x_k(t)\frac{b_k(u) - b_k(t)}{u}\frac{u-0}{\tau}$$
$$- x_k(t)\frac{s_k(u)e_k(u)b_k(u) - s_k(t)e_k(t)b_k(t)}{u}\frac{u-0}{\tau}$$
$$+ x_k(t)\frac{c_k(u)f_k(u) - c_k(t)f_k(t)}{u}\frac{u-0}{\tau}),$$

and

$$\frac{x_{k+1}(t+\tau) - x_{k+1}(t)}{\tau} =$$
$$x_k(t)\frac{(s_k(u) - e_k(u) + c_k(u)) - (s_k(t) - e_k(t) + c_k(t))}{u}\frac{u-0}{\tau}.$$

Thus, by tending τ to zero we deduce the following model for the age group population dynamics.

$$\begin{cases} \dot{x}_1(t) = A\sum_{k=1}^{n} \alpha_k(t)x_k(t) \\ \dot{x}_{k+1}(t) = A[\dot{s}_k(t) - \dot{e}_k(t) + \dot{c}_k(t)]x_k(t), \quad for\ k \in \{1,2,3,...,n-1\} \end{cases}$$

where

$$\alpha_k(t) = \dot{b}_k(t) - \dot{s}_k(t)e_k(t)b_k(t) + s_k(t)\dot{e}_k(t)b_k(t) + s_k(t)e_k(t)\dot{b}_k(t)$$
$$+ \dot{c}_k(t)f_k(t) + c_k(t)\dot{f}_k(t),$$

and A is a constant in the interval $(0,1]$.

In fact if we take $u(\tau)=\frac{\tau}{2}$ then $A=\frac{1}{2}$ and we deduce the model of section 2.

Conclusion

In the society programming sometimes we need to keep constant the numbers of some age groups. For example the age group of those who are students at universities. In this case we need to determine the function c_k, and this is possible by solving a first order linear differential equation. In fact if we want to keep constant the number of $k+1^{th}$ age group, then we only need to solve the differential equation $\dot{s}_k(t)-\dot{e}_k(t)+\dot{c}_k(t)=0$.

For example if $n=6$, $d_4(t)=\frac{e^t}{2+2e^t}$, and $e_4(t)=\frac{2+e^t+e^{\frac{t}{2}}}{2+2e^t}$ then to keep constant the number of 5^{th} age group, the rate of those who immigrate to the society and placed in the 4^{th} age group by solving the above differential equation is: $c_4(t)=\frac{e^{\frac{t}{2}}}{2+2e^t}$.

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A NEW METHOD OF EVALUATION OF BIOCENOTIC SIMILARITY

IVAN KOTLIAROV[1]

SUMMARY. An index of biocenotic similarity is proposed which is intended to evaluate structural similarity of biocenoses. Mathematical algorithm necessary to calculate this index is described.

Keywords: biocenosis, feature, difference, similarity index

Introduction

We propose a new index of biocenotic similarity (IBS) based on the well-known Sørensen index (Sørensen, 1948) (a very good overview of existing indexes can be found in Boyce and Ellison, 2001). The problem with Jacquard index is that it recognizes as similar all features shared by both biocenoses – even if their values are dramatically different.

Methodology

The methodology used to build up this index is as follows: logically, an IBS should be calculated according to the following formula:

$$IBS = \frac{2Z}{N_x + N_y}, \qquad (1)$$

Z – number of features whose value is the same for both biocenoses;
N_x – number of features of the biocenosis X;
N_y number of features of the biocenosis Y.
Obviously, the following criterion should be met:

$$0 \le IBS \le 1.$$

Let us suppose that M is the number of features that both biocenoses have in common (but the quantitative characteristics of these values are not necessarily the same). Then the following formula will apply:

$$Z = M - \sum_{i=1}^{M} f(x_i, y_i),$$

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x_i – value of the *i*-th feature of the biocenosis *X*;
y_i – value of the *i*- th feature of the biocenosis *Y*;
$f(x_i, y_i)$ – a function that meets the following requirement:

$$f(x_i, y_i) = \begin{cases} 0, & x_i = y_i \\ 1, & x_i \neq y_i \end{cases}. \qquad (2)$$

In order to write down the function $f(x_i, y_i)$ in an explicit form we will use the function sign (x):

$$\text{sign}(x) = \begin{cases} -1, & x < 0 \\ 0, & x = 0 \\ 1, & x > 0 \end{cases}.$$

Therefore,

$$f(x_i, y_i) = \text{sign}|x_i - y_i|. \qquad (3)$$

If we combine the formulae (1) and (3) then

$$IBS = \frac{2\left(M - \sum_{i=1}^{M} \text{sign}|x_i - y_i|\right)}{N_x + N_y}. \qquad (4)$$

Unfortunately, the formula (4) has a very important problem – it recognizes as similar only those features whose value is the same for both biocenoses. However, for practical reasons it is sometimes logical to consider as similar the features that have different values but this difference does not exceed a certain limit. Therefore, it would be logical to transform the formula (4) in order to take into account this practical possibility:

$$\Psi(x_i, y_i) = \begin{cases} 0, & |x_i - y_i| \leq \Delta_i^{xy} \\ 1, & |x_i - y_i| > \Delta_i^{xy} \end{cases},$$

Δ_i^{xy} – accepted value of difference between the values of the *i*-ro feature for biocenoses *X* and *Y*. This parameter shows what difference between the values will be considered as non-sufficient. The precise value of this parameter is to be defined according to the goals of the research.

The explicit form of the function $\Psi(x_i, y_i)$ is

$$\Psi(x_i, y_i) = 1 - \text{sign}\left(1 - \text{sign}\left(|x_i - y_i| - \Delta_i^{xy}\right)\right). \qquad (5)$$

The final formula of *IBS*:

$$IBS = \frac{2\left(M - \sum_{i=1}^{M} 1 - \text{sign}\left(1 - \text{sign}\left(|x_i - y_i| - \Delta_i^{xy}\right)\right)\right)}{N_x + N_y}. \qquad (6)$$

This method is especially efficient if there is a need to calculate an index of similarity of biocenoses for economical reasons (for example, for taxation). Obviously, it is hardly possible to find two absolutely similar biocenoses – but from the economical point of view two somewhat different biocenoses can be considered as similar if the difference does not exceed a certain level – which is expressed by the formula (6).

Obviously, the biocenotic distance *BD* (as a measure of dissimilarity) is equal to

$$BD = 1 - IBS\,.$$

However, it is important to remember that in case of forest taxation features of very different nature are taken into account and include all of them into just one index of similarity is not logical. It is better to calculate several partial indexes of similarity according to the formula (6) – each index measuring the similarity for a group of more or less homogeneous features (for example, wood resources, non-wood resources and hunting resources). Then an overall index of similarity can be calculated:

$$IBS_{ov} = \sqrt[n]{\prod_{i=1}^{n} ISB_i}\,,$$

ISB_{ov} – overall index of similarity of biocenoses;
n – number of homogeneous groups of features;
IBS_i – partial index of similarity of biocenoses for the i-th group of features.

In order to completely formalize the methodology of calculation of ISB it is necessary to give an algorithm of calculation of *M*.

Obviously the number of common features cannot exceed the number of features that the biocenosis with the lesser number of features has:

$$M \le \min(N_x, N_y).$$

Let us suppose for simplicity sake that

$$\min(N_x, N_y) = N_x\,.$$

The text description of each feature of the biocenosis *X* must be compared with the text description of all features of the biocenosis *Y*. Obviously, text description of any feature of the biocenosis *X* can be equal to no more than one feature of the biosenosis *Y*. In order to formalize this comparison the text function $\mathrm{EXACT}(x, y)$ can be used:

$$\mathrm{EXACT}(x, y) = \begin{cases} 1, & x = y \\ 0, & x \neq y \end{cases}.$$

Therefore,

$$M = \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} \mathrm{EXACT}(x_i, y_j) \cdot$$

So this algorithm represents a fully formalized method of calculation of the proposed index of biocenotic similarity.

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=== REVIEW ===

OXIDATIVE STRESS IN PLANTS

GYÖNGYI SZÉKELY[1]

SUMMARY. During their life cycle, plants are continuously exposed to different types of environmental stress factors, such as extreme temperatures, drought, and high intensities of photosynthetically active radiation, air pollution and pathogens. As a direct result of stress exposure, plants' metabolic functions are affected through the increased production of reactive oxygen species. These short-lived molecules inactivate enzymes and mediate damage of the cellular components. Depending on their nature, some of them are highly toxic and are rapidly detoxified by different cellular enzymatic and non-enzymatic mechanisms. In recent years, new roles of reactive oxygen species scavenging enzymes were providing the control and regulation of programmed cell death, hormonal signalling, stress responses, and development. Although reactive oxygen species perform a wide array of roles in plant cells, there is an evident controversy regarding their roles: they are necessary for the survival of plants, performing signal transduction and gene-activating roles, but they can also be lethal when they are accumulated. During optimal growth conditions there is equilibrium between the production and destruction of reactive oxygen species determining the fate of the plant. Under unfavourable growth conditions an imbalance occur between the production and destruction of reactive oxygen species that leads to oxidative stress. Plants possess a very efficient enzymatic and non- enzymatic scavenging system against reactive oxygen species. It is also important to mention, that the capacity and activity of the antioxidant defence system play a significant role in limiting oxidative damage by destroying reactive oxygen species that are produced in excess and damage metabolic processes of the plant. Here are described the most widely distributed mechanisms of reactive oxygen species generation and removal in plants.

Keywords: oxidative stress, reactive oxygen species, enzymatic defence system, non-enzymatic defence system.

ROS-generating mechanisms in plants

When aerobic metabolism appeared, molecular oxygen (O_2) together with reactive oxygen species (ROS) came into our atmosphere by O_2 evolving photosynthetic organisms. Molecular oxygen is not harmful for cells, but the reduced or activated derivatives of oxygen, such as $^1O_2$ (singlet oxygen), $O_2^{\cdot -}$ (superoxide radical), $HO_2^{\cdot}$

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(hydroperoxyl radicals), H_2O_2 (hydrogen peroxide), and $OH^{\cdot}$ (hydroxyl radical) are highly reactive and toxic, and cause oxidative destruction of cells (Apel and Hirt, 2004). Plants possess a very efficient scavenging system against reactive oxygen species. Thus, the ROS scavenging mechanisms play a pivotal role in further evolution of aerobic organisms. During optimal growth conditions there is equilibrium between the production and destruction of ROS determining the fate of the plant. Under unfavourable growth conditions an imbalance occur between the production and destruction of ROS that leads to oxidative stress. In recent years, new roles of ROS scavenging enzymes were providing the control and regulation of programmed cell death, hormonal signalling, stress responses, and development. Therefore, ROS act as toxic by-products of aerobic metabolism and are key regulators of metabolic and defence pathways. The level of ROS in the different cellular compartments is determined by the interplay between ROS producing pathways and ROS scavenging mechanisms (Apel and Hirt, 2004).

Molecular oxygen (O_2) is produced during water oxidation by the photosynthetic electron transport chain using oxygen as electron acceptor.

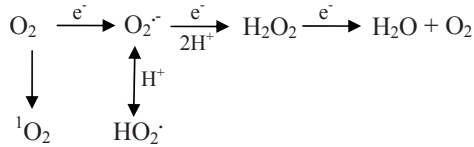
Singlet oxygen ($^1O_2$) is produced when chlorophyll pigments associates with the electron transport system and can appear as a by-product of lipoxygenase activity too. The excited singlet state of chlorophyll is used for the transfer of energy or electrons. $^1O_2$ reacts with biological molecules or transfer its excitation energy to them, thus forming peroxides (Halliwell, 1989 and Vranová, 2002). Half lifetime of $^1O_2$ is 4 μs in water and 100 μs in non-polar environment (Foyer and Harbinson, 1994).

When electrons are transported to oxygen, superoxide ($O_2^{\cdot-}$) is produced, which is not compatible with metabolism and the scavenging system has to eliminate it, while phosphoglycolate is recycled to phosphoglycerate. $O_2^{\cdot-}$ is considered a moderately reactive ROS with a half-life of 2-4 μs, thus cannot cross biological membranes and is dismutated easily to H_2O_2. $O_2^{\cdot-}$ influences the activity of metal containing enzymes by reducing quinones and Fe^{3+}- and Cu^{2+}-containing metal complexes (Vranová *et al.*, 2002).

Hydroperoxyl radicals ($HO_2^{\cdot}$) are formed from $O_2^{\cdot-}$ by protonation in aqueous solutions, cross biological membranes and extract hydrogen from polyunsaturated fatty acids and lipid peroxides, thus initiating lipid auto-oxidation (Halliwell and Gutteridge, 1989).

High levels of hydrogen peroxyde (H_2O_2) are generated in peroxisomes by glycolate oxidation, and a big part of glycolate is catabolized by catalase. Besides $^1O_2$, H_2O_2 is considered a moderately reactive molecule that can diffuse from its production site. H_2O_2 appears as a long-lived molecule, its half-life is about 1 ms. H_2O_2 can inactivate enzymes by oxidizing their thiol groups (Vranová *et al.*, 2002).

The most reactive ROS is the hydroxyl radical ($OH^{\cdot}$) that is formed from H_2O_2 by the Haber-Weiss or Fenton reactions (Halliwell and Gutteridge, 1989). When $OH^{\cdot}$ reacts with biological molecules and is accumulated in excess, it participates in the control of programmed cell death, because cells do not possess enzymatic mechanism to eliminate this highly reactive ROS (Fig. 1).



Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

Fig. 1. Formation of reactive oxygen species from O_2

Ground state molecular oxygen (O_2) is activated by energy excess, and by reversing the spin of one of the unpaired electrons, singlet oxygen ($^1O_2$) is formed. The reduction of one electron leads to the formation of superoxide radical ($O_2^{\cdot-}$), which appears in equilibrium with its conjugate acid, hydroperoxyl radical ($HO_2^{\cdot}$). Further reduction processes form hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and water (H_2O). Metal ions present in cells in the oxidized form (Fe^{3+}) are reduced in the presence of $O_2^{\cdot-}$ and catalyses the conversion of H_2O_2 to $OH^{\cdot}$ by the Fenton or Haber-Weiss reactions.

Abbreviations: $^1O_2$: singlet oxygen, $O_2^{\cdot-}$: superoxide radical, $OH^{\cdot}$: hydroxyl radical.

One of the most damaging effects of ROS is lipid peroxidation. During this, toxic compounds are accumulated, such as 13-hydroperoxy-linoleic acid and malondialdehyde (MDA). These molecules attack directly DNA, RNA, proteins, enzymes, and membranes, reduce the potential of DNA- and protein synthesis (Mittler, 2002).

Under favourable life conditions about 240 μM of $O_2^{\cdot-}$ and 0.5 μM of H_2O_2 are produced, while stress conditions (salt, drought, cold- and heat shock, heavy metals, pathogen attack, mechanical stress) increases their production up to 720 μM of $O_2^{\cdot-}$ and 5-15 μM of H_2O_2.

Table 1 summarises the potential sources of ROS in plants. Some sources are reactions of normal aerobic metabolism, such as photosynthesis and respiration, whereas others are caused by abiotic stress conditions, such as photorespiration.

Table 1.

Production and scavenging of reactive oxygen species in plants

Production of ROS	Localization	Primary ROS
Photosynthetic ET and PSI or II	Clp	$O_2^{\cdot-}$
Respiratory ET	Mit	$O_2^{\cdot-}$

Table 1. (continued)

Production of ROS	Localization	Primary ROS
Excited chlorophyll	Clp	$^1O_2$
NADPH oxidase	PM	$O_2^{\cdot-}$
Fatty acid β-oxidation	Per	H_2O_2
Xanthine oxidase	Per	$O_2^{\cdot-}$
Peroxidases, Mn^{2+} and NADH	CW	H_2O_2, $O_2^{\cdot-}$
Amino oxidase	Apo	H_2O_2
Scavenging of ROS		
Superoxide dismutase	Clp, Cyt, Mit, Per, Apo	$O_2^{\cdot-}$
Ascorbate peroxidase	Clp, Cyt, Mit, Per, Apo	H_2O_2
Catalase	Per	H_2O_2
Reduced glutathione	Cyt	H_2O_2, ROOH
Peroxidases	CW, Cyt, Vac	H_2O_2
Ascorbic acid	Clp, Cyt, Mit, Per, Apo	H_2O_2
Glutathione	Clp, Cyt, Mit, Per, Apo	H_2O_2
α-Tocopherol	Membranes	ROOH, $O_2^{\cdot-}$
Carotenoids	Clp	$^1O_2$
AOX	Clp, Mit	$O_2^{\cdot-}$

Abbreviations: AOX: alternative oxidase, Apo: apoplast, Clp: chloroplast, CW: cell wall, Cyt: cytosol, ET: electron transport, H_2O_2: hydrogen peroxide, Mit: mitochondria, $O_2^{\cdot-}$: superoxide radical, $^1O_2$: singlet oxygen, Per: peroxisome, PM: plasma membrane, PS: photosystem, ROOH: hydroperoxy compound, Vac: vacuole.

Under optimal growth conditions, ROS are mainly produced at a low level in organelles such as chloroplasts, mitochondria and peroxisomes. During stress their level of production is highly elevated. Limitation of CO_2 fixation together with over-reduction of the electron transport chain is the main cause of ROS production in chloroplasts. Over-reduction of the electron transport chain is also a major cause of ROS production in mitochondria during stress. In contrast, in peroxisomes, H_2O_2 is produced when glycolate is oxidized to glyoxylic acid during photorespiration (Mittler *et al.*, 2004 and Suzuki and Mittler, 2006).

Antioxidative defence systems of plants

Coping with oxidative stress is a well-regulated process, a balance between the oxidative and antioxidative capacities of the plant. Under normal life conditions the antioxidant defence system provides sufficient protection against ROS. During stress conditions, plants respond with increased antioxidative defence mechanisms. These defence mechanisms are located intracellularly, but are also found in the apoplasts. The antioxidant defence system of plants consists of enzymes (such as superoxide dismutase, catalase, and peroxidase) and antioxidant molecules (such as ascorbic acid, α-tocopherol, carotenoids, and glutathione).

Enzymatic defence system

Superoxide dismutase (SOD)

The first step in the antioxidative defence system of plants is represented by superoxide dismutases, a family of metal-enzymes present in all aerobic organisms, catalysing the disproportionation of superoxide to hydrogenperoxyde and molecular oxygen.

$$2O_2^{\cdot-} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Three isozymes of SOD, namely Fe-SOD, Mn-SOD and Cu/Zn-SOD have been described in different plant species. Fe-SOD has been detected predominantly in chloroplasts, but was also observed in cytosolic, mitochondrial and peroxisomal fractions. Mn-SOD is reported to be present in mitochondria and peroxisomes. There are reports describing its presence in the soluble cytosolic fraction, too. Cu/Zn-SOD was initially observed in the cytosolic fraction, but lately it was reported to be present in chloroplastic and mitochondrial fractions. Thus, it seems that all of the isozymes of SOD have been detected in most of the cellular components (Arora *et al.*, 2002).

Catalase

Catalase is a hem-containing enzyme, present in peroxisomes, glyoxysomes and mitochondria. It converts the very toxic hydrogen peroxyde to water and molecular oxygen.

$$2\ H_2O_2 \rightarrow H_2O + O_2$$

McClung (1997) reported a family of three catalase (*CAT*) genes in *Arabidopsis thaliana*, encoding individual subunits, which associate to form six isozymes. *CAT1* and *CAT3* map to chromosome one, while *CAT2* map to chromosome four. The individual isozymes and subunit mRNAs show organ-specific spatial expression. Two isozymes are detectable in roots, and all six isozymes are observable in flowers and leaves. The level of all three mRNAs is high in inflorescences and in freshly imbibed seeds,

while the mRNAs of *CAT2* and *CAT3* are abundant in leaves. The light response of the three catalase genes is different: the expression of *CAT1* and *CAT2* is increased by light, whereas *CAT3* is negatively regulated.

Peroxidases

By removing ROS, families of peroxidases play an important role in reactive oxygen detoxifying mechanism. They appear as cytochrome c peroxidases in yeast, glutathione peroxidases in mammals, ascorbate peroxidases (APX) in plants. APX, as the most important antioxidant in plants, is present in high concentrations in chloroplasts, cytosol, vacuole and the apoplastic space of leaf cells (Arora *et al*., 2002). Paradiso *et al.* (2005) reported that APX activity changes differentially under various stress conditions. If the oxidative stress conditions are moderate, a transient increase in APX activity occurs while, in the induction of cell necrosis, the activity of APX decreased gradually to cell death. Under such conditions, no alteration in the *APX* gene expression was detected, but 24 h after the generation of the oxidative stress, APX activity was significantly increased in the surviving cells in order to overcome the oxidative stress and to avoid further cell death.

Figure 2 shows the oxidation of ascorbate by APX in two steps: first producing mono-dehydroascorbate and, if not rapidly re-reduced to ascorbate and, the mono-dehydroascorbate disproportionates to ascorbate and dehydroascorbate.

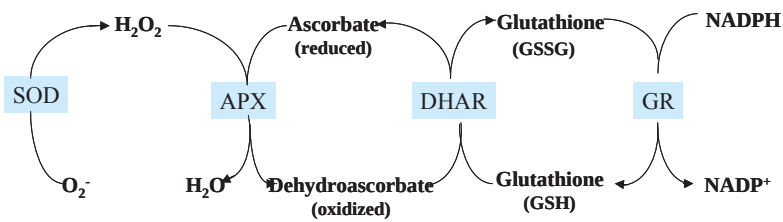


Fig. 2. Halliwell-Asada pathway, redox cycling of ascorbate in the chloroplast

Abbreviations: APX: ascorbate peroxidase, DHAR: dehydroascorbate reductase, GR: glutathione reductase, GSH: reduced glutathione, GSSG: oxidized glutathione, H_2O_2: hydrogen peroxide, NADP: nicotinamide adenine dinucleotide phosphate, NADPH: nicotinamide adenine dinucleotide phosphate hydrogen, $O_2^{\cdot-}$: superoxide radical, SOD: superoxide dismutase.

In plants, Beeor-Tzahar *et al.* (1995) described stress inducible glutathione peroxidases (GSH-PX). Another family of plant peroxidases acting as electron donors are the guaiacol peroxidases (GPX), with a key role in lignin and ethylene biosynthesis (Quiroga *et al.*, 2000).

Non-enzymatic defence system

Ascorbic acid

L-ascorbic acid (vitamin C) is an important vitamin in human diet. It is abundant in plant green tissues, where ascorbate is present in the same amount as chlorophyll. In plants, the ascorbic acid plays an important role in physiological processes, (growth, differentiation and metabolism) (Foyer, 1993), and acts as a reductant for free radicals, thus minimizing the damage caused by oxidative stress.

Tocopherol

Tocopherols, especially the most active α-tocopherol (vitamin E) have been studied mainly in mammalian tissues as membrane stabilisers and antioxidants, with a relevant role in scavenging ROS (Diplock *et al.*, 1989). Because α-tocopherol contains a benzoquinone ring and a phytyl chain, it can be located only in the cell membranes, stabilizing them. Because of its dietary importance, tocopherol concentrations have been studied thoroughly in plant tissues (200 ng g^{-1} fresh weight in potato tuber, 5 mg g^{-1} in oil palm leaflets) (Hess, 1993). Tocopherol is present in all higher plants, in both photosynthetic and non-photosynthetic tissues, but it was mainly characterised as constituent of chloroplast membranes.

Carotenoids

Carotenoids are C_{40} isoprenoids (or tetraterpenes), located in the plastids of both photosynthetic and non-photosynthetic plant tissues. In chloroplasts, the carotenoids appear as accessory pigments and play a role in light harvesting. The main protective role of carotenoids in photosynthetic tissues is direct quenching of triplet chlorophyll, thereby preventing the formation of singlet oxygen and oxidative stress.

Glutathione and glutathione reductase

The tripeptide thiol glutathione, (glutamyl cysteinyl glycine, GSH), is an important antioxidant in plants. The primary biological function of glutathione is to act as disulphide reductant to protect thiol groups on enzymes, and preventing oxidative stress in cells by regenerating ascorbate and reacting with singlet oxygen and hydroxyl radicals.

Glutathione regenerates ascorbate from dehydroascorbate via the enzyme dehydroascorbate reductase (DHAR) (figure 2). During this reaction GSH is oxidized to glutathione disulphide (GSSG). Then GSH is regenerated by glutathione reductase (GR) in a NADPH-dependent reaction. The reaction takes place in plastids and in the cytosol, which are the main centers of glutathione synthesis (Zechmann *et al.*, 2006). Glutathione reductase has been purified from different plant tissues (Smith *et al.*, 1989), and its cDNA has been cloned from pea (Creisson *et al.*, 1992).

Conclusions and perspectives

Plants are constantly exposed to ROS generated from endogenous and exogenous sources. These ROS, such as superoxide, hydrogen peroxide, and hydroxyl radical, accumulated at low levels, may function in cell signalling processes. If ROS are accumulated at higher levels, they can interact with biological macromolecules (such as DNA and RNA, proteins and lipids) causing structural and functional damage, and can participate in programmed cell death. The effects of the oxidative stress in a cell are determined by the amounts of superoxide, hydrogen peroxyde and hydroxyl radicals. Thus, the balance of superoxide dismutase, ascorbate peroxidase and catalase activities have a decisive role for suppressing toxic levels of ROS in the cell. ROS influence the expression of a large number of genes and signal transduction pathways. These findings suggest that cells have evolved strategies to utilize ROS as indicators and biological signals that activate and control different gene responses. It is presumed that a given form of ROS interacts selectively with a target molecule that perceives the increase of ROS concentration and translates this information into signals that direct the plant's responses to stress. In this way ROS can act as signalling molecules in mediating plant responses to oxidative stress. ROS are also important signal molecules in mediating plant growth and development. ROS may have a role in controlling organ number and initiation (Sagi *et al.*, 2004). Gapper and Dolan, (2006) suggest that ROS may control development by regulating cell growth. Understanding the regulation of cellular development will provide evidence in the implication of ROS in the growing process of the plants.

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INFLUENCE OF HEAVY METALS FROM THE AMBIENT AIR ON NITROGEN, PHOSPHORUS AND POTASSIUM CONTENT OF MAIZE PLANTS (CV. TURDA 200)

CRISTINA DOBROTĂ[1], RODICA MEREUȚĂ[2], RAHELA CARPA[1]

SUMMARY. Since heavy metals may cause essential nutrient deficiency and even change the concentrations of basic nutrients in plant tissues, better understanding of how heavy metal toxicity originated from ambient air interact with main macronutrients is needed. This study investigated the effects of heavy metal containing simulated rainfall on nitrogen, phosphorus and potassium content of maize plants (*Zea mays* L.*)* trying to provide an assessment of interactions and balances among foliar elements, and among soil elements.

Keywords: heavy metals, mineral nutrition, simulated rainfall

Introduction

Toxic substances dispersed in the atmosphere and diluted in precipitation cause damages especially to assimilatory tissues of plants. They impair the protective layer on leaves and penetrate into the tissues from which they leach Mg contained in chlorophylls, and other biogenic elements, primarily K, Ca and P (Tausz *et al.*, 2004). Toxic substances in the environment also decrease the potential synthesis of assimilates and disturb the mechanism of leaf stomata control (Ditmarova *et al.*, 2007).

The high concentration of heavy metals in soils or in the air is reflected by higher concentrations of metals in plants, and consequently in animal and human bodies. The study of excessive concentrations of pollutants in plant tissues has been reported in numerous publications (Greger, 2004; Zhang *et al.*, 2008).

Heavy metals can affect nitrogen metabolism at multiple levels. The first place of metal action which disturbs nitrogen acquisition by plants is connected with plasma membrane. At this level metals can alter the nitrogen transport directly, thorough the action on the specific ammonium and nitrate transport proteins or indirectly, changing the membrane composition, its fluidity and H^+ pumping. Changes

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in the intracellular concentration of nitrogen could initiate a signal transduction pathway leading to the modification of the expression of genes encoding enzymes involved in its assimilation. Heavy metals inhibition of the particular enzymes of the assimilatory pathway can alter the nitrogen uptake. Most experiments concerned only with the metals influence on the uptake processes or on the enzyme activities.

Future experiments should address the possible interferences between nitrogen transport and its acquisition in heavy metal stress. New genomic tools such as expression of specific genes encoding the nitrogen transporters and nitrogen assimilatory enzyme proteins will allow researchers to dissect the molecular complexities of metal action. This will lead to a better understanding of the complex biochemical and physiological responses observed in the metal-stressed plants and to the development of molecular strategies which improve plant resistance to the heavy metals (Klobus *et al.,* 2000).

Foliar concentrations of elements such as Mg, Ca, K, P, Mn, Pb and Zn are positively correlated with concentrations of corresponding soil elements. Our experiments try to provide an assessment of interactions and balances among foliar elements, and among soil elements. The knowledge of nutrient deficiency ranges may help diagnose foliar symptoms, but their exclusive use may overly simplify relationships between foliar symptoms and foliar elements

The aim of the present work was to investigate relations between heavy metals from the ambient air and the accumulation of the main macronutrients in different anatomical parts of plants. For this purpose, the concentrations of nitrogen, phosphorus and potassium in the tissues were measured. Researches concerning the influence of the atmospheric pollutants such as copper, zinc and lead on the mineral nutrition of maize plants may improve the knowledge in the evaluation of short time effects of the heavy metals, related to other environmental conditions.

Materials and methods

A 2-years field experiment was designed to study the effects of lead, copper and zinc on the nitrogen, phosphorus and potassium content of maize plants (cv. Turda 200).

Field experiments were conducted at Livada Research and Development Station, Northern Romania. The study took place over a 2-yr period (2006 and 2007). Sixteen experimental plots, each of 21 m^2, were installed and submitted to different treatments. All types of plots were fertilized with 300 g NPK 1:1:1 (300 kg × ha^{-1}) and 200 g NH_4NO_3 (200 kg × ha^{-1}). All the plots were surrounded by a border of 1 m. Maize was sown directly into permanent raised beds at a distance of 60 cm between rows and 35-40 cm between plants (Mereuță and Dobrotă, 2008).

For simulating the action of heavy metals from the ambient air, solutions containing Pb, Cu and Zn were applied on the leaves at 10 days after germination in the following concentrations: 0.2, 2 and $4x10^4$ ppm Cu and Zn, and 0.32, 3.2,

and 6.4 x10^4 ppm Pb respectively. Simulated rainfall was applied in amounts totaling 76 mm. The rainfall simulation system consisted of six TeeJet nozzles. (Model 1/2HH-SS50WSQ; Spraying Systems Co., Wheaton, IL) that were threaded directly into the body of an electrically operated solenoid valve. Solenoids were connected directly to a water supply pipe and were controlled by a custom-built electronic timing system. Solenoids operated on a rapid cycle in which they remained open for 1.0 s and were closed for 0.7 s, resulting in an intermittent rainfall pattern that delivered rainfall at an intensity of 76 mm h^{-1}.

Environmental characteristic were recorded for the entire period of the experiment. The analyses were made at 4-6 leaves stage, and 8-10 leaves stage corresponding at 14 and 18 days after the treatment. Sixteen plants were used for each treatment and there were four independent experiments. Plants were harvested 18 days after treatment.

Chemical analyses of tissues. Plants were separated into roots, shoots and leaves and dried at 60 ^oC. Dry plant material (0.1 g) was separately ashed at 550 ^oC and the residue was brought to standard volume with 20% HCl. The total N content was measured according to Kjeldahl. P was determined calorimetrically and K was determined on a flame photometer

Statistical analyses. Experimental design was a randomized complete block with four replications. Annual data for each parameter over the whole 2-yr period were subjected to analysis of variance (ANOVA), using a year-combined randomized complete block design according to Mc Intosh (1983). Treatment means were compared using Fisher's protected least significant difference (LSD) test at $P \leq 0.05$. The LSDs for different main effect and interaction comparisons were calculated using the appropriate standard error terms following Gomez and Gomez (1984). The Statistix v. 7.0 (Analytical Software, 2000) package was used for this purpose.

Results and discussion

The monthly temperatures and rainfall recorded at the experimental site over the 2-yr study show that the differences in temperature between the two study years were relatively modest. During the first period (April–May) the mean temperature was 4.5°C in 2006 and 6.2°C in 2007. The mean maximum temperature during this period was 22°C in 2006 and 25°C in 2007. Rainfall varied considerably between years. Mean annual rainfall varied greatly: 2006 was the wettest year (269.5 mm), followed by 2007 (180.8mm). Mean annual rainfall for the area over the last 20 yr is 255 mm; 2006 was therefore a normal year and 2007 was a dry year (Mereuță and Dobrotă, 2008).

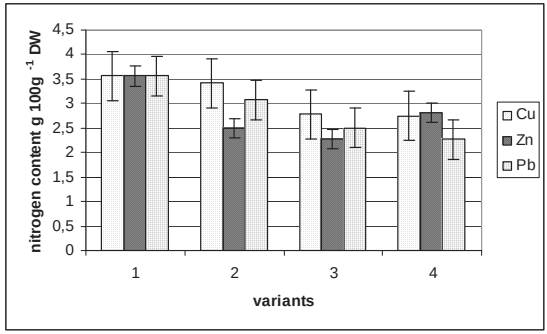

Fig. 1. Influence of Cu, Zn and Pb on nitrogen content of maize leaves (cv. Turda 200) related to applied treatment. Variants consist of: 1 - control, 2 – 0.2 ppm Cu or Zn and 0.32 ppm Pb, 3 – 2 ppm Cu or Zn and 3.2 ppm Pb, and 4 – 4 ppm Cu or Zn and 6.4 ppm Pb.

Figure 1 shows that the applied copper treatments induced a gradual decrease of the total nitrogen in the plant leaves, with an average of 0.17 units for each unit of copper. The zinc treatments induced a reduction of the nitrogen content with a mean of 0.21 units per each zinc unit applied, excepting the highest concentration where the nitrogen amount registered an increase compared to the two previous concentrations. The main trend suggested by the experiment was that all the heavy metals induced a decrease of nitrogen content of leaves, with differences related to, most probably, the mobility of the elements into the plant tissue.

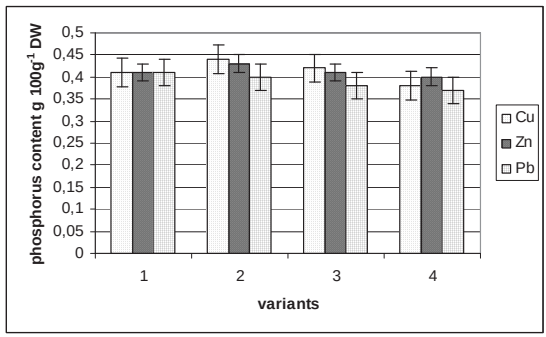

Fig. 2. Influence of Cu, Zn and Pb on phosphorus content of maize leaves (cv. Turda 200) related to applied treatment. Variants consist of: 1 - control, 2 – 0.2 ppm Cu or Zn and 0.32 ppm Pb, 3 – 2 ppm Cu or Zn and 3.2 ppm Pb, and 4 – 4 ppm Cu or Zn and 6.4 ppm Pb.

Figure 2 shows that copper and zinc treatments at low concentrations increased the level of phosphorus in maize leaves. The applied lead treatments induced a gradual decrease of the phosphorus in the plant leaves, with a mean of 0,005 units for each unit of lead. As can be seen, the heavy metals in the specific experimental conditions do not induce a significant change in the phosphorus content of the leaves.

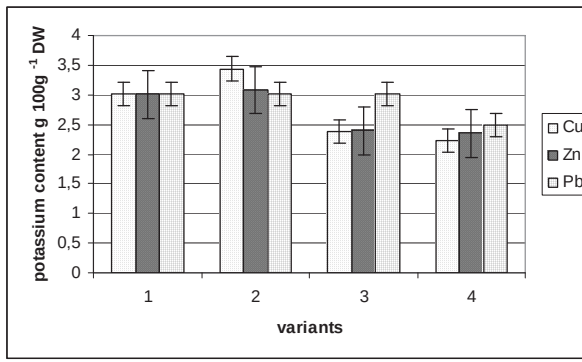

Fig. 3. Influence of Cu, Zn and Pb on potassium content of maize leaves (cv. Turda 200) related to applied treatment. Variants consist of: 1 - control, 2 – 0.2 ppm Cu or Zn and 0.32 ppm Pb, 3 – 2 ppm Cu or Zn and 3.2 ppm Pb, and 4 – 4 ppm Cu or Zn and 6.4 ppm Pb.

Concerning the influence of metals on potassium content of the leaves, as it can be seen in Fig. 3, the low concentration increased the level of potassium, with significant value for the copper appliance. At the highest concentration of metals it was recorded a decrease of the potassium content of 15% compared to control. The interesting aspect consists of the low influence of lead compared to copper and zinc on this macronutrient accumulation. Differences were recorded between the two years of the study, consisting of a higher accumulation of potassium at high temperature and low air humidity (year 2007).

The leaves capacity to absorb metals from the air is different. It depends on the air humidity (high humidity promotes leaf absorption), on the type of metal: Zn and Cu are absorbed faster in the leaves than Pb which is more adsorbed on the surface of leaves (Greger *et al*., 2004), on the pH, factor which is very important for the leaf penetrations by the water, on the moderate oxidation state of the environmental compartments (Wójcik Oliveira *et al.,* 2005).

Table 1 shows that there is an interaction between copper from the simulated rainfall and nitrogen content in plant tissues. An increase in Cu supply resulted in a very significant decrease in the concentrations of N in the roots, a significant increase in stems and a significant decrease of nitrogen in leaves.

Table 1.

Effect of Cu, Zn and Pb on plant leaf, stem and root nitrogen content after 18 days cultivation

Treatments	Leaves	Stems	Roots
	(Nitrogen g·100g^{-1} dry weight)		
Control	3,56±0.05	2.04±0.03	4,23±0.04
0.2, x10^4 ppm Cu	3,42±0.04	3.12±0.04	3,21±0.02**
2 x10^4 ppm Cu	2,78±0.04	2.59±0.04	2.43±0.05***,
4 x10^4 ppm Cu	2,75±0.05**	2.58±0.05**	2.02±0.04***,
0.2 x10^4 ppm Zn	3,76±0.05	3.16±0.03	3.02±0.04
2 x10^4 ppm Zn	2,84±0.03	2.83±0.03	2.25±0.02
4 x10^4 ppm Zn	2,81±0.04*	3.21±0.05	2.16±0.02**
0.32 x10^4 ppm Pb	3,07±0.04	2.86±0.02	3.75±0.02
3.2 x10^4 ppm Pb	2,50±0.05	2.44±0.04	3.21±0.03
6.4 x10^4 ppm Pb	2,27±0.05	1.87±0.02**	2.11±0.05**

*Mean values ± s.e. (n=4). Significant effects of heavy metal concentrations at the 0.05, 0.1 and 1 levels respectively according to ANOVA are denoted as ***, ** and *.*

It is well known that there is an interaction between nitrogen and copper - heavy dressings of nitrogenous fertilisers can depress yields in the absence of available copper; nitrogen gives increased yields only so long as there is an adequate supply of copper available. There are no available data concerning the opposite interaction and the changes of the distribution of the nitrogen among plant organs under the influence of copper.

Zn treatment influence significantly only the content of nitrogen in roots which was decreased at the highest concentration of the metal.

Published data indicated that the application of N increased the Zn concentration in wheat tops and roots in unlimed soils, and decreased it in limed soils. However, because of an increase in wheat yield, the uptake of Zn by wheat tops and roots also increased with N application (Verma and Bhagat, 1990). There are no available data concerning the opposite interaction and the changes of the distribution of the nitrogen among plant organs under the influence of zinc.

The uptake and distribution of P in plants was influenced by Zn treatment (Table 2). An increase in Zn supply resulted in a very significant decrease in the concentrations of P in the roots. The stems and the leaves were not significantly affected by the treatment. It is known that at normal Zn concentrations in the nutrient medium the content of inorganic P decreases, whereas the organic (acid-soluble)

form increases (Menser, 1985). Copper treatment did not influence significantly the distribution and the level of phosphorus in plants. Lead treatment at highest concentration decreased significantly the amount of phosphorus in stems and roots but not in plant leaves.

Table 2.

Effect of Cu, Zn and Pb on plant leaf, stem and root phosphorus content after 18 days cultivation

Treatments	Leaves	Stems	Roots
	(Phosphorus g·100g^{-1} dry weight)		
Control	0,41±0.03	0.39±0.03	1.20±0.04
0.2, x10^4 ppm Cu	0,44±0.04	0.33±0.04	1.11±0.02
2 x10^4 ppm Cu	0,42±0.05	0.31±0.04	1.18±0.05
4 x10^4 ppm Cu	0,38±0.03	0.30±0.05	1.24±0.04
0.2 x10^4 ppm Zn	0,43±0.02	0.37±0.03	1.12±0.04
2 x10^4 ppm Zn	0,41±0.02	0.29±0.03	0.89±0.02
4 x10^4 ppm Zn	0,40±0.03	0.26±0.05	0.66±0.02***
0.32 x10^4 ppm Pb	0,40±0.03	0.31±0.02	0.97±0.02
3.2 x10^4 ppm Pb	0,38±0.02	0.21±0.04	0.78±0.03
6.4 x10^4 ppm Pb	0,37±0.02	0.16±0.02**	0.53±0.05**

*Mean values ± s.e. (n=4). Significant effects of heavy metal concentrations at the 0.05, 0.1 and 1 levels respectively according to ANOVA are denoted as ***, ** and *.*

Published data indicated that Zn supply had little effect on tissue P concentration and P uptake per unit of root weight. An increase in P availability caused a significant reduction in Zn uptake per unit of root weight, and tissue concentration of Zn. The reduction in tissue Zn concentration cannot be explained entirely by a dilution effect. It is suggested that high P uptake efficiency may depress plant uptake of Zn, and therefore cause a reduction in the concentration (density) of Zn in grains of wheats grown in low P (and possibly low Zn) soils (Zhu *et al.*, 2001).

The use of phosphorus to reduce lead bioavailability is being proposed as a remedial technology for Pb-contaminated soils in residential areas. A pot study was conducted using sorghum-sudangrass (*Sorghum bicolor* L. Moench). The addition of P did not influence Pb concentrations in plant tissue and had little effect on Cd concentrations. An interaction between P source and level of P addition was found for Zn concentrations in plant tissue; concentrations increased with increasing amounts of P and decreased with increasing amounts of P from rock phosphate. Sequential extraction results suggested a reduction in Pb bioavailability from treatment with KH_2PO_4 and that P influenced the fractionations of Cd and Zn (Zwonitzer *et al.,* 2003).

The K content of the stems and leaves was not significantly affected by higher concentrations of Zn. An increase in K accumulation was detected in the roots related to the metal concentration. Copper treatment influenced significantly the accumulation of potassium in leaves but not in stems or in roots. Lead treatment in highest concentration significantly affected the content of potassium in leaves and in roots but not in the stems.

Table 3.

Effect of Cu, Zn and Pb on plant leaf, stem and root potassium content after 18 days cultivation

Treatments	Leaves	Stems	Roots
	(Potassium g·100g^{-1} dry weight)		
Control	3,01±0.03	2.98±0.02	6.28±0.03
0.2, x10^4 ppm Cu	3,44±0.02	3.15±0.04	6.17±0.02
2 x10^4 ppm Cu	2,38±0.02	3.01±0.03	6.22±0.04
4 x10^4 ppm Cu	2,23±0.03**	2.78±0.05	5.99±0.02
0.2 x10^4 ppm Zn	3,08±0.04	3.35±0.03	6.02±0.03
2 x10^4 ppm Zn	2,40±0.03	3.11±0.02	6.33±0.02
4 x10^4 ppm Zn	2,35±0.02	2.88±0.04	6.71±0.02**
0.32 x10^4 ppm Pb	3,01±0.02	2.77±0.02	5.88±0.02
3.2 x10^4 ppm Pb	3,01±0.03	2.13±0.01	5.41±0.01
6.4 x10^4 ppm Pb	2,49±0.04**	2.12±0.02	5.02±0.02**.

*Mean values ± s.e. (n=4). Significant effects of heavy metal concentrations at the 0.05, 0.1 and 1 levels respectively according to ANOVA are denoted as ***, ** and *.*

Chen *et al.*, (2007) suggested that the application of the K fertilizer could obviously restrain the uptake of Pb in an experiment conducted on wheat and that there are significant ($P < 0.05$) negative correlations between the concentrations of Pb in grains and the levels of K in soil. All the K application levels in their experiment reduced the phytoavailability of Cd and Pb. They concluded that it is feasible to apply K fertilizer (K_2SO_4) to alleviate contamination by Cd and/or Pb in soil. There are no available data concerning the opposite interaction and the changes of the distribution of the potassium among plant organs under the influence of lead.

Conclusions

Study of changes in the plant macronutrients is an important component of eco-physiological analyses. It contributes significantly to evaluation of the physiological and, consequently, health status of maize plants. In the complex chemistry of soil,

interactions between metals can be expected and these affect the uptake of the macronutrients by the plants. The role of the metal-metal interactions or metal-macronutrients may vary between different plants.

The results of analyses of nitrogen, phosphorus and potassium contained by the plant organs allow us to conclude that there is a complex interaction among heavy metals according to various internal and external factors.

At root level an increase in Cu and Zn supply resulted in a very significant decrease in the concentrations of N and an increase in Zn or Pb supply resulted in a very significant decrease in the concentrations of P compared to control.

At stem level an increase in Cu supply resulted in a significant increase of N in stems and the lead treatment at highest concentration decreased significantly the amount of phosphorus.

Our research suggests that the main trend is that the studied heavy metals induced a decrease of nitrogen content of leaves, with differences related to, most probably, the mobility of the elements into the plant tissue. Phosphorus content of the leaves was not significantly influenced by the heavy metals and lead treatment in highest concentration significantly affected the content of potassium. The interesting aspect consists of the low influence of lead compared to copper and zinc on this potassium accumulation.

An increase in Zn supply resulted in a decrease in the concentrations of P in the roots and an increase of N levels in the stems and leaves. Lower Zn translocation in aboveground parts seemed to result from Zn complexing by organic anion in the roots. This probably caused less Zn transport to the stems and leaves.

Even if there are information about the interaction between nitrogen and copper, nitrogen providing increased yields only so long as there is an adequate supply of copper available (Verma and Bhagat, 1990), there are no available data concerning the opposite interaction and the changes of the distribution of the nitrogen among plant organs under the influence of copper or lead. This was the reason for our experimental approach.

Differences were recorded between the two years of the study, consisting of a higher accumulation of potassium at high temperature and low air humidity (year 2007).

The results indicated that the mineral composition homeostasis under the stresses was important in metal tolerance At high Cu (100 μM) treatment and general nutrient deficiency treatment, the contaminated population accumulated significantly lower copper and higher phosphorus in both roots and shoots than the uncontaminated one.

Foliar concentrations of elements such as Mg, Ca, K, P, Mn, Pb and Zn are positively correlated with concentrations of corresponding soil elements. The knowledge of nutrient deficiency ranges may help diagnose foliar symptoms, but their exclusive use may overly simplify relationships between foliar symptoms and foliar elements.

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ANALYSIS OF CELL TYPES OF INFILTRATED THYROID GLANDULAR TISSUE IN OBESE STRAIN CHICKENS

ERIKA KIS[1]

SUMMARY. Hashimoto's thyroiditis is a human organ-specific autoimmune disease that affects people of all ages, with peak occurrence in women of 30-50 years of age. Chikens of the Obese strain (OS) develop, with high frequency, spontaneous autoimmune thyroiditis (SAT) characterized by circulating antibodies to autologous thyroglobin, infiltration of the thyroid gland with mononuclear cells. The early, predictable onset of SAT on OS chickens provides a unique opportunity to analyse the mechanisms initiating autoimmunity which is virtually impossible to obtain in human. All chikens from this strain develop symptoms of hypothyroidism due to SAT, and the immune system has been shown to be involved in the development of SAT. OS chickens exhibit general immune system hyperactivity, believed in part to be due to intrinsic production of cytokines. Some authors have postulated viral or bacterial infection with proteins similar to a thyroid protein, which may result in activation of thyroid-specific T cells. The evidence for an infectiouse agent as a cause of this disease, however is not convincing. The alternative hypothesis is that the thyroid epithelial cells present their own intracellular proteins to helper T cells. Once activated helper T cells (CD_4) may attract other inflammatory cells to the thyroid. The effector mechanism involved in thyroid limphocytic infiltration have been studied, but no definitive concept has yet emerged. Therefore, our studies tried to establish the histological, ultrastructural and immunohistochemical modifications of thyroid gland, and the cellular componants of the thyroid infiltrations in fowl with autoimmune thyroiditis**.**

Keywords: dendritic cells, lymphocytes, macrophages, plasmocytes.

Introduction

The Obese strain of chicknes is an excellent animal model for studies of the human autoimmune disease Hashimoto's thyroiditis, in most clinical, histological, immunological and endocrinological aspects. All chickens of this strain develop a spontaneous autoimmune thyroiditis characterized by subcutaneous fat deposits, silky feathers, massive mononuclear cell infiltration of thyroid, autoantibodies to thyroglobin and other thyroid antigens (Krömer *et al*., 1985; Hala *et al*., 1996; Pasieka, 2000; Shoko *et al*., 2009).

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The clinical and experimental data show that the infiltration of thyroid glandular tissue in the autoimmune disease, in fowls and mammals starts from the early stages of embriological development (Dietrich *et al*., 1999; Kaiser *et al*., 2002). It is assumed that the first cells invading the thyroid glandular tissue are lymphocytes T (CD_4^+) which can generated the activation of other inflammatory cells (Bagchi *et al*., 1995; Dietrich *et al*., 1999; Pasieka, 2000; Kaiser *et al*., 2002). The experimental results show that the activation of immune system are caused: on the one hand by the intensification of cytokine secretion, and on the other hand by the emergence of the immunomodulatory factor which stimulates the lymphocytes T (CD_4^+) differentiation and their migration from thymus in the thyroid (Katz *et al*., 1981).

The bibliographical data on the structure and ultrastructure of thyroid glandular tissue in the autoimmune thyroiditis in fowls are sporadic and contradictory. Therefore, we proposed to point out in this work the thyroid structural changes in the obese domestic fowl White Leghorn, to establish the degree of tissue change, as well as to identify the cell constituents of the infiltrated glandular tissue.

Materials and methods

As model of study we have used obese fowls 16 weeks old White Leghorn, who were kept under standardized bioclimatic and feeding conditions (Hala *et.al*., 1999). The animals were sacrificed by exsanguination. The thyroid gland fragments were quickly isolated and prepared for structural, ultrastructural and immunological examinations.

Histology. For structural and ultrastructural examinations, the thyroid fragments were prefixed in 4% glutaralaldehyde solution, by washing with Milloning buffer 3 successive times, postfixed in 2% osmic acid solution, by washing with Milloning buffer 3 successive times. The dehydration of samples was performed in ethylic alcohol of increasing concentration. The clarification was performed in propylene oxide and they were embedded in araldite. The semithin and ultrathin sections were obtained on a Reichert ultramicrotom. The semithin sections were stained with toluidine blue.

Immunohistochemistry. To perform the immunological studies, a part of thyroid fragments were frozen in liquid nitrogen, the other part was embedded in gelatin. For the gelatin inclusion, the fragments were fixed with 4% paraformaldehyde solution by washing on phosphate 0, 12 M buffer, dehydrated in sucrose solutions, then included in 7, 5% gelatin sucrose. To be frozen, the gelatin blocks were inserted in isopentane – 65^{o}C previously frozen in liquid nitrogen. Both the fragments frozen in liquid nitrogen and those embedded in gelatin were cut on a cryotom. The sections with thickness of 7 μm were immunological stained by the indirect immunological technique (primary monoclonat antibodies –CD_4, CD_8, Bula, anti-fowls IgG antibody, secondary antibody – biotinated anti-mouse IgG antibody, avidine - biotin peroxide complex, cloronapthol).

Results

Histological studies. The histological observations performed on semithin sections (1μm) stained with toluidine blue, revealed significant changes in the structure of the thyroid gland as compared to corresponding controls. In controls the thyroid glandular tissue is composed of follicles being in various stages of secretion (Fig. 1).

In the obese fowls groups, the thyroid glandular tissue exhibits significant changes. Thus, near the infiltrated areas the follicles containing diminished or nearly absent colloid. The follicle epithelium became thinner as it is made of squamous cells as opposed to the follicle epithelium from the controls where the cells were cuboidal. In the not infiltrated areas the thyroid follicles contain colloid, but contrary to the controls, the cells of the follicle epithelium were squamous (Fig. 2).

Histological results allowed us to identify the cell components in the infiltrated areas yet not the identification of the types of lymphocytes. They have a similar histological aspect, nevertheless they are different from the functional point of view. Their identification was achieved by means of the immunohistochemical methods.

Electronmicroscopyc studies. The electronmicroscopic examination have revealed also significant ultrastructural changes as compared to the controls. Thus in the control group the follicle epithelium is made up only of glandular epithelial cells. The follicle basal membrane is obvious. The glandular cells are cuboidal, the rough endoplasmatic reticulum is well represented, which indicates a normal secretor activity. At the apical pole, secretion grains can be noticed. The apical cell membrane shows numerous microvilli (Fig. 3).

In the obese, the follicles of the thyroid gland presents, besides the glandular epithelial cells, lymphocytes, plasmocytes, macrophages, also cells having an aspect resembling the dendritric cells (Fig. 4). The follicle basal membrane is thinner, less evident as compared to that in the controls, and are fragmented from place to place. The epithelial cells of the thyroid gland are flattened and the endoplasmatic reticulum is little developed. The apical cell membrane is almost smooth, secretions grains are not present. Among the epithelial cells there emerge epithelium-like cells with numerous extensions, having a structure resembling the dendritic cells particular to the lymphoid tissue. The basal cell membrane of these cells moves away from the follicle basal membrane. It can also be seen on the images of electronmicroscopy that the extensions of these cells are in contact with the glandular epithelial cells, with plasmocytes and T lymphocytes (Fig. 5).

In the areas thoroughly infiltrated of the thyroid gland, dilated intercellular spaces emerge in the follicle epithelium, which suggests a destruction of cellular junctions resulting in the complete or the incomplete removal of the epithelial cells. Plasmocytes, macrophages and lymphocytes emerge in the intercellular spaces in the follicle epithelium. Macrophages emerge also inside the follicle of the thyroid gland besides destructed epithelial cells. This observation was also noticed in the immunological preparations as well as those of optical microscopy. In the controls the number of cells between the follicles of the thyroid gland is insignificant (Fig. 3).

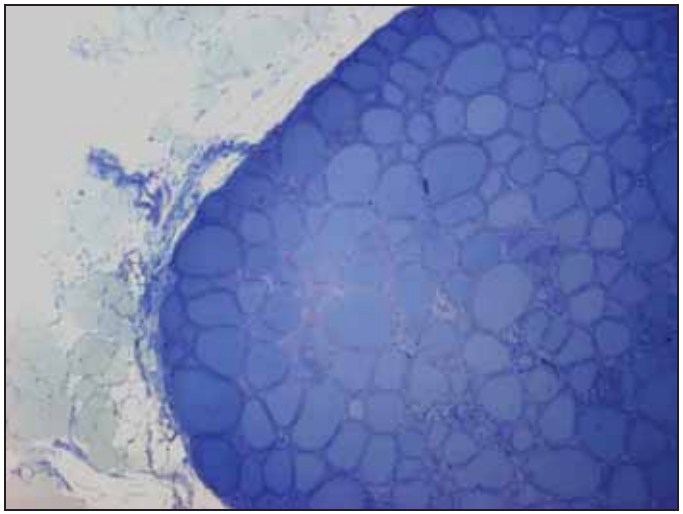

Fig. 1. Thyroid glandular tissue in controls, 10x

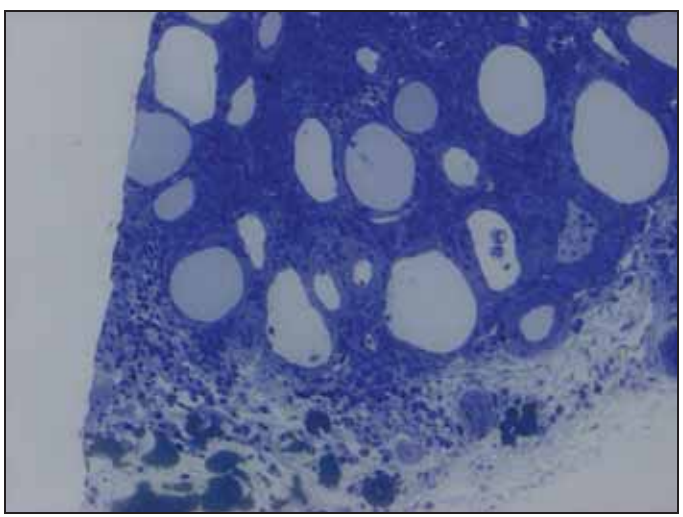

Fig. 2. Infiltrated thyroid galndular tissue in obese groups, 10x

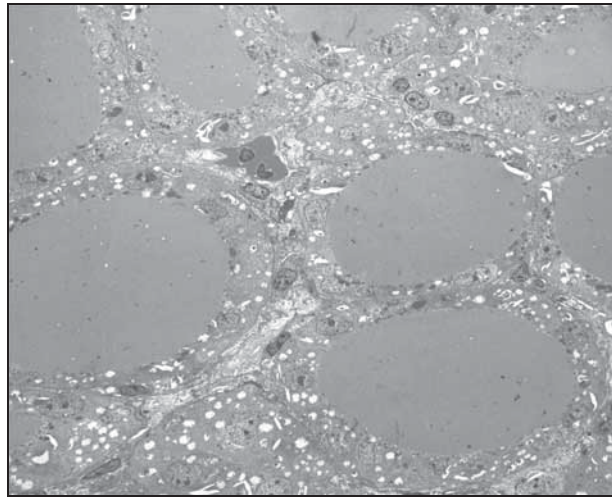

Fig. 3. Ultrastructure of normal thyroid glandular tissue, x700

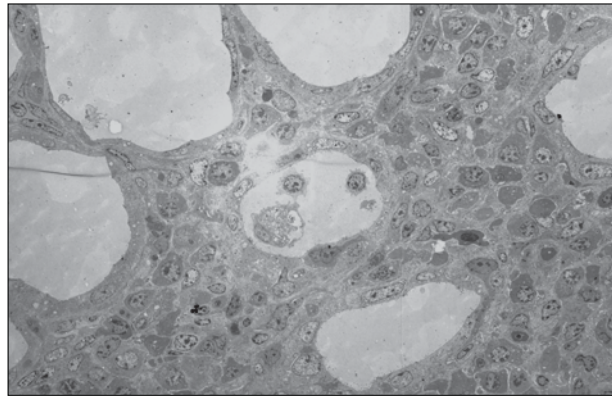

Fig. 4. Ultrastructure of infiltrated thyroid glandular tissue, x700

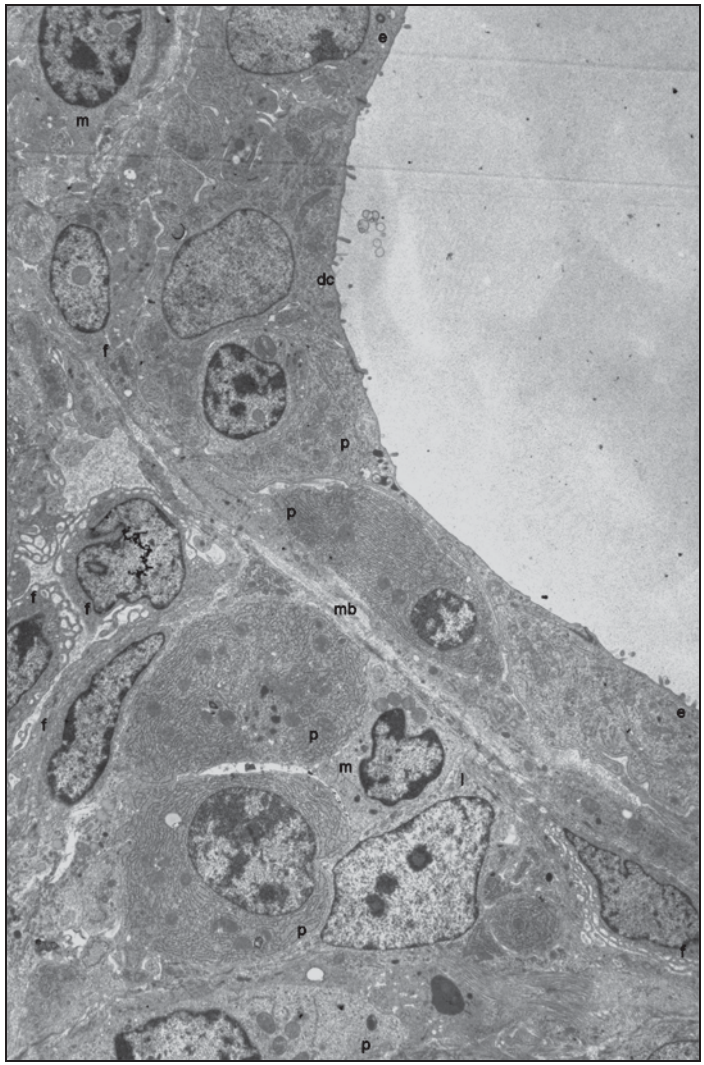

Fig. 5. Cell types of infiltrated glandular tissue, dc - dendritic cells, e – follicular epithelial cells, f - fibroblast, l - limfocite, m - macrophages, mb –basal membrane, p -plasmocite, x 3000

Immunohistochemical studies. By the immunostaining with primary monoclonal antibodies: 74.2, CD_4, CD_8, B_{ula}, anti-fowls IgG antibody (Fig. 6 A-E) the histological sections obtained from the obese fowls reveal an increased positivity (Figs. 6 f-j), while those in the control groups are negative (Fig. 6 a-e). By the immunostaining with 74.2, CD_4, CD_8 , anti-fowls IgG antibody, we have noticed that in the infiltrated areas appear macrophages (Fig. 6 Af), T-helper lymphocytes (Fig. 6 Bg), T - cytotoxic (Fig. 6 Ch) and B lymphocytes (Fig. 6 Di, Ej).

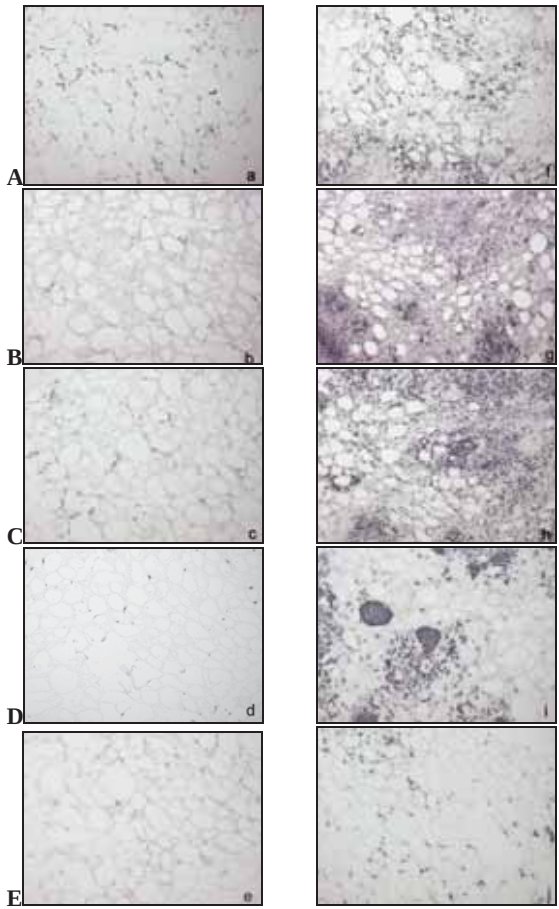

Fig. 6. Immunization with monoclonal antibodies A)74.2, B) CD_4, C) CD_8, D) B_{u1a}, E) IgG , a-e controls, f-j obese groups

Discussion

Our structural, ultrastructural and immunological results show that the glandular tissue of the thyroid gland in the obese chicken (16 weeks old), changes specific to Hashimoto's autoimmune thyroiditis. The glandular tissue of the thyroid gland is invaded by lymphocytes as well by the accessory cells of the immune system.

According to our results, in the autoimmune thyroiditis, the follicles of the thyroid gland are mostly emptied of colloid. The glandular epithelial cells are squamous, which suggest their complete inactivation. The presence of plasmocytes in a greet number around the follicles, suggests an intense secretion of antibodies, which is the expression of a strong antigenetic activity. It is unknown until now which exactly of the follicle constituents bring about the appearance of antigen, against which the body produces a high number of plasmocytes and lymphocytes and which, under normal conditions, are not present in the glandular tissue, or, it's number is very reduced. Though we have several explanations regarding the cause of glandular tissue being invaded by these cells but none of them is completely elucidated. Thus, some of the researchers assume that an antibody-antigen complex is formed in the basal cell membrane of the glandular epithelial cells but their presence is inexistent in our images of electronic microscopy. Others have the opinion that the antigen is the precursor of the thyroid hormones (thyreoglobulin) with a changed chemical structure, which causes the high number of cytotoxic lymphocytes and plasmocytes around the follicles of the thyroid gland (Stassi *et al.*, 1999). The clinical data in the case of Hashimoto's syndrome in humans show that the level of anti-thyreoglobulin antibodies is very high (Shoko *et al*., 2009)

The bibliographical data (Chen *et al.*, 2002) in the case of autoimmune thyroiditis in mouse in a primary stage show that the glandular tissue is invaded by lymphocytes, histiocytes and neutrophiles. In a more advanced stage the glandular tissue becomes fibroid. Other scientists (Yu *et al.*, 2002) assume that the first cells infiltrated among the glandular follicles are the CD_4^+ lymphocytes which stimulate the differentiation of B and T-cytotoxic (CD_8^+) lymphocytes while the T-helper (CD_4^+) lymphocytes are involved also in the fibrotic process of the glandular tissue by cytokines of type IL – 13.

The experimental results (Fässler *et al.*, 1986; Pasieka, 2000) show that the human epithelial cells in the infiltrated glandular tissue of the thyroid gland, as compared to the epithelial cells around the normal glandular tissue express the MHC II proteins. These proteins activate the T-helper lymphocytes which, at their turn, stimulate the differentiation and activation of T – cytotoxic and B lymphocytes.

Another mechanism of initiation of the thyroid autoimmune reaction was also proposed. A class of antigen presenting cells, the so-called dendritic cells was reported to be present in the thyroids of Hashimoto's patients (Lam-Tse and Drexhage, 2002). Dendritic cells (DC) are know to be the most potent antigen presenting cells of the immune system. Therefore these cells are the most likely candidates for the initiation

of the thyroid autoimmune response. DC are present in low number in normal thyroids, composing 2-3 % of the interstitial cell population. There are indications that such DC are able to proliferate, which means that not all of the thyroid DC need to be recently immigrated from the blood stream. Thyroidal DC are often in close contact with thyreocytes.

The role of dendritic cells has been reported in mice and rats which manifest the thyroid autoimmune syndrome. In the case of rats with the autoimmune syndrome, a numeric increase of these cells has been observed. As far as we are aware of, the presence and role of these cells in obese chickens was yet not signaled. In our images of electronic microscopy it has been seen that the dendritic cells are in contact with the glandular epithelial cells on the one hand while, on the other hand, with the plasmocytes and lymphocytes.

Recent bibliographical data (Shoko *et al.* 2009) show that the dendritic cells are involved in the immunological response either by means of antigens or the intensification of cytokine synthesis and the stimulation of T – helper (CD_4^+) lymphocytes differentiation. It was seen that in mice of CBA/J – type, the dendritic cell producing IL – 12 plays a role in the outburst of the autoimmune thyroiditis, stimulating the invasion of the glandular tissue with lymphocytes.

The presence of macrophages both in the follicle epithelium as well as between the thyroid follicle suggests an intense phagocytic activity. It was experimentally proved in mice of CBA/J type that, the intensification of IFN – γ synthesis by T-lymphocytes (CD_4^+) facilitates the apoptosis of glandular epithelial cells (Jin *et al.*, 2004).

The bibliographical data (Vas *et al*, 2003) related to the autoimmune disease described in man, show that T-helper lymphocytes (CD_4^+) activated stimulate the T – cytotoxic lymphocytes (CD_8^+) which are responsible for the hypothyroidism due to the destruction of thyroid epithelial cells. Our immunological results show that at the age of 16 weeks the glandular thyroid tissue in the obese chickens present the cells mentioned above having a role in the outburst of hypothyroidism.

The experimental data (Colin and Gilbert, 1996; Vasicek *et al*., 2001) show that T-helper lymphocytes (CD_4^+) are involved in the fibrosis of glandular tissue due to their capacity to produce $TG\beta_1$. $TGF\beta_1$ stimulated the diapedesis of other inflammatory cells in thyroid among which that of macrophages. It was seen in mice with the autoimmune thyroiditis that in the incipient phase of fibrosis, T lymphocytes (CD_4^+) are involved, and in a more advanced stage, the macrophages, which also synthesize $TG\beta_1$ and other cytokines (like IL_{13}). The high level of IL_{13} cytokine attracts into the thyroid the miofibroblasts and the fibroblasts, which caused the intensification of collagen synthesis. In obese mice it was immunohystochemically determined the presence of miofibroblasts (α-SMA) who have a role in the collagen synthesis. In our knowledge the presence of fibroblasts in the infiltrated thyroid glandular tissue in chickens with the autoimmune syndrome has not been yet signaled.

Conclusions

Based on the own data and those in the literature we can assume that the dendritic cells have an important role in the emergence and evolution of leukocyte infiltration of the thyroid glandular tissue in chickens which manifest the Hashimoto's autoimmune disease. It is also true that these cells are indirectly involved in the atrophy and fibrosis of the glandular tissue, by attracting the inflammatory cells, which manifest in the presence of hypothyroidism.

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THYROGLOBULIN IN THE EVALUATION OF NODULAR GOITRE AND THYROID CARCINOMA

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SUMMARY. The number of patients with nodular goitre and thyroid carcinoma worldwide has increased, mainly after Chernobyl nuclear accident. Thyroglobulin is reported as an essential tumour marker in the management of patients with differentiated thyroid carcinoma. The aim of the study was to determine the thyroglobulin level in the thyroid tissue obtained by fine needle aspiration from thyroid nodules or cervical lymph nodes and to improve the specificity of the method in the diagnosis of cancer. In this retrospective study we used the database of the Institute of Oncology "Prof. Dr. I. Chiricuţă" in Cluj-Napoca (Romania) from the decade 1998-2007; a number of 1803 cases of malign thyroid tumors with the mean age of 47 years and 15,778 cases of nodular goitre with the mean age of 42.1 years. We analyzed the evolution of the incidence of this pathology. We performed 600 fine needle aspirations; among them we selected 35 cases with the serum and also the tissue level of thyroglobulin determined on electrochemiluminescence method. We analyzed the results and the correlation between the serum thyroglobulin, tissue thyroglobulin, their ratio with serum, the total protein and the histological results after the surgery. The conclusion of the study was that a simple and very accessible method, such as fine needle aspiration, could improve the differential diagnosis of thyroid nodules.

Keywords: nodular goitre, thyroglobulin, thyroid carcinoma

Introduction

In the last decade, the number of patients with nodular goitre and thyroid carcinoma in South-Eastern Europe is dramatically increased, mainly due to the high ionizing radiation exposure after Chernobyl catastrophic event (Pacini and DeGroot, 2001). Thyroglobulin (Tg) is reported as an essential tumour marker in the management of patients with differentiated thyroid carcinoma (DTC). Serum level of Tg is of crucial importance as indicator of early local recurrence and/or distal metastasis of DTC (Van Herle, 1973; Irimie *et al.*, 2003). The aim of the study was to determine the Tg level in the thyroid tissue obtained by fine needle aspiration (FNA) from thyroid nodules or cervical lymph nodes and its relevance in the diagnosis of cancer (AACE).

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Patients and methods

Patients: In this study we used the database of patients with nodular goitre and thyroid carcinoma being in the evidence of the Institute of Oncology "Prof. Dr. I. Chiricuță" in Cluj-Napoca (Romania) in the decade 1998-2007. It was a retrospective study of the incidence of this pathology following the nuclear accident from Chernobyl. In this period were diagnosed and treated in the Institute a number of 1803 cases of malign thyroid tumors with the mean age of 47 years (standard deviation of 14 years) and with a female to male ratio of 7.3 to 1. The number of patients with thyroid nodular disease is 15,778 cases in the mentioned period with the mean age of 42.1 years. All these patients underwent the diagnosis and the therapeutic strategies (thyroidectomy, radioiodine therapy and hormonal therapy) in the Institute of Oncology "Prof. Dr. I. Chiricuță". The follow-up of the patients with DTC included the routinely measurements of serum level of Tg and antibodies against Tg, in a period of rising level of thyroid stimulating hormone (TSH) by interruption of hormonal suppression.

Fine needle aspiration (FNA): In the last 2 years of our study we introduced the FNA of thyroid nodules and the determination of Tg level from the tissues. The needle used is thinner than the one for drawing blood (25G 0.5 X 16 mm), and is attached to a syringe in a syringe holder that allows the operator to apply suction easily. Cells from thyroid lesion were extracted through this thin needle. These cells were smeared (spread) on glass slides, stained with hematoxilin-eozin and examined under the microscope. After examining all the slides, the pathologist made a cytological diagnosis and issued a written report. For a number of 600 cases of FNA we used the standard classification of the FNA results in five groups (Thy 1-5) according to the British Thyroid Association (Gharib and Goellner, 1993).

Thyroglobulin (sTg): The serum level of this tumor marker was determined in an authorized commercial laboratory (Synevo, Cluj-Napoca) by the electrochemiluminescence method, using a Roche kit. The cutoff value of the test was 78 ng/ml.

Thyroid Tissue Thyroglobulin (tTg): The tTg from the cytological specimen obtained by FNA was analyzed in the same laboratory and with the same kit, after an ultrasonic centrifugation at 10,000 x g for 20 minutes. The protein supernatant was isolated and analyzed by electrochemiluminescence on an Roche Elecsys ® System.

We analyzed the results and correlation between the sTg, tTg, the ratio sTg/serum total proteins, tTg/ serum total proteins and the histological results after surgery. Due to the lack of a normal distribution, statistical analysis was carried out with FNA Excel version 5.0 for Windows using the Spearman Rank Order Correlation and the Mann-Whitney's test. The observed differences were considered statistically significant if the probability of chance occurrence was $p < 0.05$.

Results

This retrospective study of the incidence of thyroid carcinoma following the nuclear accident from Chernobyl shows a clear rise of the number of cases of thyroid carcinoma according to the worldwide tendency. From 1970 till 1997, in 28 years were recorded in the Institute 578 cases of thyroid cancer. In the next 10 years (1998-2007) 1803 cases of malign thyroid tumors were diagnosed and treated in the Institute (Fig. 1). The mean age was 47 years (with standard deviation of 14 years); ranging between 6 and 90 years, and the average age was 51 years. The sex distribution and the histological types are presented in Figures 2 and 3. The number of patients with thyroid nodular disease was 15,778 cases in the mentioned period with the mean age of 42, 1 years and the female/male ratio of 7.8/1 (Fig. 4).

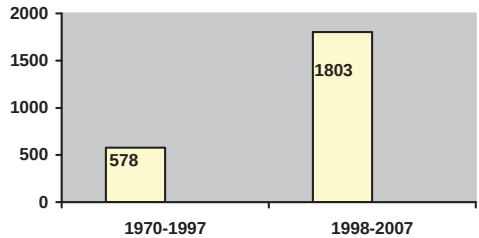

Fig. 1. Thyroid cancer incidence during two periods (1970-1997 and 1998-2007) as diagnosed and treated in the Institute of Oncology (Cluj-Napoca, Romania)

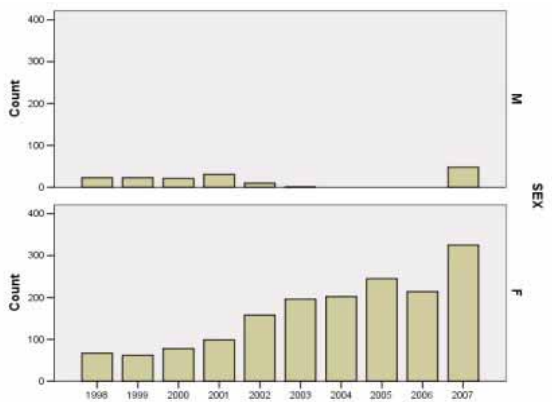

Fig. 2. Sex distribution of thyroid cancer diagnosed and treated in the Institute of Oncology (Cluj-Napoca, Romania) between 1998-2007

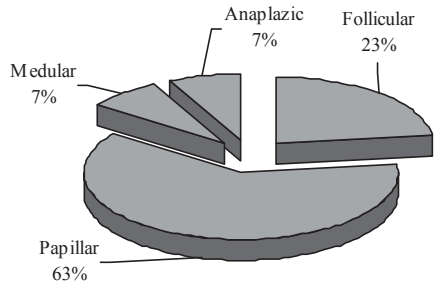

Fig. 3. Histological distribution of thyroid cancer in the 1803 patients diagnosed with thyroid cancer between 1998 and 2007 at the Institute of Oncology

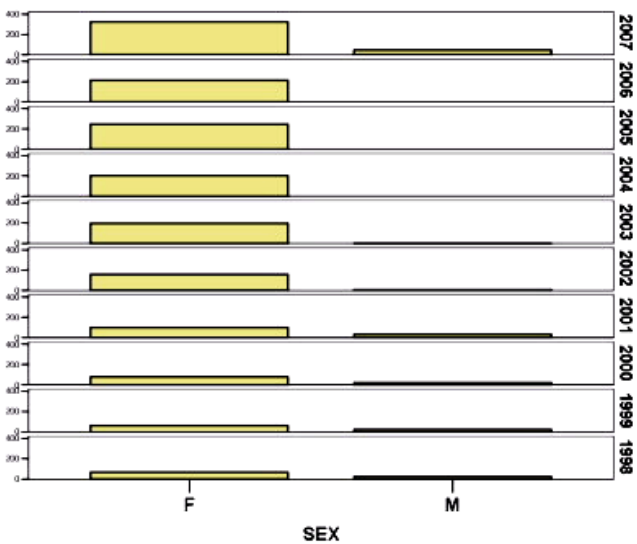

Fig. 4. Sex distribution of nodular goitre diagnosed in 15,778 patients recoded at the Institute of Oncology (Cluj-Napoca, Romania) between 1998-2007

A number of 35 patients have been analysed by FNA and the sTg and tTg have been determined. All patients were treated surgically with one of the following methods: total thyroidectomy (17 cases), subtotal thyroidectomy (10 cases) and cervical lymph node dissection (8 patients). Diagnosis of the disease was established on the basis of clinical, laboratory and histopathological findings. The clinical and histological data for these patients are shown in Table 1. The histological tumor classification was made according to the World Health Organization criteria (Robin and Wittekind, 2002).

The group of 35 patients was selected according to typical clinical, ultrasound and scintigraphic criteria, being suspicious of malignant thyroid nodules or lymph nodes. The results of FNA divided the group into five cytological classes as following: Thy I (undetermined) - 1; Thy 2 (benign cells) - 15; Thy 2 (follicular cells) - 3; Thy 4 - (suspicious malignant cells) - 10 and Thy 5 (malignant cells) - 6 patients, respectively. The clinical exam revealed a single or dominant nodule, “cold ” at scintigraphy, hypoechogenic at ultrasound.

Table 1.

General characteristics of 35 patients with FNA and Tg measurements

Parameter		**Number of patients**
Age	< 45 years	11
	> 45 years	24
Clinical features	solitary nodules	14
	multinodular goiter	10
	diffuse goiter	4
	not known	3
	palpable lymph node	4
Histologic characteristics		
Simple goiter	colloid diffuse goiter	4
	colloid micro- and acrofollicularis	2
Toxic nodular goitre	colloid nodules goiter	1
	colloid diffuse goiter	4
Graves-Basedow disease	parenchymatous toxic goiter	3
	colloid micro- and macrofollicularis	1
Metastatic lymph nodules from thyroid differentiated carcinoma		4
Hürthle adenoma	“Hürthle cell” adenoma	3

Tabel 1. (continued)

Parameter		Number of patients
Malignant tumors	papillary	9
	follicular	3
	anaplastic	1
Tg concentration in serum	normal < 78 ng/ml	9
	high > 79 ng/ml	22
	low < 10 ng/ml	4
Tg content in the tissue of FNA	normal 50–100 mg/ml	10
	high > 100 mg/ml	18
	low < 10 ng/ml	7
Complementary treatment	^{131}I therapy	12
	thyroid hormone	35

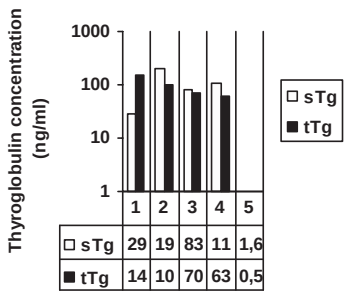

Fig. 5. Mean values of thyroglobulin content in thyroid tissues and serum in patients with different diagnosis.
1 – benign diseases (n-15); 2 – metastatic lymph nodes from thyroid differentiated c1arcinoma (n = 4), 3– papillary thyroid carcinoma (n =9), 4 – folliculary thyroid carcinoma (n =3), 5 – anaplastic thyroid carcinoma (n =1)

The analysis of the Tg revealed a significant amount of tTg (197 ±8.42 ng/ml, with normal values being 50–100 ng/ml) and high sTg serum concentration (103.45 ± 9.66 ng/ml, with normal values < 78 ng/ml) in the metastatic nodules from thyroid differentiated carcinoma. In anaplastic cancer, the content of tTg was very low (1.63 ng/ml) and sTg- 0.54 ng/ml in serum. The mean value of tTg content was 83.76 ± 24.33 ng/ml in tissue and 70.23 ± 21.89 ng/ml in serum for the patients with follicular thyroid carcinoma. (Fig. 5).

Discussion and conclusions

Thyroglobulin is a recognized and uncommonly useful marker in thyroid neoplasm. This protein is an important prognostic factor indicating the presence of metastatic disease, and a rise in the serum Tg in patients with known metastases indicates progression of the disease. Since the first work of Van Herle *et al*. (1973), several papers on Tg concentration have been published but only a few publications evaluated the Tg content in thyroid tissue. In the cases of anaplazic neoplasms, the Tg levels in thyroid tissue were undetectable. This was probably caused by the absence of the expression of mRNA Tg. We examined patients with benign thyroid and Tg concentration and we detected a normal or slightly elevated level of thyroid Tg amount.

The Tg serum concentration was elevated in connection with various thyroid disorders, e.g. goiter, differentiated thyroid carcinoma and in metastatic lymph nodes from thyroid differentiated carcinomas. As shown in Fig. 5, the serum Tg is higher in metastatic disease than in the thyroid tissue of FNA, also in the case of follicular carcinoma. In the benign tumors, the tissue level of Tg is higher than in serum level. The assay of thyroglobulin in serum is of major importance for the follow-up of patients with thyroid carcinoma. Differentiated thyroid carcinomas of epithelial origin generally produce thyroid hormones and thyroglobulin. Follicular carcinomas usually cause higher serum levels than papillary carcinomas (Irimie *et al.*, 2006). Undifferentiated carcinomas produce thyroglobulin only occasionally.

In conclusion, no significant differences in the amount of sTg and tTg were noted in cases of papillary carcinomas, an increased synthesis of large amounts of both sTg nd tTg may appear in thyroid lymph node metastasis and a ratio sTg/tTg < 1 strongly suggests benign thyroid nodules.

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BACTERIAL COMMUNITY STRUCTURE IN THE POLLUTED SOILS FROM CLUJ COUNTY (NORTH-WEST ROMANIA)

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SUMMARY. Microorganisms either independently or in association with other organisms play a very important role to the plant nutrients recycling. Soil pollutant concentrations above certain limits disrupt the ecosystems and as a result the nutrient cycling process. The paper presents the seasonal evolution of the bacterial community structure in the most representative polluted soils from Cluj County: urban roadside soil, industrial polluted soils including metallurgy and refractory products manufacturing sector, soils affected by zootechnical residues, soil contaminated with hexachloro-cyclohexane pesticide residue and soil polluted by uncontrolled dumping of municipal solid waste. The following 7 ecophysiological groups were investigated: aerobic heterotrophic bacteria, ammonifying bacteria, nitrifying bacteria (ammonium and nitrite oxidizing bacteria), denitrifying bacteria, iron-reducing bacteria and sulphate reducing bacteria. Enumeration of viable soil microorganisms was accomplished by serial dilution and plate count technique (aerobic heterotrophic bacteria) or most probable number method (all other analyzed bacteria). Based on the obtained results, the bacterial indicator of the soil quality (BISQ) was calculated for each soil sample. The BISQ values ranged between 2.047 and 3.708, indicating a moderate density of the bacterial groups. Quantitative analysis of soil microbial populations shows a marked decrease in total culturable numbers of the different microbial groups of the contaminated soil samples. Aerobic heterotrophic bacterial populations were particularly sensitive to pollution. Organic matter decomposition and nitrogen mineralization, which are carried out by various groups of microorganisms, have also been affected by increased pollutants concentration in the soils. A negative correlation between the bacterial indicators of soil quality and heavy metals was established.

Keywords: bacterial communities, bacterial indicator of soil quality, soil pollution

Introduction

The mineralization of organic matter is carried out by a large community of organisms and involves a wide range of metabolic processes. Changes in the composition or activity of microbial communities due to pollution might have lasting

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effects on ecosystem functioning (Perry *et al.*, 1989; Winding *et al.*, 2004). Heavy metals affect the growth, morphology and metabolism of microorganisms in soils, through functional disturbance, protein denaturation or the destruction of the integrity of cell membranes (Leita *et al.* 1995). The response of microorganisms to heavy metals has been measured in soil by estimating the size of microbial biomass (Brookes and McGrath 1984, Brookes 1995), respiration and enzyme activities (Nannipieri *et al.*, 1994). Microbial parameters appear very useful in monitoring soil pollution by heavy metals.

The aims of the present study are the investigation of the effects of pollutants (heavy metal, pesticides) on microbial biomass and an evaluation of the impact of contamination with pollutants on the diversity of soil microbial communities. This approach may permit an evaluation of the status of polluted ecosystems, while providing insight about the functional diversity of soil microbial communities of undisturbed habitats.

Materials and methods

Soil samples. Eight sampling sites were selected in order to cover the main sources of pollution in Cluj County: traffic (urban roadside area from Unirii Square), industrial activities from the manufacturing sector of non-metallic materials (Casirom Turda) and metallurgy (Combinatul de Utilaj Greu Cluj-Napoca – CUG Cluj-Napoca), industrial farm animal production (Popesti chicken farm and Bontida swine farm), industrial waste disposal (hexachlorocyclohexane waste deposit from Turda) or household waste (Pata Rat landfill from Cluj-Napoca). The control samples were taken from the Cheile Turzii Nature Reserve, one of the most important protected wildlife sites in Cluj County. Individual soil cores were taken with a PVC core sampler (at a depth of 0-20 cm) from three different places and mixed together to prepare a composite sample for each site. The composite samples were used for all subsequent analyses. All operations connected to the bacteriological analyses were carried out under sterile conditions. The content of the soil in dry substance was established by drying parts of samples at 105°C for three days.

Physical and chemical analyses. The pH values were determined with ProfiLine 197 handheld pH meter; conductivity with HANNA HI 993310 handheld conductivity meter, soils humidity was determined by the gravimetric method at 105^0C, soil organic matter by sulfochromic oxidation method (ISO 14235:2000 Soil quality – Determination of organic carbon by sulfochromic oxidation) and organochlorine pesticides concentrations by gas chromatography-mass spectrometry. Gas chromatography was performed using a Hewlett Packard HP6800 series equipped with a Micromass AutoSpec Ultima mass spectrometer (Micromass Ltd., Manchester, UK). The column was a BPX-25 fused silica capillary column, 0.22 mm i.d. × 30 m with a 0.25-mm film thickness (SGE International Pty Ltd.). Heavy metals concentrations were analyzed with atomic absorption spectrophotometer type Varian SpectrAA 880.

Microbiological analyses. The following 7 ecophysiological groups of bacteria were analyzed: aerobic heterotrophic bacteria, ammonifying bacteria, nitrifying bacteria, denitrifying bacteria, iron-reducing bacteria, and sulphate reducing bacteria.

A solid nutrient medium containing meat extract, 0.3 g; agar-agar, 2 g; peptone, 1 g; NaCl, 0.5 g and 100 ml distilled water, was used for enumeration of the total number of *aerobic heterotrophic bacteria* (Atlas, 2004). The pH was adjusted to 7.5, prior sterilization at 120ºC for 1 hour.

Ammonifying bacteria were revealed in liquid culture medium containing peptone, 2 g; NaCl, 0.5 g; 100 ml distilled water. The pH was adjusted to 7.9 before sterilization at 120ºC for 1 hour.

Ammonium oxidizing bacteria were cultured on a liquid culture medium containing Winogradsky's salt solution (0.4 g K2HPO4, 0.13 g NaCl, 0.13 g MgSO4·7H2O, 1.52 mg MnSO4·H2O, 0.5 g NH4NO3) diluted 1:20, 100 ml; ammonium sulphate $(NH_4)_2SO_4$, 0.05 g, calcium carbonate ($CaCO_3$), 0.1 g (Drăgan-Bularda medium, 2000). The medium was sterilized at 110 ºC for 20 minutes.

For *nitrite-oxidizing bacteria*, the following liquid culture medium was used: Winogradsky's salt solution diluted 1:20, 100 ml; sodium nitrate ($NaNO_3$), 0.1 g; calcium carbonate ($CaCO_3$), 1 g (Drăgan-Bularda medium, 2000). The medium was sterilized at 110 ºC for 20 minutes.

A liquid culture medium consisted of: potassium nitrate (KNO3), 0.2 g; glucose, 1 g; CaCO3, 0.5 g; *Winogradsky's salt solution*, 5 ml and distilled water, 95 ml; pH, 7.2 (De Barjac culture medium) (Pochon, 1954) was used to enumerate *denitrifying bacteria*. The medium was sterilized at 112 ºC for 20 minutes on three successive days.

Iron-reducing bacteria were cultured in a liquid culture medium that contained glucose, 2 g; asparagines, 0.5 g; yeast extract, 0.05 g; dibasic potassium phosphate (K_2HPO_4), 0.3 g; monobasic potassium phosphate (KH_2PO_4), 0.08 g; potassium chloride (KCl), 0.02 g; distilled water 100 ml, iron oxide ($Fe_2O_3 \cdot 3H_2O$), 0.1g; pH, 7.0 (Ottow medium, 1968). The medium was sterilized at 105 ºC for 1hour on three successive days.

Sulfate reducing bacteria were revealed in a liquid culture medium with Na-lactate, 0.5 g; asparagines, 0.2 g; dibasic potassium phosphate (K_2HPO_4), 0.1 g; trace metal solution (0.15 g $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$); distilled water, 100 ml (Van Delden medium) (Allen, 1957). The medium was sterilized at 105 ºC for 1 hour on three successive days.

Total viable counts of culturable aerobic heterotrophic bacteria were obtained by preparing six successive 10-fold serial dilutions of 1 g of fresh soil samples (in order to obtain a small number of colonies on each plate) and surface plating on to sterile nutrient agar in triplicate. After incubation (30°C for 7 days), the counts obtained were multiplied by the dilution factor to obtain the number of colony forming unit per gramme of soil.

Except for the aerobic heterotrophic bacteria (where the plate count method was used), the most probable number (MPN) method was applied to estimate the number of soil microorganisms, using dilution series and multiple tubes per dilution (five tubes for each of the six successive 10-fold dilutions). Dilution was made until a readable count was obtained. After incubation, the most probable number of bacteria was calculated according to the statistical table of Alexander (1965).

Statistical analysis. The results obtained were examined statistically with a one-way analysis of variance (ANOVA). The least significant differences were calculated with the Tukey`s test at a significance level of p=0.05 and p=0.01. Pearson`s correlation coefficient r was used to describe the degree of linear association between the number of bacteria and soil physico-chemical properties.

Results and discussion

Physical and chemical analyses were carried out during the summer of 2008 in order to establish the effects of the pollutants on the microbiological characteristics of the soil. The physico-chemical characteristics of the analysed samples are presented in the Table 1.

Tabel 1 shows the presence of heavy metals in all of the soil samples which were analysed. The normal limits for Zn, Cr, Cu, Pb, Cd, Ni and Co are: 100 ppm (Zn), 30 ppm (Cr), 20 ppm (Cu, Pb and Ni), 15 ppm (Co) and 1 ppm (Cd).

The concentrations of Zn, Cu, Pb, Ni and Co were higher than the normal limits in the samples collected from Combinatul de Utilaj Greu Cluj-Napoca (Zn, Cu, Pb, Ni), Pata Rat deposit (Zn, Ni), Unirii Square (Cu, Pb), Turda hexachlorocyclohexane deposit (Cu, Pb, Co) and Popesti (Co).

The highest concentrations in heavy metals were detected in the samples collected from Turda hexachlorocyclohexane deposit: Pb (64.6 ppm), Co (46.8 ppm), Cu (59.1 ppm) and Pata Rat deposit: Zn (188.7 ppm) and Ni (23.8 ppm).

In all soil samples, which were analysed, the concentrations of Cr and Cd were significantly below the normal limit. The samples collected from Cheile Turzii presented the lowest content of heavy metals.

Table 1.

Physico-chemical characteristics of the soil samples

Sample location	pH	C µS/cm	H %	OM %	HCH ppm	Heavy metal (ppm)						
						Zn	Cr	Cu	Pb	Cd	Ni	Co
CUG Cluj	6.8	322	7.33	4.68	-	132.4	26	43.5	55	0.2	22.7	5.7
Casirom	7.95	145	20.86	3.51	-	36.7	18.4	11.5	16.1	0.2	12.6	2.8
Unirii Square	6.66	189	5.65	4.77	-	50.4	26	28.8	37.1	0.7	16.6	3.1

Table 1. (continued)

Sample location	pH	C µS/cm	H %	OM %	HCH ppm	Heavy metal (ppm)						
						Zn	Cr	Cu	Pb	Cd	Ni	Co
Bontida	7.83	115	11.95	8.59	-	40.9	11.8	7.4	15.8	0.2	9.18	8.7
Popesti	7.85	188	20.3	7.82	-	37.9	7.9	16.6	14.5	0.2	15.2	35.2
Pata Rat deposit	7.45	82	10.74	6.78	-	188.7	6.9	10.2	5	0.5	23.8	5.8
HCH deposit	7.77	195	13.22	5.71	473	94.0	10.4	59.1	64.6	0.4	13.8	46.8
Cheile Turzii	7.82	173	10.82	11.38	-	16.3	5.3	5.2	8.7	0.3	9.9	0.2
Normal limit	**-**	**-**	**-**	**-**	**0.005**	**100**	**30**	**20**	**20**	**1**	**20**	**15**

C – conductivity, H – humidity, OM – organic matter, HCH - hexachlorocyclohexane, Normal limit – according to the Order number 756/1997 of the Ministry of Waters, Forests and Environmental Protection *Reglementation regarding evaluation of environment contamination*

A high concentration of organochloride pesticide (hexachlorocyclohexane) was detected in Turda hexachlorocyclohexane deposit, much more over the normal limit which represents an alarm signal for human and environmental health.

Microbiological analyses were carried out in each season during 2008 on soil samples taken from the same eight samples which were analyzed from physical and chemical point of view. The samples were taken from a depth of 20 cm.

Six ecophysiological groups were present in all the analyzed samples: aerobic heterotrophic bacteria, ammonifying bacteria, ammonium oxidizing bacteria, nitrite oxidizing bacteria, denitrifying bacteria and iron-reducing bacteria. In the soil from Cheile Turzii, Bontida and Popesti was a large number of aerobic heterotrophic bacteria of order 10^4 - 10^6 colony-forming unit (CFU)/g dry matter soil. The number of bacteria belonging to the other groups was much smaller.

In the order of their abundance, the aerobic heterotrophic bacteria (10^4 - 10^6 CFU/g dry matter soil) were followed by the ammonifying bacteria (10^3 - 10^4 cells/g dry matter soil), ammonium oxidizing bacteria (10^2 - 10^4 cells/g dry matter soil), denitrifying bacteria (10^1 - 10^3 cells/g dry matter soil) and nitrite oxidizing bacteria (10^1 - 10^3 cells/g dry matter soil). The smallest numbers were observed for the iron-reducing bacteria (10^1 – 10^2 cells/g dry matter soil).

The microbiological analyses showed lack of the sulphate reducing bacteria in the soils from Combinatul de Utilaj Greu Cluj-Napoca, Casirom, Unirii Square, Popesti, hexachlorocyclohexane deposit and Cheile Turzii protected area. The sulphate reducing bacteria were present only in 2 soils (Bontida and Pata Rat), but their number was the lowest (10^{-1}-10^1 cells/ g dry matter soil), as compared to the other bacterial groups.

The general bacterial potential of the soils was appreciated on the base of the bacterial indicators of soil quality (BISQ) values, taking into account the number of bacteria belonging to each ecophysiological group (Muntean, 1995-1996).

$$BISQ = 1/n \times \Sigma \log_{10} N$$

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

Figure 1 presents the variation of the bacterial indicator of soil quality for each soil sample during the year 2008.

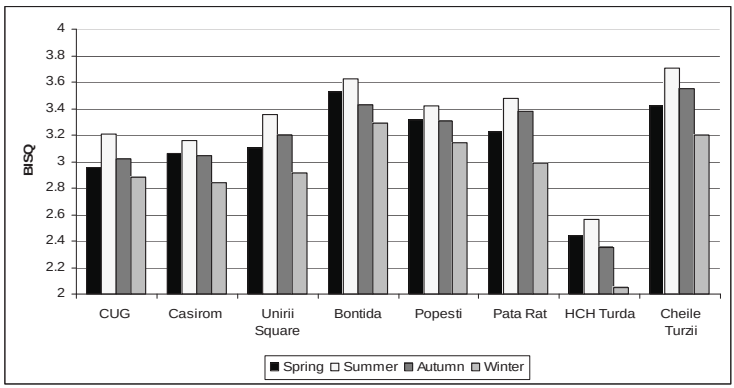

Fig. 1. Seasonal variation of the bacterial (BISQ) indicators of soil quality in the polluted soils from Cluj County

In all soil samples the highest values of BISQ were recorded during the summer due to the presence of nutrients and high temperatures and the lowest values of BISQ were recorded in the winter. The maximum value was recorded in summer in Cheile Turzii (3.7080) and the minimum in winter, in the area of the Turda hexachlorocyclohexane deposit.

Another obvious observation was the low level of the bacterial potential of the soil from the Turda hexachlorocyclohexane deposit area compared to the other soil samples. In Turda area was also recorded the highest concentration of heavy metals and pesticides. BISQ values from the soil samples affected by industrial pollution (Combinatul de Utilaj Greu Cluj-Napoca, Casirom Turda) and heavy traffic from Unirii Square were lower than the ones recorded in the disposal area of the municipal household waste from Pata Rat or the zootehnichal area at Popesti and Bontida. BISQ

had the maximum value in the control sample Cheile Turzii, area unaffected by pollution, where the content of heavy metals was the lowest, which reveals an active and balanced microbial community. The high value for BISQ in the household waste landfill was probably due to the organic material input which can stimulate the development of microorganisms. Also, the high values of BISQ in Popesti and Bontida were the consequences of the stimulating effect of natural fertilizer (dung) which was used on the land in those areas, along with the loss of used waters produced by the farms caused by the un-appropriate sewerage system.

In the hierarchy of the polluted soils, based on the values of the bacterial indicator of soil quality, the soil from Cheile Turzii protected area was situated on the first 8 positions and on the last one was situated the soil from hexachlorocyclohexane deposit area (Table 2).

Table 2.

Hierarchy of the polluted soils according to the BISQ values

Position	Sample location	BISQ
1	Cheile Turzii	3.471
2	Bontida	3.467
3	Popesti	3.296
4	Pata Rat	3.266
5	Unirii Square	3.143
6	Casirom Turda	3.028
7	CUG Cluj-Napoca	3.016
8	Turda hexachlorocyclohexane deposit	2.349

The inhibiting effects of pollutants upon the dimension and the activities of bacterial populations are also sustained by correlation analyses between the physical and chemical characteristics and the microbiological properties. The statistical analysis of the collected data revealed positive correlations between all eco-physiological bacterial groups and the quantity of organic substances. Statistically significant positive correlations were detected between the organic matter content and the number of aerobic heterotrophic bacteria ($r = 0.97$, $p < 0.01$), ammonifying bacteria ($r = 0.83$, $p<0.01$) and denitrifying bacteria ($r = 0.84$, $p<0.01$).

These analyses showed a parallel evolution of the bacterial populations involved in the nitrogen cycle (ammonifying bacteria, nitrifying bacteria and denitrifying bacteria). Significant correlations were found between the number of ammonifying bacteria and ammonium oxidizing bacteria ($r=0.92$, $p<0.01$); ammonifying bacteria and nitrite-oxidizing bacteria ($r = 0.86$, $p<0.01$); ammonium oxidizing bacteria and nitrite-oxidizing bacteria ($r = 0.87$, $p<0.01$); ammonium oxidizing bacteria and denitrifying bacteria ($r = 0.82$, $p<0.05$); nitrite-oxidizing bacteria and denitrifying bacteria ($r = 0.73$, $p<0.05$).

Negative correlations were recorded between the content of organic substances and the concentration of heavy metals but these were not statistically significant.

The correlation analyses between the physical and chemical properties and the microbiological properties confirmed the inhibiting effect of heavy metals on the dimension of the bacterial populations. Negative correlations were observed between the size of the aerobic heterotrophic bacteria, ammonifying bacteria, ammonium oxidizing bacteria, nitrite oxidizing bacteria, denitrifying bacteria and the concentrations of all seven heavy metals. Were found statistically significant negative correlations between the density of aerobic heterotrophic bacteria and the concentrations of copper ($r = -0.79$, $p<0.05$) and lead ($r = 0.72$, $p<0.05$). The study data also showed significant negative correlations between the density of ammonifying bacteria and the concentrations of copper ($r = -0.84$, $p<0.01$) and lead ($r = - 0.76$, $p<0.05$).

Conclusions

The results of the elaborated study revealed a seasonal variation of the bacterial density with maximum values in the summer and minimum values in the winter, fact that illustrates the development and the intensity of bacterial activities in deep correlation with the temperature conditions.

The physical and chemical oscillations have determined a series of specific particularities of the bacterial populations. The key factors which have controlled the density of soil bacteria were the content of organic substances and the concentration of the pollutants.

In the order of their abundance, the aerobic heterotrophic bacteria (10^4 - 10^6 CFU/g dry matter soil) were followed by the ammonifying bacteria (10^3 - 10^4 cells/g dry matter soil), ammonium oxidizing bacteria (10^2 - 10^4 cells/g dry matter soil), denitrifying bacteria (10^1 - 10^3 cells/g dry matter soil) and nitrite oxidizing bacteria (10^1 - 10^3 cells/g dry matter soil). The smallest numbers were observed for the iron-reducing bacteria (10^1 – 10^2 cells/g dry matter soil).

The harmful effect of the pollutants was obvious, in all the eco-physiological groups the recorded values in polluted areas being lower than in the control sample area, which is unpolluted. The presence of pollutants, even at low concentrations, had inhibitory effect on soil microorganisms. For this reason, the soil pollution will lead most probably to a reduction in the decomposition and nutrient cycling rates.

The obtained results confirm the fact that bacteria are sensitive to the pollution with hexachlorocyclohexane pesticide and heavy metals. The accomplished analyses on the polluted soils from Cluj County have shown that these compounds have more powerful inhibiting effects on aerobic heterotrophic bacteria and ammonifying bacteria than on iron-reducing bacteria and sulphate reducing bacteria.

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ASSESSMENT OF ENZYMATIC ACTIVITIES IN CONTAMINATED SOILS FROM CLUJ COUNTY (NW ROMANIA)

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SUMMARY. Phosphatase, catalase, urease, actual and potential dehydrogenase activities were determined in the 0–20 cm layers of the most representative polluted soils from Cluj County: urban roadside soil, industrial polluted soils including metallurgy and refractory products manufacturing sector, soils affected by zootechnical residues, soil contaminated with hexachlorocyclohexane (HCH) pesticide residue and soil polluted by uncontrolled dumping of municipal solid waste. In order to have insights about complex microbial processes in habitats, these enzymological researches have been completed with physical and chemical analyses which make possible the appreciation of pollutants influence on the activity of soil microbial communities. The phosphatase, catalase, actual and potential dehydrogenases activities have been studied in eight soil samples; including one unpolluted site (Cheile Turzii protected area). The studied activities were detected in all the 8 zones, with differences noticed only regarding the intensity of the processes. Based on the absolute values of the enzymatic activities, the enzymatic indicators of soil quality (EISQ) were calculated. The EISQ values ranged between 0.032 and 0.3, indicating a low or a moderate intensity of the enzymatic activities. Soil enzyme activities were strongly intercorrelated. Soil enzyme activities were positively correlated with soil organic content and negatively correlated with soil heavy metals concentrations.

Keywords: enzymatic activities, enzymatic indicators of soil quality, polluted soil

Introduction

As a result of the activity of microorganisms secreting enzymes, a series of processes is initiated, which provide nutrients for plants. The activity of enzymes is dependent on the physico-chemical properties of the soil (temperature, texture, pH, organic matter content, mineral composition). In spite of numerous factors modifying the action of soil enzymes (for example soil temperature, humidity, pH) their activity - in the opinion of many authors – is a reliable indicator for the fertility and quality of soils and they are sensitive bio-indicators of early changes in the soil environment

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(Lee *et al.*, 2002; Hu and Cao, 2007; Garcia-Ruiz *et al.*, 2008). Soil enzymes are considered to be sensitive indicators of contamination because of their role in organic matter cycling and regulation of nutrient pools (Visser and Parkinson, 1992).

Like other microbial processes any change in the soil quality can disturb soil enzymatic activities (Trasar-Cepeda *et al.*, 2000; Przybulewska and Nowak, 2004; Drăgan-Bularda and Samuel, 2006). Soil enzymes used as a measure of microbial diversity, give an indication of change, but not an absolute measurement of any specific group of organisms. Some enzymes activities serve as a measure of the microbial intra-cellular activity (e.g. dehydrogenase, catalase), which typically reflect general microbial activity in soil (García *et al.*, 1997; Carmiña *et al.*, 1998), while others reflect extra-cellular activity (e.g. phosphatase, urease) in the soil and therefore can be used as a simple metal toxicity test (Rogers and Li, 1985). Different enzymes are reported to respond differently to contamination (Kandeler, 1996). In general, the degree of enzyme inhibition varies depending on the concentration and form of the pollutant, the investigated soil and the assayed enzymes (Nannipieri, 1994). Intra-cellular rather than extra-cellular enzymes may be good predictor of soil contamination; these enzymes may be an indirect measure of the amount of biomass or at least its state of activity (Khan, 2000).

The aims of the present study are: to investigate the effect of pollutants (heavy metals and pesticides) on two oxidoreductases (catalase and dehydrogenase) and two hydrolases (involved in the cycling of N - urease and P - phosphatase), to examine the potential use of enzyme activities to differentiate soils with different degrees of pollution and to draw inferences about the impact of pollutants on the diversity of soil microbial communities. This approach may permit an evaluation of the status of polluted ecosystems.

Materials and methods

Soils analysed. Eight points for sample assay were selected in order to cover the main sources of pollution in Cluj county: traffic (Unirii Square), industrial activities from the manufacturing sector of non-metallic materials (Casirom Turda) and metallurgy (Combinatul de Utilaj Greu Cluj – CUG Cluj-Napoca), zootechnical activities (Popesti and Bontida), industrial waste disposal (hexachlorocyclohexane deposit from Turda) or household waste (Pata Rat landfill from Cluj-Napoca). The control samples were taken from the Cheile Turzii Nature Reserve. The depth of sampling was 20 cm. The samples were collected in each season during 2008.

Physical and chemical *analyses.* The pH values were determined with handheld pH meter, conductivity with handheld conductivity meter, soils humidity was determined by the gravimetric method at 105^0C, soil organic matter by sulfochromic oxidation method (ISO 14235:2000 Soil quality – Determination of organic carbon by sulfochromic oxidation), organochlorine pesticides concentrations by gas chromatography and heavy metals concentrations were analyzed by atomic absorption spectroscopy.

Enzymological analyses. The following five enzymes were studied: phosphatase – activity expressed in mg phenol/g dry matter soil (Drăgan-Bularda, 2000); catalase – activity expressed in mg splitted H_2O_2/g dry matter soil (Drăgan-Bularda, 2000); urease – activity expressed in mg NH_4/g dry matter soil (Drăgan-Bularda, 2000); actual and potential dehydrogenase – activities expressed in mg formazan/g dry matter soil (Drăgan-Bularda, 2000). The analytical data serves as the base for calculating the enzymatic indicator of the soil quality (EISQ) (Muntean *et al.*, 1996; 2006).

Statistical analysis. All analyses were carried out in triplicate. The values reported for enzymatic activities are averages of the three determinations expressed on an oven-dried soil basis (105^0C)**.** The results obtained were interpreted using *Student's t-test*. Pearson`s correlation coefficient *r* was used to describe the degree of linear association between enzyme activity and soil physico-chemical properties.

Results and discussion

Physical and chemical analyses were carried out during the summer of 2008 in order to establish the effects of the pollutants on the enzymatic activities of the soil microorganisms.

In all samples the presence of all analysed heavy metals was detected. The normal limits for Zn, Cr, Cu, Pb, Cd, Ni and Co are: 100 ppm (Zn), 30 ppm (Cr), 20 ppm (Cu, Pb and Ni), 15 ppm (Co) and 1 ppm (Cd), according to the Order number 756/1997 of the Ministry of Waters, Forests and Environmental Protection *Reglementation regarding evaluation of environment contamination.*

The concentrations of Zn, Cu, Pb, Ni and Co are higher than the normal limits in samples collected from Combinatul de Utilaj Greu Cluj-Napoca (Zn, Cu, Pb, Ni), Pata Rat (Zn, Ni), Unirii Square (Cu, Pb), Turda hexachlorocyclohexane deposit (Cu, Pb, Co) and Popesti (Co).

The highest concentrations in heavy metals were detected in samples collected from Turda hexachlorocyclohexane deposit: Pb (64.6 ppm), Co (46.8 ppm), Cu (59.1 ppm) and Pata Rat deposit: Zn (188.7 ppm) and Ni (23.8 ppm) .

In all analysed soil samples the concentrations of Cr and Cd are much more under the normal limits.The samples collected from Cheile Turzii have presented the lowest content in heavy metals.

We have detected a high concentration of organochloride pesticide (hexachlorocyclohexane) in Turda hexachlorocyclohexane deposit, much more over the normal limit which represents an alarm signal for human and environmental health.

Enzymological analyses were carried out in each season during 2008 on soil samples taken from the same eight points that has been analyzed from physical and chemical point of view. The samples were taken from a depth of 20 cm.

The results of the enzymological analyses are presented in figures 1-4. In all the samples analyzed were observed the presence of each of the five studied enzymes and the intensity of these activities varied within larger limits. The values of all enzymatic activities were higher in the summer and smaller in the winter.

The phosphatase activity ranged from 0.21 to 7.95 mg phenol/g dry matter soil. The intensity of phosphatase activity was significantly higher in uncontaminated soils, compared to the polluted soils through disposal of solid municipal waste, industrial activities or road traffic. The highest value of phosphatase activity was registered in Bontida area (7.95 mg phenol/g dry matter soil) due to organic phosphorus input from zootechnical activities. Phosphatase activity was lowest in winter, in hexachlorocyclohexane deposit area (0.21 mg phenol/g dry matter soil), as a result of high heavy metals and hexachlorocyclohexane concentrations.

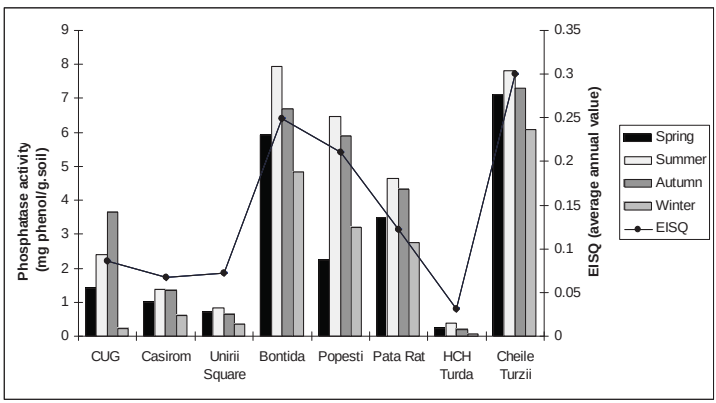

Fig. 1. Soil phosphatase activity (mg phenol/1 g soil/24h)

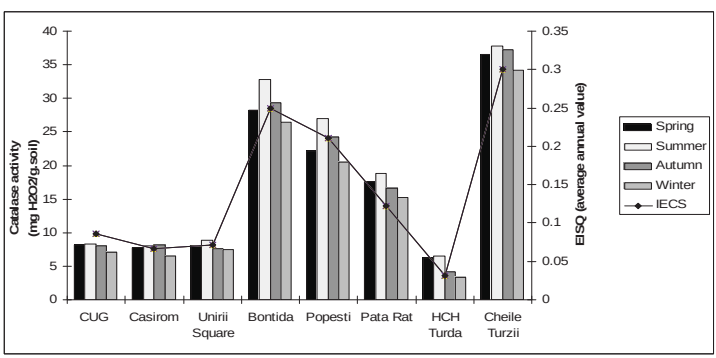

Fig. 2. Soil catalase activity (mg H_2O_2/1 g soil/24h)

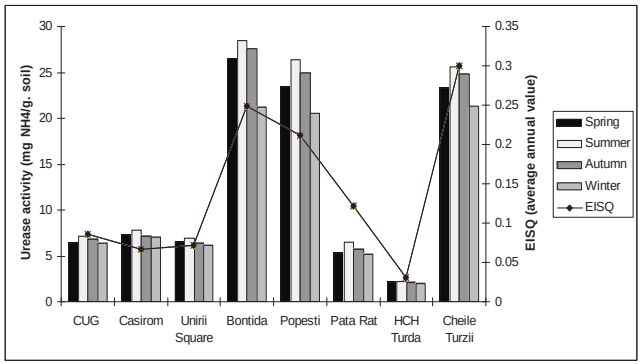

Fig. 3. Soil urease activity (mg NH_4/1 g soil/24h)

In summer, the most intense catalase activity was mesured in Cheile Turzii protected area (37.82 mg splitted H_2O_2 /g dry matter soil), Bontida (32.74 mg splitted H_2O_2 /g dry matter soil) and Popesti (26.95 mg splitted H_2O_2 /g dry matter soil). The minimum value of the catalase activity was recorded in hexachlorocyclohexane deposit area (3.31 mg splitted H_2O_2/g dry matter soil), in the winter of 2008.

The maximum values of urease activity were achieved summer in the soils from Bontida (28.47 mg NH_4/ g dry matter soil) and Popesti (26.36 mg NH_4 /g dry matter soil), due to the land application of animal manures containing nitrogen, phosphorus and potassium (NH_4, P_2O_5 and K_2O). Most of the nitrogen in animal manure is in ammonium (NH_4^+) and organic forms. The minimum value of the urease activity was recorded in the soil from hexachlorocyclohexane deposit area (2.0 mg NH_4/g dry matter soil), in winter.

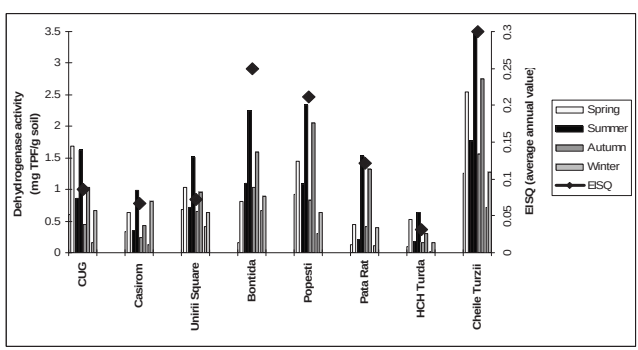

Fig. 4. Soil dehydrogenase activity (mg TPF/1 g soil/24h)

The actual and potential dehydrogenase activity has registered low values in all the samples analyzed. The reduction of 2,3,5 -triphenyltetrazolium chloride (TTC) in uncontaminated soils was higher than in industrial and road traffic polluted soils. The most intense dehydrogenase activity was measured in Cheile Turzii protected area (1.77 mg triphenylformazan/g dry matter soil) and the lowest value of the dehydrogenase activity was recorded in the soil from hexachlorocyclohexane deposit area (0.01 mg TPF/ g dry matter soil).

The substrate additions (glucose) increases the reduction of 2,3,5-triphenyl-tetrazolium chloride. In the presence of glucose the reduction of TTC was the highest in the soil from Cheile Turzii protected area (3.45 mg TPF/g dry matter soil) and the lowest in the soil from hexachlorocyclohexane deposit area (0.16 mg TPF/g dry matter soil).

Because dehydrogenase is not active independently of the parent microbial cell as extracellular enzymes in soil, the measurement of the dehydrogenase is a good overall indicator of microbial activity. Some researchers (Casida, 1964; Oliveira and Pampulha, 2006) suggested that dehydrogenase assay is a useful indicator for evaluating the effect of toxic metals on soil microbial activity. Other investigations have suggested that dehydrogenase assay is a more sensitive indicator of heavy metal effects on soil microbiological properties than other soil parameters (Lenhard, 1963; Oliveira and Pampulha, 2006; Dungan, 2006). The results of the study suggest a good relationship between dehydrogenase inhibition and heavy metal contamination. In winter, the dehydrogenase activity in Turda hexachlorocyclohexane deposit was only 9.6% of that of the reference site.

One might notice that the minimum values of each activity were achieved in the soil from the Turda deposit area. The maximum values for the dehydrogenase and catalase activities were registered in the soil from Cheile Turzii protected area and the phosphatase and urease activities register the maximum value in the soil from Bontida zone.

As compared with the data in the literature (Drăgan-Bularda *et al.*, 1995; Lee *et al.*, 2002), we may consider that the phosphatase, urease and especially catalase activities are intense, while the dehydrogenase activities have lower values.

The enzymatic indicator of the soil quality (EISQ) was calculated based on the absolute values of each enzymatic studied activity (Muntean *et al.*, 1996). The enzymatic indicator of the analyzed habitats quality offers an overall image on the intensity of the enzymatic activity and, implicitly, of the general biological activity in the analyzed soils. The enzymatic indicator may have values ranging between 0 (when no real activity of any of the studied enzymes is detected) and 1 (when all the activities have real individual values equal to the maximum theoretic values).

The enzymatic potential of soils, defined by the values of the quality enzymatic indicator is represented in Fig. 5.

Based on the results and in comparison with the data in the speciality literature (Pasca *et al.*, 1993; Drăgan-Bularda *et al.*, 1995), can be considered that the analyzed soils from Cheile Turzii, Bontida and Popesti have an appreciable

biological potential when compared to the others; the soils from polluted areas Pata Rat, Combinatul de Utilaj Greu Cluj-Napoca, Unirii Square and Casirom Turda have lower values of the enzymatic indicator, which set the basis for this appreciation.

Only the Cheile Turzii soil exceeds the 0.3 value of the EISQ. The value of soil from the polluted areas Combinatul de Utilaj Greu Cluj-Napoca, Unirii Square and Casirom Turda is lower than 0.1 of the EISQ.

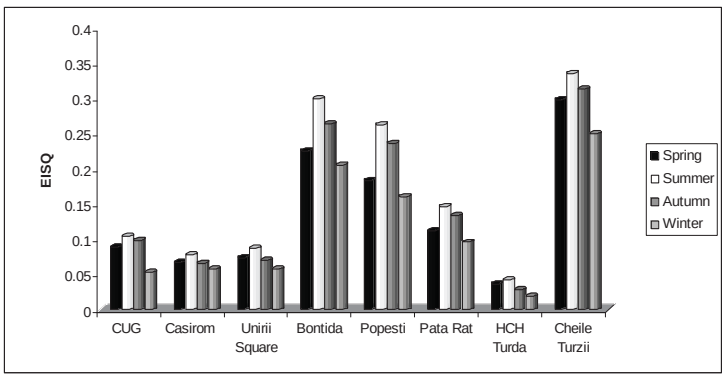

Fig. 5. The enzymatic potential of soils

Table 1.

Hierarchy of the polluted soils according to the EISQ values

Position	Sample location	EISQ
1	Cheile Turzii	0.30
2	Bontida	0.249
3	Popesti	0.211
4	Pata Rat	0.122
5	Combinatul de Utilaj Greu Cluj-Napoca	0.086
6	Unirii Square	0.072
7	Casirom	0.067
8	Turda hexachlorocyclohexane deposit	0.032

In the hierarchy of the polluted soils, based on the values of the enzymatic indicators (Table 1), the soil from Cheile Turzii protected area was situated on the first 8 positions, showing that there exists an active balanced community of microorganisms and on the last one was situated the soil from hexachlorocyclohexane deposit area, uncovered with plants, due to high hexachlorocyclohexane and heavy metal concentration.

The inhibiting effects of heavy metals upon the dimension and the activities of bacterial populations are also sustained by correlation analyses between the physical and chemical parameters and the microbiological properties.

Positive correlations were detected between all enzyme activities analyzed, including the enzymatic indicators of soil quality, and organic matter content. Statistically significant correlations were found between the organic matter content and phosphatase activity ($r = 0.818$, $p<0.01$), catalase activity ($r= 0.875$, $p<0.01$), actual dehydrogenase activity ($r = 0.678$, $p<0.01$) and potential dehydrogenase activity ($r = 0.721$, $p<0.01$). A significant positive correlation was also found between the organic matter content and EISQ ($r = 0.919$, $p<0.01$).

The correlations analyses between the physical and chemical properties and the soil enzyme activities confirmed the inhibiting effect of heavy metals on the dimension of the bacterial activities. Negative correlations were observed between the soil enzyme activities and the concentration of heavy metals. Statistically significant negative correlations were detected between EISQ and the level of all analyzed heavy metals: Cu ($r = -0.699$, $p<0.01$), Pb ($r = -0.658$, $p<0.01$), Cr ($r = -0.582$, $p<0.01$), Zn ($r = -0.453$, $p<0.01$), Ni ($r = -0.49$, $p<0.01$), Cd ($r = -0.375$, $p<0.01$) and Co ($r = -0.225$, $p<0.05$).

Conclusions

The phosphatase, catalase, urease and dehydrogenase activities were registered in all the samples of soil, at higher values in the unpolluted areas, than in the industrial polluted ones. Soil enzymatic activities were significantly lower in the soil from the hexachlorocyclohexane deposit, compared to other soil sampling points, due to high hexachlorocyclohexane pesticide and heavy metal concentration.

The enzymatic potential of soils, defined by the values of the enzymatic indicators of soil quality decreases in the following order: control soil from Cheile Turzii protected area > soil from zootechnical area Bontida > soil from zootechnical area Popesti > soil from the Pata Rat urban deposit > soil from industrial CUG zone > soil from Unirii Square > soil from industrial Casirom zone > soil from hexachlorocyclohexane deposit.

The correlation analyses between the physical and chemical properties and the enzymes activity have proved the inhibiting effect of heavy metals and hexachlorocyclohexane pesticide on the dimension and activity of the bacterial population. There was a negative correlation between the intensity of these activities and the concentration of pollutants. The results presented in this study showed a strong correlation ($r = +0.919$) between enzymatic activities and organic matter content.

The Cheile Turzii protected area soil proved to be a good soil from enzymological point of view. Enzymatic indicators of soil quality reached the highest values: EISQ = 0.30. This value indicates the presence of high enzymatic potential in that protected area, as compared to the other analyzed zones.

Our measurements showed that high concentrations of pollutants have negative impact on soil enzyme activities. The most sensitive enzyme activity was the dehydrogenase. The toxicity exerted by heavy metals and hexachlorocyclohexane pesticide decreased enzyme activities of soil, perhaps as a result of the suppression of sensitive parts of the microbial community.

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===REVIEW===

THE ANAMMOX PROCESS AND ITS APPLICATIONS IN WASTEWATER TREATMENT

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SUMMARY. Natural nitrogen cycling is at present seriously disturbed by human activities. The main sources of nitrogen pollution are the industrial streams and municipal wastewater. The biotechnological treatment of such streams represents a viable alternative to the classic technologies of reducing the pollutants level released to the water, soil or atmosphere. In the present paper, the current knowledge on anaerobic ammonium oxidation (anammox), a recently described biological process, is discussed in regard to its biotechnological potential in nitrogen removal from wastewater. The process is compared to conventional biological nitrogen removal and the main modes of operation are also given. The paper is concluded with an overview of the state of the art of application on full scale.

Keywords: Anammox bacteria, dinitrogen gas, granular sludge reactor, nitrification-denitrification, autotrophic nitrogen removal, membrane-based system

Introduction

Human acceleration of the nitrogen cycle is one of the most immediate aspects of global change (Vitousek *et al.*, 1997). Human activity now contributes between 30% and 45% of all the nitrogen fixed on Earth, by all processes, on land and in the oceans (Howarth, 2004). Nitrogen is an essential element for the growth of all organisms, and is the element most limiting to primary productivity in many ecosystems.

The vast majority of nitrogen on Earth is present as molecular N_2 in the atmosphere and dissolved in the world's oceans. Nitrogen pollution leads directly to acidification of soils and water (through nitrification), eutrophication and loss of biodiversity in terrestrial and aquatic ecosystems. Human wastes can be considered as a source of reactive nitrogen to the environment and wastewater flows are expected to grow in the future as an increasingly large proportion of the world's population lives in cities (Howarth, 2004). Thus, wastewater treatment can reduce nitrogen pollution by releasing less nitrogen to the environment.

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Nitrogen removal in wastewater treatment can be accomplished by microbial processes such as nitrification followed by denitrification, which are conventional treatment methods. The efficiency of nitrification-denitrification treatment depends on spatial and/or temporal separation, due to differentiation in process conditions and microorganisms involved. Nitrification is usually carried out by aerobic, autotrophic bacteria, which oxidize ammonium to nitrate via nitrite (Van Loosdrecht and Jetten, 1998) thus requiring high oxygen levels. Subsequently, by denitrification NO_2^- and NO_3^- are reduced to molecular nitrogen (N_2) by anaerobic denitrifying bacteria (Knowles, 1982).

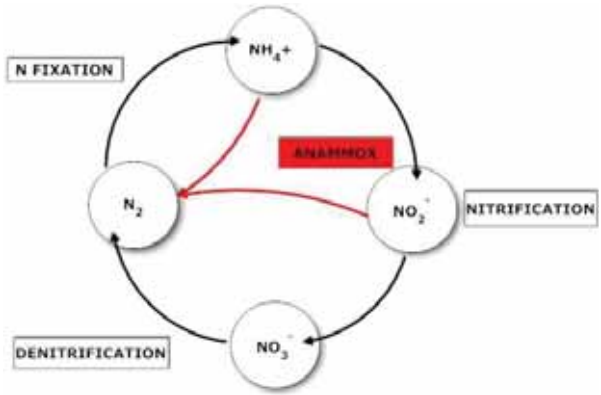

Fig. 1. Global nitrogen cycle, including the anammox process.

The Anammox process

In the past two decades, changes have occurred in the "classical" nitrogen cycle, by the discovery of novel processes involved in nitrogen conversion: denitrification by autotrophic nitrifiers, a process that involves regular ammonium oxidizing bacteria which reduce nitrite under oxygen limitation (probably to N_2O) (Siegrist *et al.*, 1998), and anaerobic ammonium oxidation -Anammox, (Mulder *et al.*, 1995). The first process is, from an environmental point of view, not desirable, because of the N_2O release to the atmosphere, although it needs no COD source and has low oxygen requirements (Van Loosdrecht *et al.*, 2004). An overview of the nitrogen cycle including the anammox process is shown in Fig. 1.

The Anammox process involves the oxidation of ammonia with nitrite as the electron acceptor to yield dinitrogen gas. The theoretical basis of this process was postulated by Broda (Broda, 1977), whose thermodynamic calculations predicted the existence of the chemolithoautotrophic microorganisms capable of catalyzing such exergonic (Equation 1, -357 kJ/mol) reaction (Jetten *et al.*, 2001).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (1)$$

NH_4^+, NO_2^- (and HCO_3^-) are the main substrates in the anammox process and the stoichiometry of the reaction, determined experimentally (Strous *et al.*, 1998), can be expressed by the following reaction:

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.066CH_{1.8}O_{0.5}N_{0.2} + 0.26NO_3^- + 2.03H_2O \quad (2)$$

Intermediates in the anammox process are hydrazine (N_2H_4) in combination with hydroxylamine (NH_2OH) or (and) nitric oxide (NO) (Strous *et al.*, 2006). Hydrazine is a reactive, explosive and highly toxic compound (Schalk *et al.*, 2000), rarely observed as an intermediate in nature or in biological nitrogen conversions (Jetten *et al.*, 1999).

Several years after the identification of the anammox process in wastewater, the role of anammox bacteria in the nitrogen cycle has been investigated for different environments: the anoxic basin of the Black Sea (Kuypers *et al.*, 2003), continental shelf sediments (Dalsgaard and Thamdrup, 2002), the anoxic water column of Goldo Dolce, Costa Rica (Dalsgaard *et al.*, 2003), sediment samples from geographically and biogeochemically distinct environments gave evidence that the anammox microorganisms are widely distributed (Penton *et al.*, 2006). In a global context, the anammox reaction accounts for a significant part of oceanic N_2 production (Devol, 2003), although the relative contributions of the anammox process and denitrification are still unknown (Ward *et al.*, 2009).

Biological nature of the anammox process

The microbiological nature of the process was confirmed by Van de Graaf (Van de Graaf *et al.*, 1996), but the purification of the responsible bacteria proved to be difficult and could only be accomplished by density gradient centrifugation (Strous *et al.*, 1999a). The phylogenetic identity of anammox bacteria was determined by molecular techniques based on 16S ribosomal RNA gene, and they form a monophyletic group within the phylum Planctomycetes, (Schmid *et al.*, 2000, Jetten *et al.*, 2009). The sequencing of the genome of "*Kuenenia stuttgartiensis*" (Strous *et al., in press*) directly from an unpurified mixed culture confirmed this identity.

Physiology and phylogeny

As a specific characteristic of Planctomycetales, anammox bacteria lack peptidoglican in their cell wall and show a specific internal cell compartmentalization (Lindsay *et al.*, 2001). The main compartment of anammox bacteria is the anammoxosome, thought to be the site of catabolic reactions (Van Niftrik *et al.*, 2004). Possibly serving as an adaptation to the toxic intermediates of the catabolic reactions, the bilayer membrane of the anammoxosome consists of unique ladderane lipids, which increase the density and impermeability of the membrane (Sinninghe Damsté *et al.*, 2005). The anammoxosome is surrounded by the riboplasm (containind ribosom-like particles and the nucleoid), which is surrounded by the paryphoplasm (Fuerst, 2005).

The chemolithoautotrophic anammox bacteria are (in)famous for their low growth rates and slow doubling time of several days (Strous *et al.*, 1998; Tsushima *et al.*, 2007; Van der Star *et al.*, 2008), they show high affinity for substrates NH_4^+ and NO_2^- ($\leq 10^{-4}$ M), are reversibly inhibited by oxygen and reversibly or irreversibly inhibited by nitrite (dependent on the concentration) (Strous *et al.*, 1999b).

Several genera of anammox bacteria are known, from freshwater and marine environments. Since no anammox bacteria could yet be obtained in pure culture all species have a Candidatus status. "*Brocadia anammoxidans*" (Strous *et al.*, 1999a), "*Brocadia fulgida*" (Kartal *et al.*, 2008), "*Kuenenia stuttgartiensis*" (Schmid *et al.*, 2000), "Anammoxoglobus propionicus" (Kartal *et al.*, 2007), "*Jettenia asiatica*" (Quan *et al.*, 2009), "*Scalindua brodae*", "*Scalindua wagneri*" (Schmid *et al.*, 2003) were identified and enriched in wastewater or wastewater-derived systems. "*Scalindua arabica*" (Woebken *et al.*, 2008) and "*Scalindua sorokinii*" were discovered in marine environment, the latter always being the dominant anammox species (Schmid *et al.*, 2007).

Sequence similarity between different anammox species is relatively low (below 91%). The significant phylogenetic distances between the anammox species indicate clear niche differentiation, but so far only for *Scalindua sorokinii* (oceanic oxygen minimum zones) and *Anammoxoglobus propionicus* (addition of propionate) an indicative niche could be determined (Kartal *et al.*, 2007; Schmid *et al.*, 2003).

Applications of the anammox process

System configurations used for enrichment of anammox bacteria

The choice of experimental configuration for enrichment of anammox bacteria must consider the low growth rate (0.03-0.07/day) of these microorganisms (Strous *et al.*, 1998; Tsushima *et al.*, 2007; Van der Star *et al.*, 2008). Thus, systems with efficient biomass retention based on the capacity of anammox bacteria to form biofilms or to grow as flocks or granules can be the best option. Especially in biofilm systems, high biomass concentrations and high volumetric loads can be achieved (Abma *et al.*, 2007).

The anammox process was first discovered in a denitrifying fluidized bed reactor in which the microbial community grows as a biofilm on sand particles (Mulder *et al.*, 1995). Because of inefficient biomass retention, variations of the biofilm structure and stratification only 64% enrichment was achieved (Van de Graaf *et al.*, 1996). Biofilm formation and efficient biomass retention are combined in sequencing batch reactors, which prevents stratification, allows continuous mixing inside the reactor and high rate ammonium removal (Fux *et al.*, 2002). Due to strong selective conditions, 74% enrichment was achieved (Strous *et al.*, 1998). Several other biofilm-based systems for anammox enrichment were used: rotating biological contractors-RBC, (Egli *et al.*, 2003; Pynaert *et al.*, 2002; Schmid *et al.*, 2000; Siegrist *et al.*, 1998), fixed bed reactors (Fux *et al.*, 2004), gas-lift reactors (Sliekers *et al.*, 2003).

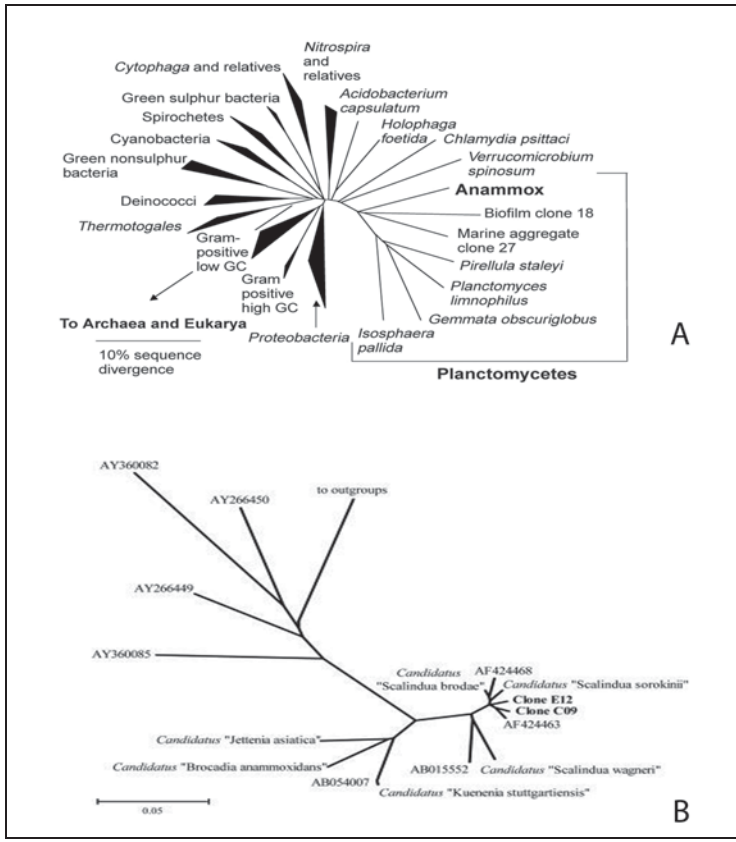

Fig. 2. (A)Phylogenetic position of the lithotroph responsible for anaerobic ammonium oxidation within the domain Bacteria, based on 16S rRNA phylogeny (Strous *et al.*, 2002) and (B) phylogenetic diversity of anammox species (Quan *et al.*, 2009).

An alternative method to achieve high biomass retention is represented by membrane-based systems. In these systems, biomass retention is not based on settling of biomass, the effluent being withdrawn via a membrane impermeable for microbial cells (Van der Star *et al.*, 2008). An indication for fast growing anammox bacteria, with doubling time of 5.5-7.5/day was recently obtained (Van der Star *et al.*, 2008), achieving 97.6% enrichment purity. Because of the absence of a selective pressure

for settling and under the right conditions, planktonic cells (rather than granules) formed in this reactor. The potential for high purity (no significant activity from the side population), fast growing (high biomass production), planktonic cells (easy cell disruption, DNA isolation etc) makes the membrane bioreactor a particularly promising technique for the study of the microbiology of the anammox process. However, for full scale application of this technology, disadvantages are membrane costs, fouling and high energy consumption. Because MBR retains all other particles in suspension, the anammox sludge could be highly diluted, decreasing the specific activity of the biomass (Van Loosdrecht *et al.*, 2001) but the retention capacity could favor the start-up for the low growth rate anammox bacteria (Trigo *et al.*, 2006).

Basic configurations

Two basic configurations are in place for ammonium removal using the anammox process. In both processes, first partial nitritation takes place:

$$2NH_4^+ + 1.5O_2 \rightarrow NH_4^+ + NO_2^- + H_2O + 2H^+ \quad (3)$$

The formed ammonium nitrite is subsequently converted in the anammox process

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (4)$$

Because both types of microorganisms involved in the process are autotrophic, the configuration of this one reactor partial nitrification-anammox system eliminates the need for external carbon addition giving rise to the following overall reaction.

$$NH_4^+ + 0.85O_2 \rightarrow 0.435N_2 + 0.13NO_3^- + 1.3H_20 + 1.4H^+ \quad (5)$$

Both processes can be performed in the same reactor or in separate reactors and have been used extensively both on full scale and lab scale. Because the anammox process is ideal for wastewater containing high ammonium loads and low COD (Jetten *et al.*, 1997; Strous *et al.*, 1997b), the nitritation anammox process is often used for the treatment of effluents of anaerobic (sludge) digestion. The one- and two-reactor configurations are discussed below:

Two reactor Nitritation-anammox process

The two reactor nitritation-anammox process (Fig. 2) is a promising technology allowing the removal of NH_4^+ from wastewater, without the necessity of adding other chemical compounds. The possibility of combining anaerobic ammonium oxidation with a preceding partial nitritation step was tested by van Dongen (Van Dongen *et al.*, 2001) in a chemostat configuration at slightly elevated temperature (ca 30^{o}C).

Within this system without biomass retention, a dilution rate higher than the maximal grow rate of NO_2^- oxidizing bacteria, but lower than NH_4^+-oxidizing bacteria is imposed. In these conditions nitrite is the stable end product of nitrification (Hellinga *et al.*, 1998). Thus, the moment half of the NH_4^+ in converted, the alkalinity of the water is almost depleted, determining a pH drop and preventing the formation of NO_3^- by stopping the nitrification (Jetten *et al.*, 2001).

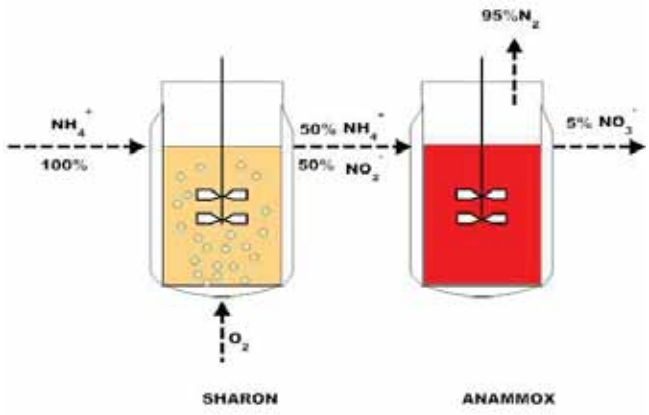

Fig. 3. The two-reactor nitritation-anammox process for highly ammonium concentrated waste streams (after Jetten *et al.*, 2002)

One reactor nitritation-anammox process

The one-reactor nitritation-anammox process (termed, amongst others: CANON, deammonification or OLAND, see Van der Star, 2007 for an overview) is based on occurence of the nitriation and anammox process in one reactor. In continuously operated biofilm systems, the nitritation takes place on the aerobic outside of the biofilm or granule, and the anammox process in the anoxic inner parts (Fig. 3). Approximately 50% of NH_4^+ is oxidized to nitrite by aerobic ammonium-oxidizing bacteria (AOB) in micro-aerophilic conditions, offering the substrate for anammox activity (Third *et al.*, 2005b). Critical to the operation of one-reactor processes is obtaining conditions favorable for AOB and anammox bacteria, but not favorable for NOB. The allowable oxygen level to obtain such conditions depends on the mode of operation and is strongly dependent on granule size. When granules are large enough, higher oxygen levels can be allowed leading to stabler operation (Abma *et al.*, 2009). The nitritation-anammox system can be maintained for 30 days, without irreversible damage, in an ammonium limited environment (Third *et al.*, 2001), but longer periods are likely to result in conditions which are unfavorable for anammox and AOB, but which would favor undesired NOB. Besides continuously operated systems described above, it is also possible to alternate aerated and non-aerated phases, thus separating the nitritation and anammox process also in time (Wett, 2007).

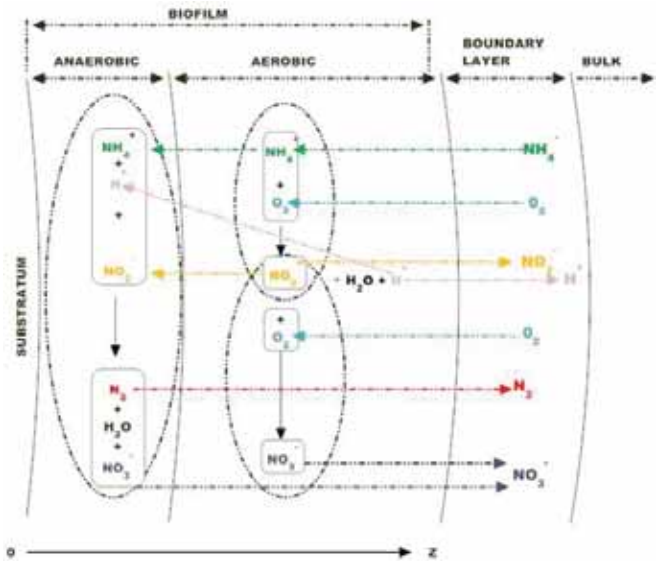

Fig. 4. Schematic model of the cooperation between aerobic and anaerobic microorganisms in a nitritation-anammox biofilm (after Hao *et al.*, 2002).

Denitrification-anammox process

The denitrification-anammox process was the configuration in which the anammox process was initially discovered (Mulder *et al.*, 1995) and can be used for anaerobic oxidation of ammonium in anaerobic, denitrifying conditions. The essential characteristic of the process, compared to already existent anammox applications, is the combination of partial denitrification (nitrate reduction to nitrite) by using HS^- as electron donor with the anammox process.

$$4NO_3^- + HS^- \rightarrow 4NO_2^- + SO_4^{2-}$$
$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (6)$$

The process, proposed by Mulder (2004) is currently investigated at lab-scale conditions (Kalyuzhnyi *et al.*, 2006).

Full scale application

After successful lab-scale testing the two-reactor nitritation-anammox process was scaled up (Van der Star *et al.*, 2007) using a granular sludge type system in which the biomass forms dense well settling granules. The advantages of such configuration

are high specific surface area allowing high volumetric loading rates (10 kg-N/m^3/d in Rotterdam (The Netherlands) in a system treating reject water), no need for carrier material, better mixing and dispersion of granules (Abma *et al.*, 2007). Continuous mixing of anammox reactors is essential for avoiding inhibition by high nitrite levels or sulphide formation (Strous *et al.*, 1999b). Several 2-reactor processes are now operational worldwide (Van der Star *et al.*, 2007).

The one-reactor process was applied on full scale in granular configuration (Abma *et al.*, 2009), moving bed (Ling, 2009) and sequencing batch configuration (Joss *et al.*, 2009, Wett, 2009) and currently more than 10 reactors are operational. The highest conversion was achieved in granular sludge reactors (2 kg-N/m^3/d)

With the plans for several very large full scale reactors (10,000 kg-N/d) in 2010, the use of anammox reactors is expected to grow even more in the next years. It is, therefore, likely that the currently installed cumulative capacity of 7 ton-N /d will double in 2010 (Van der Star *et al.*, in press). The "novel" anammox process is, therefore, well on track to become "conventional technology" in the next years.

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MICROBIAL COMMUNITIES IN PERCOLATING WATER IN CAVES OF PĂDUREA CRAIULUI MOUNTAINS (NW ROMANIA)

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SUMMARY. Due to the absence of light belowground, and thus the consequent lack of photosynthetic plants, microorganisms and dead organic materials transported from the surface provide the basis of any groundwater food web. We aim to relate the abundance of microbial assemblages to the presence and density of groundwater fauna in the epikarst of Pădurea Craiului Mountains (NW Romania), and to determine whether the water dripping from the unsaturated zone of karst can be regarded as microbiologically and chemically clean. For that, we determined the density of aerobic heterotrophic bacteria, coliform microorganisms, and various microbial physiological groups (*i.e.* iron reducing bacteria, ammonifying bacteria, and denitrifying bacteria) in samples of water dripping in three caves. Our analyses revealed that the sampled groundwater is not contaminated by surface microflora, *i.e.*, it is free of coliform microorganisms (*Enterobacteriaceae*). Although the estimation of the abundance of aerobic heterotrophic bacteria showed relatively low numbers of viable cells, other physiological tests revealed relatively larger numbers of iron reducers, ammonifying and denitrifying bacteria. The relative large number of microorganisms involved in the nitrogen cycle can be related to ammonification and denitrification processes occurring in the above-karst soils through which percolating water passes. In general, the abundance of microorganisms was larger in locations where there was a larger population density of groundwater fauna, and conversely, at locations with lower density of groundwater fauna, fewer microorganisms were detected.

Keywords: cave, groundwater fauna, microorganisms, Pădurea Craiului Mountains, unsaturated zone

Introduction

Microorganisms and dead organic materials transported from the surface provide the basis of any groundwater ecosystem. Microorganisms were only recently detected in the upper-most layer of karst, but their role is poorly understood in this habitat. Besides the decomposition of organic matter processes, which occur in all natural habitats, in karst, microorganisms represent food for larger organisms, such as

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groundwater fauna. The upper part of the unsaturated zone of karst is often seen as an ecotone between soil and karst ecosystems (Gibert *et al.*, 1997). It contains thus elements of both surface and subsurface environments. As microbial densities are in general larger and microbial activity more intense in soils than in underground habitats, this ecotone can be a good source of microorganisms and other organic materials, and thus energy, which are transported by percolating water to lower levels of karst.

In contrast to microorganisms, groundwater fauna in the unsaturated zone is better documented, from the largest amphipods to the smallest copepods (Delay, 1968; Galassi, 2001; Moldovan *et al*., 2007; Pipan and Brancelj, 2001; Pipan, 2003; Pipan and Brancelj, 2004; Rouch, 1968; Sket *et al*., 2004). On the contrary, the only indication of the presence of microorganisms in epikarst, the upper part of the unsaturated zone (Klimchouk, 2004), is that of Gerič and coworkers (2004), where bacteria of various types were detected, and a relation was established between the abundance of bacteria and the number of collected meiofauna.

The aim of our research is to relate the abundance of microbial assemblages to the presence and density of groundwater fauna in the epikarst of Pădurea Craiului Mountains (NW Romania). Our aim is also to determine whether the water that percolates through the epikarst in the belowground environments, such as the caves, is chemically and microbiologically clean.

Materials and methods

Site description and sampling. Pădurea Craiului Mountains are located in NW Transylvania (Romania); they are mainly formed of carbonatic rocks and largely karstified with many caves (Rusu, 1988). Physico-chemical measurements and sampling of fauna and microorganisms were carried out in three caves, in one sampling point in each cave. Two of the caves (*i.e.* Peştera de la Vadu Crişului and Peştera Ungurului) are located at an altitude of 305 m in the gorges formed by Crişul Repede River, in the vicinity of the village of Şuncuiuş, at 3.75 km distance from each other (Fig. 1). Peştera cu Apă din Valea Leşului is located at an altitude of 650 m on Leşului Valley, a left tributary of Iadului River (Fig. 1). The water used for microbiological analyses was sampled directly in sterile bottles. Sampling of fauna in percolating water was performed according to the method described by Brancelj (2004). Basically, the water dripping from the cave ceilings was collected during one month via a large funnel into buckets that were foreseen with 100 μm mesh-sized planktonic nets, allowing the excess of water to pass through, but retaining the debris with the animals.

Physico-chemical measurements. Before sampling, physico-chemical parameters (*i.e.* pH, temperature, electrical conductivity) were measured in the field using a Multiparameter Hanna Combo instrument (Hanna Instruments Inc., Woonsocket, RI, USA).

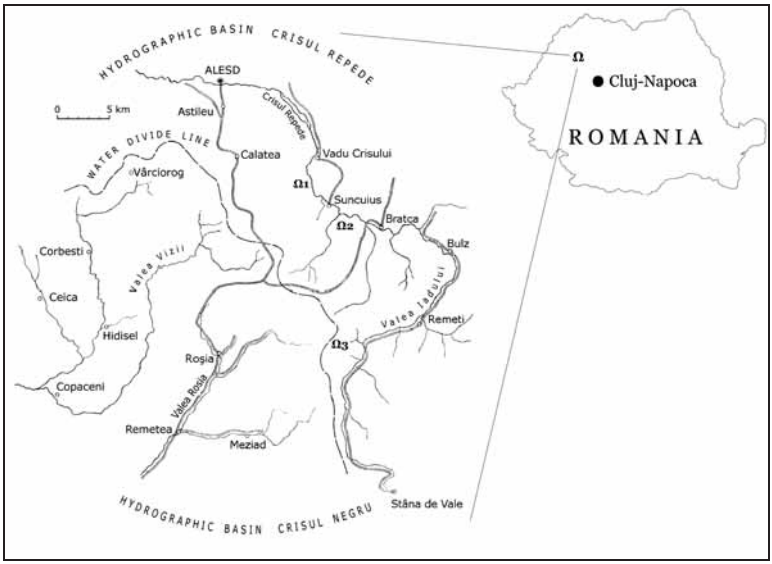


Fig. 1. Map of the study area with the localization of the three caves (Ω_1 - Peştera de la Vadu Crişului; Ω_2 - Peştera Ungurului; Ω_3 - Peştera cu Apă din Valea Leşului) in Pădurea Craiului Mountains

Culture-based quantification of bacteria. Cultivation of microorganisms was initiated within 24 h of sampling.

The medium used for quantification of aerobic heterotrophic bacteria had a pH of 7.5 and contained (g/l) peptone (5), yeast extract (1), $FePO_4$ (0.1) and agar (12). For each cave, 0.2 and 0.4 ml of percolating water were inoculated in triplicates onto Petri dishes. The cultures were incubated in the dark at 30˚C and the colonies grown onto medium were counted after 7 days of incubation. The number of viable cells was calculated as colony forming units (CFU) by averaging the numbers obtained in each of the inoculated plates.

The density of Fe-reducing bacteria, ammonifying bacteria and denitrifying bacteria, was calculated by estimating the most probable number (MPN) according to the method described by Lorch *et al*. (1995). The medium used had a pH of 7.0 and contained per liter of water 1 g K_2HPO_4, 0.8 g KH_2PO_4, 0.2 g KCl, 0.2 g $Fe_2O_3 \times 3H_2O$, 0.5 g yeast extract, 5 g peptone and 20 g glucose. A tenfold serial dilution was made for each sample in this medium up to a dilution of 10^{-3} in triplicates. The cultures were incubated 48 h in the dark at 30˚C. The formation of Fe^{2+} ions, and thus the

presence of iron reducing bacteria, was detected by adding 1 ml of the iron chelator α-α' dypirydil to 9 ml of culture. The cultures containing iron reducers colored in red after the treatment with α-α' dypirydil.

The medium used for determining the density of ammonifying bacteria was the peptone water (pH 7.9), which contained 2 % peptone and 0.5 % NaCl. A tenfold serial dilution was made for each sample in this medium up to a dilution of 10^{-3} in triplicates. The cultures were incubated 48 h in the dark at 30˚C, and MPN was calculated like in the case of iron reducing bacteria. Ammonifying bacteria decompose the amino acids present in peptone and produce ammonia. After incubation, 1 ml of Nessler reagent (0.09 M solution of potassium tetraiodomercurate in 2.5 M potassium hydroxide) was added to 1 ml of culture. The Nessler reagent colors the cultures where ammonia formed in yellow or orange.

The density of denitrifying bacteria was determined by estimating the MPN like in case of iron reducing and ammonifying bacteria. The medium used for the growth of denitrifying bacteria had a pH of 7.2 and contained per liter of water 2 g KNO_3, 5 g $CaCO_3$, 10 g glucose and 10 ml of Winogradsky solution. After 48 h of incubation in the dark at 30˚C, the presence of nitrites formed as a result of nitrates reduction was detected by adding 0.1 ml Griess II reagent (a solution of 0.5 g sulfanilic acid in 150 ml acetic acid 30 %) to 1 ml of culture. Griess II reagent colors the cultures where nitrates formed, and thus denitrifying bacteria were present, in red.

After determining the four physiological groups of microorganisms (*i.e.* aerobic heterotrophic bacteria, Fe-reducing bacteria, ammonifying bacteria and denitrifying bacteria), the bacterial indicator of water quality (BIWQ) was calculated according to formula proposed by Muntean (1995-1996):

$$\mathrm{BIWQ} = \frac{1}{n}\sum \log_{10} N$$

where *n* is the number of analyzed physiological groups of bacteria and *N* is the number of bacteria in each physiological group.

For determining the density of coliform microorganisms, a presumptive and a confirmation test were performed. The medium used for performing the presumptive test was the lauryl sulfate broth (LSB), simple and double concentrated. The simple-concentrated medium (LSBs) contained (per liter of water) 20 g peptone, 2.75 g Na_2HPO_4, 2.75 g NaH_2PO_4, 5 g NaCl, 5 g lactose and 0.1 g sodium lauryl sulfate $[CH_3(CH_2)_{11}OSO_3Na]$. The double-concentrated medium (LSBd) contained the same ingredients as for LSBs but in double the amounts.

Before inoculation, Durham tubes were inserted upside-down in the test tubes containing the media. From each sample, 10 ml of percolating water was inoculated in five test tubes, each containing 10 ml LSBd; 1 ml of water was inoculated in five test tubes, each containing 10 ml LSBs; 0.1 ml of water was inoculated in five tubes, each containing 10 ml LSBs, and five test tubes were not inoculated and used as controls. The cultures were incubated 48 h in the dark at 37˚C. After incubation, the test tubes

where visible gas bubbles formed inside the Durham tubes, and thus the lactose was fermented by supposedly present coliform microorganisms were noted as positive and used latter for estimating the density of coliform microorganisms according to the statistical table of McCrady (McCrady, 1937).

The cultures in all positive tubes were subjected to a second test in order to certify whether the microorganisms responsible for the fermentation of lactose with gas production in the Durham tubes belonged to coliform bacteria. The medium used for the confirmation test (the eosin methylene blue medium) had a pH 7.0 and contained (g per 100 ml of water) peptone (1), lactose (1), K_2HPO_4 (0.2), soluble eosin (0.04), methylene blue (0.0064) and agar (2).

IMViC tests. The IMV*i*C tests are biochemical assessments used for differentiating the coliform bacteria, members of the *Enterobacteriaceae*. This family includes many pathogen bacteria that normally form the gut flora of humans and animals, and have in the environment a fecaloid origin.

Indole test. The medium used to perform this test was the peptone water (pH 7.9) that contained 1.5 % NaCl and 6.0 % peptone. From each sample, 1 ml of water was inoculated in 10 ml of peptone water. After 24 h incubation in the dark at 37˚C, 0.25 ml of Kovács reagent (indole reagent) was added to each test tube in order to test whether indole formed following tryptophan hydrolysis. Kovács reagent was prepared by dissolving 5 g of p-dimethylaminobenzaldehyde in 75 ml amylic alcohol, followed by the addition of 25 ml of concentrated HCl.

Methyl red reaction. This test follows the capacity of certain microorganisms to metabolize glucose with stable acids production. The medium used for this test (Clark and Lubs medium) had a pH 7.5 and contained (per 1 l of water) 5 g peptone, 5 g glucose and 5 g K_2HPO_4. From each sample, 1 ml of water was inoculated in 10 ml of medium. After 24 h incubation in the dark at 37˚C, 0.25 ml of methyl red reagent was added to each test tube. Methyl red reagent was prepared by dissolving 0.1 g methyl red in 300 ml ethylic alcohol followed by the addition of 200 ml of water. The methyl red reagent will color all cultures where acids formed (pH < 4.4) in red.

Voges-Proskauer reaction. Some microorganisms are capable of metabolizing glucose, but do not produce sufficient amount of stable acids needed to lower the pH under 4.4. For this type of microorganisms, the final product following glucose metabolism is the acetylmethylcarbinol (CH_3-CO-CHOH-CH_3). In presence of alkaline compounds and oxygen, the acetylmethylcarbinol oxidizes to diacetyl (CH_3-CO-CO-CH_3). The subsequent reaction of dyacetil with the residual peptone in the medium will lead to a fluorescent-pink color. The medium used to perform this test was, like for the methyl red reaction, the Clark and Lubs medium. From each sample, 1 ml of water was inoculated in 10 ml of medium. After 24 h incubation in the dark at 37˚C, each test tube was amended with 50 µl of 2 % solution of $FeCl_3$ in order to accelerate the reaction. 5 ml of 10 % KOH solution was then added to each test tube. After another incubation time of 1 h in the dark at 37˚C, a pink or red color will indicate the presence of acetylmethylcarbinol.

Citrate test. The aim of this test was to determine whether the microorganisms present in percolation water are capable of using the citrate as unique carbon source, as members of *Enterobacteriaceae* are known to have this capacity. The Simmon's medium (pH 6.8) used to perform this test contained (g to 1 l of water) NaCl (5), $MgSO_4$ (0.2), $NH_4H_2PO_4$ (1), K_2HPO_4 (1), sodium citrate (2.8) and agar (30). After dissolving these ingredients, the medium was colored with 4 ml solution of bromothymol blue (pH indicator). 0.1 ml from each sample was inoculated on Petri dishes containing Simmon's medium, and the cultures were incubated 72 h in the dark at 30˚C. If the citrate was used, alkaline (pH > 7.6) or acid (pH < 6.0) products will form, and the medium (initially green) will turn into blue or yellow, respectively.

Results and discussion

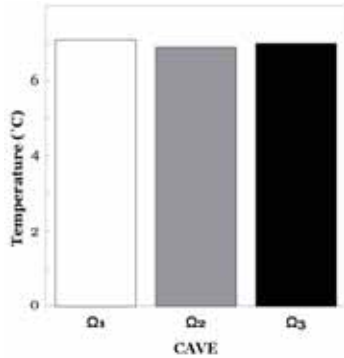
Physico-chemical measurements. The physico-chemical parameters of percolating water had comparable values in all three caves (Fig. 2). The groundwater temperature ranged between 6.9 and 7.1˚C, the pH had values between 8.9 and 8.94, and electrical conductivity between 163 to 169 µS/cm.

Culture-based quantification of bacteria. The density of aerobic heterotrophic bacteria estimated in the percolation water in the three caves had comparable values to those obtained in other similar studies (Gerič *et al*., 2004; Mulec *et al*., 2002). The largest density of aerobic heterotrophic bacteria (Fig. 3a) was registered in Peştera cu Apă din Valea Leşului (294 CFU/ml), while the lowest density of this type of microorganisms was obtained for Peştera de la Vadu Crişului (12 CFU/ml). 110 CFU/ml were obtained for Peştera Ungurului.

The density of iron reducing bacteria (Fig. 3b) was largest in Peştera cu Apă din Valea Leşului and Peştera Ungurului (360 cells/ml). Only 1 cell/ml was obtained for Peştera de la Vadu Crişului.

The number of ammonifying bacteria (Fig. 3c) was highest in Peştera cu Apă din Valea Leşului (1.1×10^5 cells/ml). The density values of this type of microorganisms were 1.5×10^4 cells/ml for Peştera Ungurului and 4.3×10^2 cells/ml for Peştera de la Vadu Crişului. The density of denitrifiers (Fig. 3d) was largest in Peştera cu Apă din Valea Leşului (1.1×10^5 cells/ml). The same number of denitrifying bacteria (2.9×10^4 cells/ml) was obtained for both Peştera Ungurului and Peştera de la Vadu Crişului. The large number of microorganisms involved in the natural cycle of nitrogen can be related to the ammonification and denitrification processes occurring in the soils above the caves, which water washes percolating towards lower layers of karst.

Bacterial indicator of water quality was calculated on basis of the obtained density values of the various bacterial physiological groups. BIWQ (Fig. 4) suggested that the cleanest water is in Peştera de la Vadu Crişului, while the largest microbial charge was registered in Peştera cu Apă din Valea Leşului.



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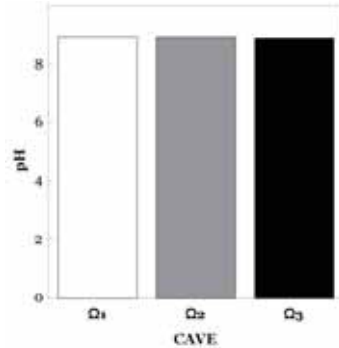

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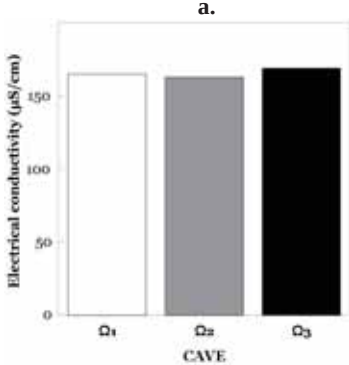

c.

Fig. 2. Physico-chemical parameters of percolation water (a - temperature, b - pH and c - electrical conductivity) measured in Peştera de la Vadu Crişului (Ω_1), Peştera Ungurului (Ω_2) and Peştera cu Apă din Valea Leşului (Ω_3)

Coliform bacteria. For determining the presence and density of coliform microorganisms, a presumptive and a confirmation test were performed. After the presumptive test, which was based on gas formation in the Durham tubes due to lactose fermentation by supposedly present coliform bacteria, only two test tubes were recorded as positive. The cultures in these two test tubes were inoculated onto Petri dishes containing the eosin methylene blue medium. This confirmation test revealed no coliform-like growth, suggesting that the water dripping in the three caves is free of coliform bacteria. The IMV*i*C tests (*i.e.* indole test, methyl red reaction, Voges-Proskauer reaction and citrate test) were all negative, suggesting that members of *Enterobacteriaceae* (coliform bacteria) are absent in the water dripping from the upper part of the unsaturated zone of karst in the three caves.

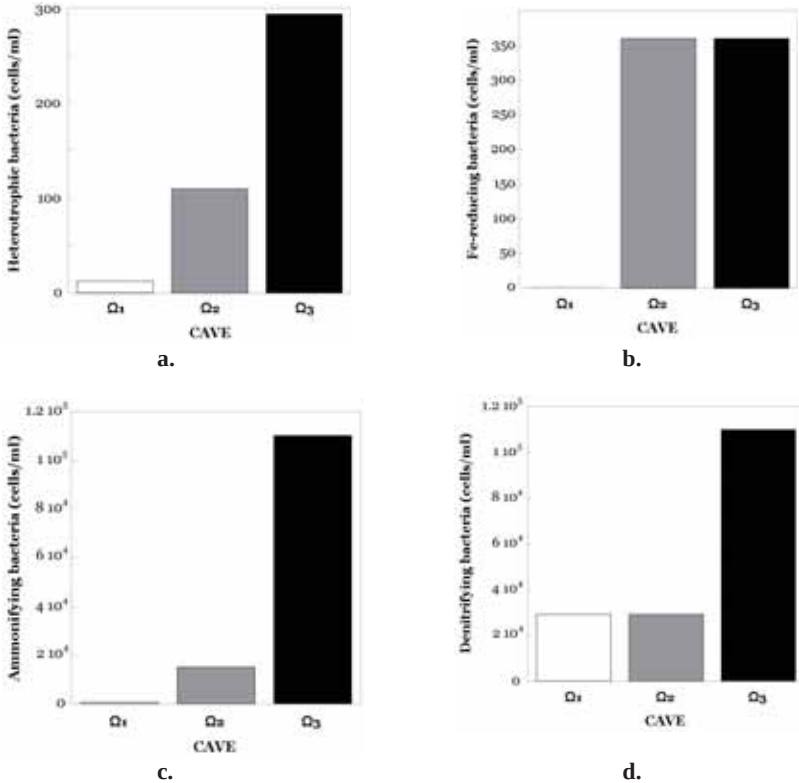

Fig. 3. Density (in cells/ml) of different bacterial physiological groups (a - aerobic heterotrophic bacteria; b - iron reducing bacteria; c - ammonifying bacteria; d - denitrifying bacteria) determined in percolation water in Peştera de la Vadu Crişului (Ω_1), Peştera Ungurului (Ω_2) and Peştera cu Apă din Valea Leşului (Ω_3)

These tests revealed that the water dripping in the three caves is clean; it is free of microorganisms with a fecaloid origin, organisms that are generally responsible for the microbiological pollution of groundwater or other environments. Measurements of the electrical conductivity indicated also that the percolation water is rather clean, as organically polluted groundwater has in general higher electrical conductivity values (Christensen *et al*., 2001).

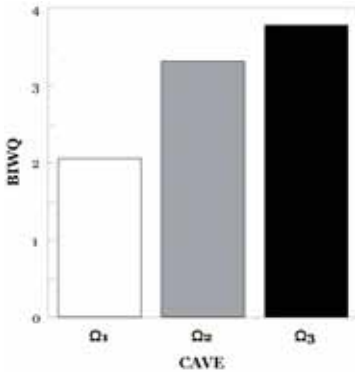

Fig. 4. Bacterial indicator of water quality (BIWQ) calculated for the water dripping in Peştera de la Vadu Crişului (Ω_1), Peştera Ungurului (Ω_2) and Peştera cu Apă din Valea Leşului (Ω_3)

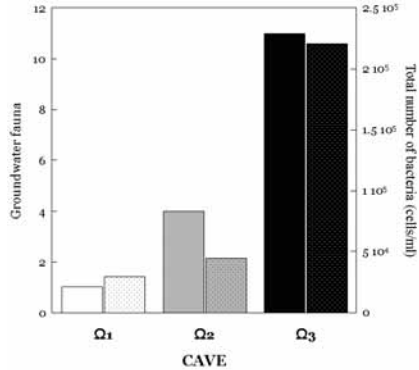

Fig. 5. Relation between the abundance of groundwater fauna and density of bacteria in percolation water in the three studied caves (Ω_1 - Peştera de la Vadu Crişului; Ω_2 - Peştera Ungurului; Ω_3 - Peştera cu Apă din Valea Leşului). Empty columns indicate the groundwater fauna, and dotted columns represent the bacteria

Groundwater fauna. Groundwater fauna was collected at the same locations in each cave where sampling of water for microbiological analyses was performed. The abundance of groundwater fauna (mainly crustaceans of the *Copepoda*) was larger in locations where a larger density of microorganisms was obtained and, conversely, fewer microorganisms were detected in places with lower density of groundwater invertebrates (Fig. 5). In groundwater food webs, microorganisms and dead organic matter are the basis of all food chains, because light and photosynthesizing plants are absent in the subsurface. Due to the scarcity of resources in the subsurface, groundwater animals are in general omnivorous or detritivorous (Gibert *et al.*, 2009) and microorganisms are good part of their diet. Groundwater animals seem thus to agglomerate more in places where more food (microorganisms) is present.

The water in caves, such as that dripping from the unsaturated zone of karst, feed the aquifers below and must be protected against both surface and subsurface human disturbances. Clean drinking water sources, such as the caves, are desired in present times, when pollution of the environment in general, and contamination of waters in particular, are obvious phenomena due to increased human population density, causing an increase in water demand, agricultural practices and landscape alteration, industrial activities, mining, electricity production, increase in urban area and demand for public drinking water, tourism and climate change (Danielopol *et al.*, 2003).

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===JOURNAL REVIEW===

AQUACULTURE, AQUARIUM, CONSERVATION & LEGISLATION – INTERNATIONAL JOURNAL OF THE BIOFLUX SOCIETY

PIOTR EPLER[1]

In 2008 a new, published in Romania, journal appeared on the European market of scientific periodicals. It is *Aquaculture, Aquarium, Conservation and Legislation – International Journal of the Bioflux Society* (AACL Bioflux). Scope of the journal covers aquaculture, fisheries, fish genetics and improvement, aquarium sciences, ichthyology, aquatic ecology, conservation of aquatic environment, and legislation. For the present moment there were published two issues of the Vol. 1 (2008) and one issue of the Vol. 2 (2009). Since 2009 the journal is going to be published quarterly.

All papers were written in English, and each article was reviewed by two independent specialists, at least one from abroad. An editor page was prepared correctly and very carefully. Special attention should be addressed to high quality of all figures and photographs printed in colours. All articles published were of high scientific quality and fitted directly within the scopes of the journal.

In **Vol. 1 (1)** there were three papers devoted to genus *Poecilia*: one concerning genetic basis of phenotypic variability in *P. reticulata*, second dealing with some aspects of behaviour in *P. parae*, and third on relationships among predators, water vegetation and colour patterns in *P. sphenops*. Another work was devoted to genetic basis of stress resistance in some fish species. There were also papers on: ichthyofauna of Bucharest City; systematics of the gudgeons (Gobioninae) in Europe; ecosystem services and water management within the Fizeş River drainage;

quality of some aquatic organisms that are consumed by humans; and Romanian efforts in order to improve water-related legislation (AACL Bioflux 2008*a*).

In **Vol. 1 (2**) there were ten articles included. These were devoted to aquatic parasitology, non-native species management (two papers), some aspects of water ecology, concentration of omega-3 fatty acids in fish meat, monitoring of water quality, contamination by heavy metals, and review of endangered fish species of the world. Special attention should be addressed to continuation of discussion on genetics of the guppy (*Poecilia reticulata*) (AACL Bioflux 2008*b*) that was developed in the Vol. 2(1) (AACL Bioflux 2009).

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Vol. 2 (1) consisted of nine scientific articles. One dealt with growth rate of two important fishes of Polish riverine ecosystems and two others with growth rate and body-mass related factors during the starvation in a non-native species in Romania. Other authors discussed the usage of geothermal energy resources. Two papers concerned various legislative aspects of fisheries and aquaculture. Another paper was devoted to evolution patterns of the brown trout (*Salmo trutta*). Furthermore, an interesting study of cytochrome *b* nucleotide sequences was demonstrated. Also the discussion on guppies' genetics was continued.

After analysis of materials presented in these three issues of AACL Bioflux it must be said that Editorial Board has chosen very interesting, on-time scientific topics, with high impact on conservation of water environment in a broad sense (i.e. inland waters, geothermal waters, wetlands), as well as certain aspects of water-related legislation, and exploitation of resources. A special attention should be also addressed to well-documented reviews of some aspects of fisheries and ichthyology.

Summarising, we think that it is very good that a journal such AACL Bioflux has appeared on international market of scientific periodicals. We strongly encourage submitting scientific manuscripts to this journal, and we hope that AACL Bioflux would reach an appropriate Impact Factor in the nearest future.

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