

Institute of Biology Bucharest

**The Anniversary Conference**  
of the Institute of Biology  
*50 Years of Academic Research*  
*in Biology*

**Book of abstracts**

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**UNIVERSITY OF BUCHAREST**

**The Anniversary Conference of the Institute of Biology**  
***50 Years of Academic Research in Biology***

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**Institute of Biology Bucharest**

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**(The book is dedicated to 50<sup>th</sup> anniversary of Institute of Biology Bucharest)**

**ARS DOCENDI  2010  
UNIVERSITY OF BUCHAREST**



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## INTRODUCTION

The Institute of Biology Bucharest of the Romanian Academy is the host of a wide panel of research topics in biology, in agreement with the scientific directions of the European Union and National Strategy for Research-Development. The main research areas, developed in three departments, concern the conservation and management of biodiversity, the bioremediation of polluted environments, and the microorganisms from extreme and hostile environments. Several of our studies are performed in the frame of the Romanian Academy program “Biodiversity into frame of global climate change and sustainable development”. Besides the scientific achievements, these topics open a dialog between the scientific community and the society, in order to strengthen their relations.

The current research projects of the *Ecology, Taxonomy and Nature Conservation* Department concern (1) the biodiversity and the impact of global climate change on terrestrial ecosystems from Romania, (2) the impact of hydrotechnical modifications in Danube Delta, on various biotops within the frame of sustainable development, (3) the taxonomy and corology of macromicetes, lichens, bryophytes and superior plants from Romania, and (4) the identification and characterization of habitats and endemic endangered/rare plant species of national interest.

The research projects of the *Microbiology* Department focus on the study of microorganisms able to grow and develop in extreme and hostile environments, in the presence of high salt concentrations, high temperatures or low pH values, aiming to the identification and molecular characterization of new strains of applicative potential in nanotechnologies, medicine and bioremediation.

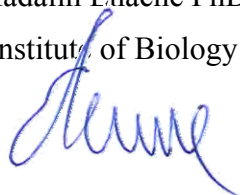
The research teams from the *Plant and Animal Citobiology* Department are involved in projects concerning the *ex situ* conservation of the natural genofond by developing cell and vegetal tissue data banks of conservational and biotechnological values. In addition, some research projects are directed on the study of cellular and molecular interactions at the interface tumor-peritumoral stroma *in situ* and in experimental models.

*The Anniversary Conference of the Institute of Biology Bucharest – 50 Years of Academic Research in Biology* is a major opportunity for giving an overview of our recent results and research directions, covering various biological domains.

We warmly appreciate the support from the Romanian Academy and the National Authority for Scientific Research for this scientific event that will contribute to the development of scientific contacts and dialogue with researchers worldwide.

Mădălin Enache PhD

Director of the Institute of Biology Bucharest



**INVITED LECTURES  
AND  
ORAL PRESENTATIONS**





## YOU ARE STRESSED, I'M STRESSED AND THE PLANTS ARE STRESSED: IS THERE A COMMON STRESS-CONCEPT?

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**Introduction:** There is an increasing interest to analyze big areas of biotopes in order to get information about the health of its vegetation. The goal is to detect early effects of stress. The optical techniques today are so highly developed that remote sensing for these purposes become possible. Reflection and fluorescence signals can be detected in a passive manner that means with the sun as only light source. With the amplitude of reflectance and fluorescence as a function of wavelength, incident light intensity and time, we have enough information to classify the biological state and the biological behavior of a big vegetation area, relatively to a **stress concept**. Technically the data acquisition from satellites is available. A theory of behavior of plants in a given environment is still awaited, even so more and more stress concepts are formulated.

**The JKB-Trilogy:** As a first approach every very complex biological system is reduced to the three symbols J-K-B, the so called JKB-trilogy. J stands for all energetic inputs from the environment into the biological system. K stands for all molecules and rate constants of the biological system and represents this way the architecture of the organism. B stands for the dynamic "Behavior" of the system. It is a measure of the overall flow level in the dissipative structure or open system.

The JKB-trilogy therefore represents:

The environmental	INPUT TERM	<b>J</b>
The hardware	EXTENSIVE TERM	<b>K</b>
The behavior	INTENSIVE TERM	<b>B</b>

**The State Change Concept:** The biological activity is a function of B and K which both depend on J. However J is a direct function of the environment. Already with this very simple JKB-trilogy it is possible to formulate a state-change of a biological system, what represents an adaptation process upon a stress. The idea is that for any given conformation K, it is possible to adjust the relative flow level B between zero and unity by changing the input parameter J which is a function of the environmental parameter I. Therefore the efficiency of the system as output flux per input flux is a function of B with one or more values of B where the efficiency is

optimal. As long as K is constant, the system can only work according to its state function (efficiency versus B).

**Sub-optimality as Driving Force for Adaptation:**

The concept of the state-change theory is that every SUBOPTIMALITY creates a force which alters the statistical distribution of the microstates what appears as a change in the conformation of the system. Every change in K to K' is called a state change. To the new state change function f(K) there belongs a new efficiency function f(B) with a new optimum.

- Moving within one state function corresponds to intensity changes of the biological activity (B-changes), leading to sub-optimality.
- Moving from one state function to another corresponds to conformational changes (K-changes, leading to optimality

Changing the environmental parameter from  $I_1$  to  $I_2$  and back to  $I_1$  (e.g. clouds to full sunshine to clouds again) forces the system into sub-optimality and therefore into a state change cycle. That means the way of adaptation to new conditions is different to the way of re-adaptation to the original conditions.

**The Stress Concept:** Every state change is a consequence of **stress**. The following definitions are used for the stress concept:

**STRESSOR** is called every factor which provokes a **STRESS**

**STRESS** Is called every condition established which forces a system away from the thermodynamic **OPTIMAL** state.

**OPTIMAL** state is called when a biological system is in full **HARMONY** with its Environment.

**HARMONY** of a biological System with its environment is achieved when the system does not tend to change any activity or conformation what so ever, considering: **ECOSYSTEMS – SATABILITY – DISSIPATIVE STRUCTURES – MINIMUM ENTROPY PRODUCTION.**

**STRAIN** is any physical or chemical change produced by a **STRESS**.

The stress concept deals always with the interaction of the environment and the biological system in this environment. The stress concept given here is based on non-equilibrium thermodynamics and dissipative structures. It offers the possibility of analytical description and quantification. Nevertheless this concept is in full agreement with the definitions of stress given by W.L. Larcher of the University of Innsbruck (Naturwissenschaften 74, 158-167 (1987).

### Correlations between stress concept Larcher (left) and Strasser (right)

Level of organization versus the organized hardware and conformation term K

Destabilization versus Stability means no change which corresponds to the thermodynamic optimal state or harmony with the given environment. Destability means thermodynamic suboptimality manifested so that the actual value of B is not the optimal value of B. Destabilization = B change from an optimal to a suboptimal state.

Normalization versus Conformational adaptation. Suboptimal B creates a driving force which changes the former optimal K until B and K are again optimal (or normal). Therefore: Normalization = K change from suboptimal to optimal.

Driving Force versus Suboptimality. Difference of the actual state and the optimal state the system is searching for, relatively to the given environmental conditions.

The essence of the optimalization theory is that it is always the **changed environment** which determines **the future** optimal state of the biological system. If the new environment requires unrealistic state values, then the state change driving force will push the system in direction of the unrealistic attraction point. The adaptive capacity however is then overtaxed and permanent damage or even death may result (destructive element of stress according to Larcher). If the attraction point is within realistic limits, adaptation and improved resistance will result (constructive element of stress according to Larcher).

**In short:** Any stress is a force established by an environmental change, so that the stressed system has to undergo conformational changes to approach optimality relatively to the new environmental conditions. Stress can be considered as a driving force to increase Biodiversity even without genetic changes.

# THE MICROBIOLOGY OF THE DEAD SEA: CULTURE-DEPENDENT AND CULTURE-INDEPENDENT STUDIES IN AN INCREASINGLY EXTREME ENVIRONMENT

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The Dead Sea, located on the border between Israel and Jordan, currently contains around 348 g/l salts. Divalent cations (2 M Mg, 0.5 M Ca) dominate over monovalent cations (1.5 M Na, 0.2 M K), with Cl (6.5 M) and Br (0.1 M) as main anions. Sulfate is low (4 mM), and the pH is about 6. The lake's water balance is negative, and during the past decade the level has dropped over one meter per year (current level: about -424 m). The water is supersaturated with Na, and massive amounts of halite precipitate to the bottom. The lake has an area of about 630 km<sup>2</sup> and a maximum depth of 300 m.

Biological monitoring since 1980 has shown that blooms of the unicellular green alga *Dunaliella* and halophilic Archaea of the family *Halobacteriaceae* develop only following significant dilution of the upper water layers after very rainy winters. Such events occurred in 1980 and even more dramatically in 1992, when up to  $3.5 \times 10^7$  Archaea per ml in the diluted upper 5-10 meters of the water column colored the lake red. Species isolated from the lake include *Haloferax volcanii*, *Haloarcula marismortui*, *Halorubrum sodomense*, and *Halobaculum gomorrense*. After 1996 *Dunaliella* was no longer observed and prokaryote numbers remained low.

To characterize the residual microbial community in the lake we concentrated biomass from a large volume of brine in February 2007 for environmental genomic analyses. The results were compared to the metagenome of microbial bloom material collected in 1992, kept frozen for 16 years. The 16S rRNA archaeal phylotypes recovered from the 2007 sample were diverse, with phylotypes distantly related to the genera *Halorhabdus*, *Haloplanus*, *Natronomonas*, and others. *Halorhabdus* sp. was also recovered in culture. However, the 1992 bloom sample was very homogeneous, with a single cluster related to *Halobacterium salinarum*. It is thus shown that even in one of the most extreme environments on Earth the microbial communities are dynamic, showing strong shifts in species composition as conditions for life become increasingly adverse.

## **HIGH PRESSURE IN BIOCHEMISTRY AND BIOLOGY**

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The influence of pressure on biomolecules results from the changes in volume which are associated to their different properties like conformational changes, monomer association-dissociation processes, denaturation, and/or catalytic activities. These effects are associated to changes in hydration of these molecules and can be either beneficial or deleterious. They are thermodynamically linked to temperature variations. Their reversibility depends on the degree of pressure and on the duration of exposure.

The impact of pressure on living cells and organisms is the complex result of these effects on their molecular constituents. Some organisms are living permanently under high pressure and are adapted to it. This is the case of the microorganisms and pluricellular organisms which thrive around the volcanic deep-sea vents. Some compensatory effects of pressure and temperature might have contributed to their adaptation. There are convincing indications that life might have emerged on earth in those high-pressure / high-temperature environments. Even the physiology of organisms living at atmospheric pressure has to deal with pressure effects. This is documented for instance in the cases of animal and human joints, tall trees growth and even in some genes expression. In the past years numerous apparatus were devised in order to perform most biochemical and biophysical measurements under pressure.

## DON'T CRACK UNDER PRESSURE ! IS THE MAJORITY OF PROKARYOTES PIEZOPHILIC?

D. PRIEUR

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Among the physical parameters that influence life in the deep-sea, hydrostatic pressure is unique since it is constant for a particular location. It is a function of the weight of liquid (seawater) above a given area, and is expressed in  $\text{kg cm}^{-2}$ , bars, atmospheres or megaPascals (MPa). Hydrostatic pressure increases with depth (100 bars or 10 MPa per kilometer). At the bottom of the Marianna Trench (11 km depth) hydrostatic pressure reaches 110 MPa : imagin a small car, just put on one of your hand nails ! Several Prokaryotes adapted to these cold and pressurized conditions have been isolated and described.

Micro-organisms living at deep-sea hydrothermal vents are exposed to a constant hydrostatic pressure, but a rather sharp temperature gradient. At depth, when temperature exceeds  $100^{\circ}\text{C}$ , water remains liquid because of hydrostatic pressure. For this reason, at least, microbiologist have investigated deep-sea hydrothermal vent ecosystems and described a variety of hyperthermophilic micro-organisms. However, although characteristic of the deep-sea, hydrostatic pressure has rarely been considered.

Enrichment cultures carried out under elevated hydrostatic pressure and high temperatures allowed to isolate piezophilic (including obligate) hyperthermophiles such as *Thermococcus barophilus* or *Pyrococcus yayanosii*. Surprisingly for almost all hyperthermophiles with a piezophilic behaviour studied so far, optimum pressure for growth was always above pressure existing at sampling depth, suggesting these organisms could inhabit the sub-seafloor. Recent data from microbiological studies of deep marine sediments are in favour of this hypothesis. Taking into accounts estimations that showed that most of the Prokaryotes on Earth were located beneath the sea floor, one can ask the question: Is the majority of Prokaryotes piezophilic?

**BIOGEOGRAPHY AND GENETIC DIVERSITY OF  
HYPERTHERMOPHILIC ARCHAEA, *VULCANISAETA*  
*DISTRIBUTA* AND RELATED TAXA**

T. ITOH

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The genus *Vulcanisaeta* was created in 2002 to encompass two hyperthermophilic archaeal species, *V. distributa* and *V. souniana*, isolated from several hot spring areas in eastern Japan. The *V. distributa* strains, which were all isolated from spouting hot spring or heated solfataric soil samples, are classified into the 16S rRNA gene-based subgroups, which are congruent with the geographic locations as well. On the other hand, the *V. souniana* strains, isolated from pipelined hot spring water, allocate in a different lineage. This may indicate that the geographic separation and the environmental difference promote the genetic diversification, leading to the speciation, among members of the genus *Vulcanisaeta*. In order to verify the hypothesis, we have tried to isolate strains from other locations, and conducted phylogenetic analyses on the 16S rRNA, *radA* and DEAD/DEAH box helicase genes. The comparison of gene organization of the 23S rRNA gene downstream regions (ca. 12k bases) was also conducted for several strains. The respective phylogenetic analyses on the 16S rRNA, *radA* and helicase genes revealed that the *V. distributa* strains and allied strains, including newly isolated strains from Hokkaido to Kyushu, could be differentiated biogeographically. However, the phylogenetic positions of the strains from Hokkaido and Akita hot springs were contradictory in the 16S rRNA/*radA* gene-phylogenies, implying that there have been functional constraint or homologous recombination in the genes of these strains. The genetic relatedness between *V. distributa* and *V. souniana* by the DNA-DNA hybridization study and the gene organization comparison, allowed us to deduce that the two species have diverged from the common ancestor more recently. Those results would underpin the usefulness of these *Vulcanisaeta* strains as model organisms to study the allopatric microbial speciation.



**THE *Aquifex aeolicus* ATC-DHO COMPLEX:  
A THERMOSTABLE NANOSCALE REACTOR CATALYZING  
CONSECUTIVE REACTIONS**

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In chemistry, a one-pot synthesis is the ideal because sequential reactions are carried out in a single chamber without the necessity to isolate and purify the intermediates, thus greatly increasing efficiency and minimizing waste. A large pyrimidine biosynthetic complex comprised of aspartate transcarbamoylase (ATC) and dihydroorotase (DHO) in *Aquifex aeolicus* is a biological analog of the chemist's reactor. Biochemical and structural studies have shown that the *A. aeolicus* enzymes assemble to form a 480 kDa dodecameric complex. X-ray crystallographic analysis revealed an extraordinary structure. The DHO-ATC complex is comprised of six copies each of ATC and DHO arranged to enclose an enormous central chamber. The active sites of both DHO and ATC are located in the interior of the complex, so that substrates and products must diffuse in and out of the central chamber via narrow access channels. The reactor is fed by another large complex, carbamoyl phosphate synthetase (CPS), which associates with DHO-ATC to provide the labile substrate, carbamoyl phosphate. The architecture of the complex may represent a new class of protein assembly; a one-pot nanoscale reactor so named because it shields the intermediates from the solvent and provides direct transfer of intermediates between the active sites of enzymes catalyzing consecutive reactions.

## **DIVERSITY OF CARBAMOYL PHOSPHATE SYNTHETASES IN PROKARYOTES**

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Carbamoyl phosphate synthetases (CPS), the starting point catalysts for both pyrimidine nucleotides and arginine biosynthetic pathways, present a wide variety of structural and functional characteristics associated with the organism type, environmental conditions, the role in the cell, pathologies, etc. These factors determine the presence or absence of the enzyme in the cell, the subunit composition and/or cellular location, the protein size and particularities at the primary structure level, kinetics and interactions with other enzymes, allosteric and posttranslational regulation. Studies concerning CPS adaptation to various stress conditions and phylogenetic evolution cover both prokaryotic domains, Archea and Bacteria, unraveling basic aspects on structure and functional mechanisms of this very diverse class of enzymes and particular adaptation mechanisms to cope with extreme environmental conditions such as temperatures around 100°C. CPS involvement in pathologies related to ammonia metabolism disfunction, such as urea cycle disorders and cancer, has recently extended from eukaryotes to microbial world, by the putative implication of CPS from of the major archaeon in human gut in controlling energy harvest related to obesity. This methanogen presents a unique CPS enzymatic system that contributes to ammonium metabolism via two biosynthetic pathways, containing the smallest hypothetical missing link in the evolution of CPS.

## A MISSING LINK IN THE EVOLUTION OF CARBAMOYL PHOSPHATE SYNTHETASES IS A PUTATIVE TARGET IN OBESITY PREVENTION

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The critical role of the major archaeon in human gut *Methanobrevibacter smithii* in improving the efficiency of our digestive system was recently stated. Key-enzymes involved in ammonium assimilation appear to be essential for controlling this process. *M. smithii* possesses two carbamoyl phosphate synthetases (CPS) that catalyze the precursor of both arginine and pyrimidines biosynthetic pathways. These enzymes, containing one of the smallest synthetase subunit, represent a unique CPS system that might represent a missing link in the evolution of this class of enzymes and a putative target for controlling human obesity-related processes.

The genome of *M. smithii* contains a *carA* gene, coding for glutaminase subunit (GLN), and three *carB* genes coding for synthetase subunits (SYN). These SYN are very different in size, SYN1 of 1058 residues, and two small size subunits of 391 residues (SYN2) and 367 residues (SYN3), respectively. These hypothetical polypeptides show conserved active site aminoacids. Remarkably, *M. smithii* SYN2 and SYN3 appear to be the smallest CPS subunits identified so far that might reconstitute the “large” synthetase subunit of most CPSs.

Cloning of the three genes *carA*, *carB1* and *carB3* was performed by PCR amplification and insertion in pRSET expression vector and expressed in *E. coli* BL21(DE3). Purification of all three CPS subunits by affinity chromatography is currently under way.

Corroboration of the structural and functional properties of these archaeal CPSs might lead to identify one of the ancestral forms of this class of enzymes, with medical impact.

## RAMAN SPECTROSCOPY DETECTION OF THE HALOARCHAEA IN SALT CRYSTALS

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The interest for the determination and detection of microorganism with different physical methods (Rösch et al., 2006) is increasing due to the necessities of identifying rapidly the bioterrorism attacks and epidemics and to identify microorganisms in the subsurface of other planets in the frame of future missions, in specially for Mars (Ellery et al., 2004) where halite rocks were discovered (Gooding, 1992). The instruments sensibility are increasing and the new equipments and tables of detectable compounds spectra obtained by this method, showed that is a very quickly, useful and easy to use method for identification of different bacteria and archaea from environmental samples without a complicated sample preparations.

For this purpose, the authors used a microbial biomass (different haloarchaeal strains) obtained by centrifugation at 6000 rpm 5 minutes and washed twice with saline buffered solution 4M, resuspended in the same solution and spreaded aliquots of 40 µl saline solution-archaea mix on sterilized quartz disks. After complete drying, we performed investigations with Raman spectroscopy (Raman Dilor XY spectrometer with laser excitation at 514.5 nm wavelength, coupled with a confocal microscope BX40-Olympus Corp., Japan) and Raman Infrared Spectroscopy (Fourier Transformed Near Infrared Raman Spectroscopy using an equipment Bruker IFS 66 + FRA106 with laser excitation at 1064 nm (Bruker, Germany), obtaining the Raman spectra with intense peaks at 1505, 1153 and 1000 cm<sup>-1</sup> wavelength characteristic for C<sub>50</sub> carotenoid compounds contained by these microorganisms.

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## MORPHOLOGICAL CHANGES IN *Magnetospirillum gryphiswaldense* EXPOSED TO TEMPERATURE AND PH STRESSES

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One of the most well characterized examples of biologically controlled mineralization is the formation of intracellular magnetic nanoparticles by magnetotactic bacteria. In this study, we investigated the pH and temperature effect on the physiology and biomineralization ability of the magnetotactic bacterium *Magnetospirillum gryphiswaldense*. Scanning and transmission electron microscopy studies revealed that exposure to adverse conditions (pH variations and extreme temperatures) induced significant changes in cellular length and morphology. Filamentous and supercoiled cells were observed at pH 8.0 and 9.0. Such filamentous forms were rarely observed in pH 7.0 long-time cultures. Bacterial cell diversity and morphology can be correlated with a given level of pH. The results obtained seek to relate the cell adaptation of magnetotactic bacterium *Magnetospirillum gryphiswaldense* to transitory environmental stress.

## COLOURED FUNGI IN SPACE: PROJECT MANAGEMENT AND PRELIMINARY SCIENTIFIC RESULTS

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The project “Growth and survival of coloured fungi in space – CFS-A” is based on experiments both on the Earth and in Space inside of microcapsules made by experts from Romanian Institute of Space Science (ISS). Microbiology activities and imaging of spores at optic and electron microscope are done by experts from Romanian Institute of Biology (IBB) and National Technical University of Athens (NTUA). The main activities can be grouped in: experiment documentation, preflight tests and experiments, flight experiment, post flight studies.

*Experiment documentation* contains a lot of documents to assure the security of flight, astronauts and space environment. Romanian partners together with European Space Agency (ESA) experts, were preparing the following official documents (some of them specific and other common for Soyuz taxi and ULF shuttle): CFS-A - Experiment Scientific Requirements (ESR); The Minute of the SURE CFS-A meeting (RO)=Meeting in Bucharest on 13-14 March 2007; Partnership agreement “Growth and survival of coloured fungi in space – CFS-A”, Experiment in the SURE project the contract number RITA 026069 (AO-2006-022); ESA-SURE - Agreement regarding the implementation of the experiment “Growth and survival of coloured fungi in space - CFS PART A - CFS” on board of The International Space Station under THE SURE PROJECT; Technical Specification of the Experiment (TS-EX) (100); Technical Specification of the Equipment (TS-EQ) (200); Main Schedule; Upload Item List; In-flight Biohazard Materials Approval Form; Recommended Data Format for Biological Material; Hazardous Materials Documents; Payload Integration Agreement for CFS-A; International Space Station - Standard Payload Integration Agreement.

*Pre flight tests and experiments* had been done by Romanian partners either in their institutions or in the institutions recommended by ESA. According to the design of experiment it has to be done in Base/Ying biocontainers available from ESA. Institute for Space Science from Bucharest had been involved in design and realization of microcapsules for biological experiment. It was choose polycarbonate as material,  $\Phi = 60$  (for living cultures) and 30 mm (for dried spores samples) as size; it was also

responsible by all activities involved for integration of microcapsules with biological material in biocontainers. Microcapsules had been tested for: compatibility with biological material, resistance to sterilization, transparency for photos taken in flight (IBB, Romania), resistance to vibration and shocks (Zarma, Bremen, Germany), leaking (Astrium, Bremen, Germany), contamination (BIOTESC, Zurich, Switzerland) oxygen content in microenvironment, and rubber toxicity (IBB).

Biological materials were represented by living cultures of *Ulocladium chartarum* being different ages and dried spores of *Ulocladium chartarum*, *Aspergillus niger*, *Cladosporium herbarum* and *Basipetospora halophila* fixed on silica, iron and polycarbonate wafers

After performed Experimental Scientific Test (EST) which was an experiment done according to Space scenario but in simulated conditions at BIOTESC, Zurich, and Astronauts training (Cologne, Germany), CFS-A was well prepare for flight. Unfortunately the flight had been postponed two times; the closest scenario is planned in October 2010.

The main scientific results got during EST at BIOTESC (Zurich) were the following:

- The growth of colonies is not affected by simulated microgravity due to the fact that substrate mycelium is attached to the nutrient medium and aerial mycelium is not involved in growth but in sporulation. Using photos taken before launch, at Flight Day 4, Flight Day 8 and after landing, it can be measured rate of growth, active area involved in colony growth and sporulation process
- Rate of growth is different because it is in connection with age of the colony. Nevertheless takes place for all ages used in experiment but it is highest in non integrated conditions
- All biological processes are going on in simulated microgravity conditions starting with germination, growth and branching of hyphae ending with sporulation. That is a real prove of fungal growth not only on Earth but also in simulated microgravity conditions.
- The highest rate of growth according to the active zone situated at the edge of colony was found up to Flight Day 4. Then it became slower due to bursting hyphae and moving structures considered to be bacteria according to their moving in cytoplasm, vacuoles, around the hyphae (after cell wall is cracked) and Gram-color staining (Gram negative bacteria round shape). Microscopically observations revealed that the growth of submerge mycelium is going on in the depth of nutrient according to very long and low branched hyphae; getting the edge of microcapsules suddenly are branched and become visible as small colonies.
- 0 day old culture can be considered as a pattern to demonstrate that in microgravity conditions a fungal colony is able to grow and to colonize a

substrate. It demonstrates that spores are able to germinate to generate hyphae and mycelium to become mature to make spores and to assure species survival.

- In Random positioning Machine samples, optical microscopy revealed abnormal shape of spores, enlarged hyphae unable to branch, random branching, oblique and horizontal position of conidiophores.

- Spores viability had been not affected by simulated microgravity conditions. New cultures obtained from integrated cultures and dried spores samples had normal rate of growth.

*Post flight studies* will be focused on: measurements of the rate of colony growth in real microgravity conditions, distribution of submerged mycelium in nutrient medium, assessment of sporulation, presence of mobile structures, microscopically changes etc, the effect of cosmic radiation and microgravity on dried spores samples exposure for short (maximum 14 days) and long time(several months) in International Space Station etc



## **THE FUTURE PROTOCOL ON ACCESS AND BENEFIT SHARING TO THE CONVENTION ON BIOLOGICAL DIVERSITY - SCIENTIFICALLY AND TECHNICAL CONSIDERATIONS**

M.M. ANTOFIE

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Adopted in 1992, the Convention on Biological Diversity (CBD) is recognizing in the context of Art. 15 the need for provisions development regarding the access to genetic resources as a baseline for the Access for Benefit Sharing (ABS) international regime a tool for the implementation of the third objective of the Convention. An estimation regarding the species diversity states that all over the world exists at present about 1.75 million species described whereas the estimates of the total number of species vary between 7 and 20 million (Groombridge & Jenkins 2000). These numbers are very important for the very ambitious goals of the CBD making almost impossible the implementation of the provisions of the annex 1 of the Convention. Furthermore the economy of ecosystems the so called “green economy” will be based also on the ABS international regime development. Developing an ABS regime at international level become more and more complex as this regime involves also different other issues which are already covered by other international instruments. The adoption of the CBD creates already challenges to the FAO which before 1991 considered that the genetic resources are belonging to the world heritage and which was modified through the adoption of the FAO Resolution 3/91 according to the provisions of art. 15. 1 of the CBD which states that the sovereignty rights of countries over their natural resources and states that the authority to determine access to genetic resources rests with the national governments and is subject to national legislation. Actually the 1992 year was shifting the world vision regarding the people’ rights over their natural resources creating new challenges, barriers and opportunities for the world development. According to the provisions of the CBD all Parties have obligations regarding their own right to determine the conditions upon which their resources could be accessed. An international regime such as the ABS – will grant the access to genetic resources and benefit-sharing arising out of the utilization of these resources within their jurisdiction through bilaterally negotiated contracts on the basis of mutually agreed terms (MATs) and prior informed consent (PIC) (art. 15.2). Further, presently there are negotiations taken by the ABS Working Group under the CBD, initiated in 2004, which is mandated by the COP9 to finish the ABS

Protocol by the COP 10 in Nagoya Japan in line with the Bonn Guidelines (adopted at the 6th Conference of the Parties of the CBD, 2002). It is important to underline that under the “Bonn Guidelines”, access should only be granted subject to an adequate knowledge of the situation (PIC), the agreement of mutual terms (MAT), for sustainable use, and with the balanced and equitable sharing of the benefits derived from the use of genetic resources. Under debates are today some important provisions of the draft ABS Protocol: [1] tools for compliance, [2] misappropriation of genetic resources and or traditional knowledge versus misuse, [3] acquisition of genetic resources, [4] international access standards, mechanism for exchange information at international through the CBD clearing house mechanism, [5] ABS certificates and bioprospecting [6] patent disclosure, [7] intellectual property rights in relation with the traditional knowledge related to the genetic resources, [8] access to justice and so on. However now it is agreed that the international ABS regime must give recognition for compliance to the role of codes of conduct, that it should promote existing codes and include a mechanism for identifying those codes that Parties regard as best practice such as: [1] Code of Ethics of the International Society of Ethnobiology (IES), [2] The International Plant Exchange Network (IPEN), [3] The Principles on Access for Genetic Resources and Benefit Sharing for participating Institutions for botanical gardens, [4] Rules and provisions by national institutes and organizations elaborated [5] The Belgian Coordinated Collections of Micro-organisms launched Micro-Organisms Sustainable use and Access regulation International Code of Conduct (MOSAICC) and [6] The International Federation of Pharmaceutical Manufacturers and Associations (IFPMA) who established a set of Guidelines for IFPMA members on Access to genetic resources and Equitable Sharing of Benefits Arising out of their Utilization. Romania as a Party to the CBD has the obligation to implement the provisions of Art. 15 and all the other Convention’s provisions in relation with the national legislation and complying with all international instruments. Presently Romania did not develop an ABS regime and neither the European Union (EU). Still the EU adopted the Bonn Guidelines and Romania doesn’t. Couples of countries all over the world developed such a regime but which is not complete or fully operational because of the complexity of interrelationship with other international instruments or multilateral environment agreements. According to our opinion as the ABS protocol’s procedures will be more analytical and more complex the more it will be hard to be applied to the poor countries or with economy in transition. The new created barrier will create opportunities for new financial mechanisms development shifting the funds for capacity building to be implemented in these countries for all domains covered by the sustainable development concept. Still remains the question if the mega-

biodiversity rich country will be really helped to reach the MDG as the Gabala Declaration doesn't mention the need for the ABS Protocol adoption just before the COP10 of the CBD. Moreover, the adoption of a very complex ABS Protocol will issue new barriers against the poorest in economy but richer in biodiversity (e.g. the proper implementation of art. 8 j. of the CBD in relation with the traditional knowledge).

## UNRAVELING THE SECRETS OF SPONTANEOUS FOOD FERMENTATIONS

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The earliest production of fermented foods was based on spontaneous fermentation of agricultural raw materials and was due to the development of the microbiota, such as yeasts and lactic acid bacteria, naturally present in the raw materials. The quality of the end-products was dependent on the microbial load and spectrum of the raw materials. Spontaneous fermentation has been optimized through backslopping, *i.e.*, inoculation of the raw materials with a small quantity of a previously performed, successful fermentation, resulting in dominance of the best-adapted microbial strains. It represents a way, be it unconsciously, of using a selected starter culture to shorten the duration of the fermentation process and to reduce the risk of fermentation failure. Spontaneous fermentation and backslopping are still in use worldwide. They further represent a cheap, reliable, and durable means of food preservation in general and particularly contribute to the artisan character of fermented food products. The direct addition of selected starter cultures to raw materials on an industrial basis, from the end of the 20<sup>th</sup> century, has been a breakthrough in the processing of certain fermented foods and beverages, resulting in a high degree of control over the fermentation process and standardization of the end-products.

Before a starter culture can be developed, the natural fermentation process of the raw materials has to be understood in detail. The Research Group of Industrial Microbiology and Food Biotechnology of the Vrije Universiteit Brussel focuses on several spontaneous food fermentation processes, including sourdough fermentation, cocoa bean fermentation, and spontaneous fermentation of vegetables and fruits. Vegetable and fruit fermentations are studied in collaboration with the Romanian Institute of Biology in Bucharest. For all these studies, microbiological culture-dependent and culture-independent analyses on time-dependent samples are performed. Culture-dependent analysis involves the selective isolation of cultivable microorganisms present in the samples, followed by identification of the picked up colonies through (GTG)<sub>5</sub>-PCR fingerprinting (bacteria) or M13-PCR fingerprinting (yeasts) and/or multi-locus sequence analysis. Culture-independent analysis encompasses denaturing gradient gel electrophoresis of PCR amplicons of targeted rRNA gene fragments

(universal and/or group-specific primers) of genomic DNA directly isolated from the samples and sequencing of DNA bands cut out of the gels, functional gene microarray analysis, and metagenomics. The combination of these microbiological analyses with meta-metabolomics on the same samples of the microbial ecosystems involved helps to unravel the secrets of spontaneous food fermentation processes, including detection of new species and/or pathways (metabolites).

Sourdough develops by spontaneous fermentation of yeasts and lactic acid bacteria in mixtures of cereal flour(s) and water, with the lactic acid bacteria being mainly responsible for the acidification of the dough and the yeasts for the leavening action; both yeasts and lactic acid bacteria are responsible for flavor formation as well. Usually, a stable microbial community arises during periodic refreshments of the flour/water mixture, closely depending on external factors such as temperature and pH, propagation cycles, dough hydration and yield, and the type of cereal. For instance, independent of the flour type, a three-stage evolution of communities of lactic acid bacteria results in the dominance of *Lactobacillus fermentum* and *Lactobacillus plantarum* during laboratory sourdough fermentations. Dominating yeasts are *Candida glabrata* and *Wickerhamomyces anomalus*. The succession and final composition of these microbial communities could be correlated with tolerance toward acid stress and carbohydrate metabolism (maltose metabolism and fructose-into-mannitol conversion) and amino acid conversions (e.g., arginine-into-ornithine conversion) for energy generation and cofactor recycling. However, in bakeries, the in-house microbiota determines the microbial community composition to a large extent. Belgian artisan bakery sourdoughs are composed of *Lactobacillus paralimentarius*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus sanfransiscensis*, *Saccharomyces cerevisiae*, and *Wickerhamomyces anomalus*.

Cocoa-based products are to be considered as fermented foods. The raw materials, cocoa beans, undergo a spontaneous fermentation carried out traditionally and usually close to the site of harvest in the rainforest of cocoa-producing regions. Fermentations may be performed in heaps or boxes according to different farming practices. In the cocoa pods, the beans are embedded in a mucilaginous white pulp, mainly consisting of pectin, citric acid, and the main carbohydrates sucrose, glucose, and fructose. Once the pulp-bean mass is exposed to the environment by cutting the cocoa pods and removal of beans and pulp from the pods, a spontaneous fermentation starts, during which successive microbial activities of yeasts (mainly *Hanseniaspora opuntiae*, *Pichia kudriavzevii*, and *Saccharomyces cerevisiae*), lactic acid bacteria (mainly *Lactobacillus plantarum* and *Lactobacillus fermentum*), and acetic acid bacteria (mainly *Acetobacter pasteurianus*) lead to the formation of a range of metabolic end-products,

such as ethanol, lactic acid, and acetic acid, which are precursors for cocoa flavor and color formation. Again, the nature of the metabolites is reflected in the carbohydrate degradation and amino acid conversion routes followed. Farming practices and environmental parameters, such as cocoa pod harvest, bean selection and pre-processing, and duration of the fermentation, are of crucial importance for a successful cocoa bean fermentation process, independent of the region or fermentation method. Well-fermented dry cocoa beans determine the quality of the chocolate products made thereof.

Spontaneous fermentations of vegetables and fruits are a durable means of food preservation in Eastern Europe, including a note of gastronomic excellence. Fresh vegetables are salted and sealed off from air to initiate a spontaneous fermentation process, which is mainly carried out by lactic acid bacteria, the species of which depend on the salt level, pH, and temperature of fermentation. The lactic acid bacteria most frequently isolated are *Lactobacillus brevis* and *Lactobacillus plantarum*, independent of the vegetable type. Glucose and fructose, the main vegetable carbohydrates, are completely converted into lactic acid, acetic acid, and mannitol. In addition, flavor compounds are produced that enhance the taste of these products as compared with vegetables preserved in vinegar.

Knowledge on the microbial succession and/or metabolites produced during spontaneous fermentations will contribute not only to the development of starter cultures for bulk production but also to innovation purposes targeting new processes and/or functional products.

## DIVERSITY AND POTENTIAL APPLICATIONS OF LACTIC ACID BACTERIA ISOLATED FROM ROMANIAN FERMENTED DAIRY PRODUCTS

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Lactic acid bacteria (LAB) constitute a heterogeneous group of bacteria that are traditionally used to produce fermented foods and beverages. The large-scale industrial production using specific starter cultures and modern equipment improved the technological quality of dairy products, but at the same time limited their biodiversity as well as the organoleptic variation of the end products. Therefore, an increasing demand exists for new strains that show desirable effects on the product characteristics. Artisan dairy products are a candidate source of such microorganisms. Traditionally fermented dairy products are still a very important part of the daily food in Romania, especially in the countryside. Due to their artisan and region-dependent handling, these products can be a source of interesting LAB, either new taxa or strains with specific functional properties, which might be used for the development of new starter cultures and innovative food products. For example, exopolysaccharides (EPS) or EPS-producing LAB strains might be applied to improve the mouthfeel and texture of fermented foods, in particular of fermented milks and cheeses. On the other hand, many LAB strains were shown to contribute to the food spoilage retardation. Their preservative effect is mainly due to the acidic conditions created during fermentation, but also due to some anti-microbial compounds that are produced, including bacteriocins.

In our studies we have identified the LAB species occurring in Romanian raw milk and in traditionally prepared dairy products and we have selected some LAB strains with potential biotechnological applications (strains producing bacteriocins or exopolysaccharides).

The LAB diversity of Romanian raw milk and traditionally fermented dairy products is characterized by lactococci, leuconostocs, and enterococci. Consequently, Romanian fermented milks can be assigned to the group of mesophilic fermented milks, which is typical for Scandinavian and Central and Eastern European countries. Among the enterococci, the new species *E. saccharominimus* was found. During this study, the synonymy of two *Lecunostoc* species was also proven.

The newly isolated strains were screened further for bacteriocins- and EPS production. *Lactobacillus acidophilus* IBB 801, isolated from an artisan dairy product, was found to produce a small bacteriocin, acidophilin 801. This bacteriocin has an estimated molecular mass of less than 6.5 kDa. It displays a narrow inhibitory spectrum, being active towards only related lactobacilli, but including two Gram-negative bacteria (*Escherichia coli* Row and *Salmonella panama* 1467). The antibacterial activity is insensitive to catalase, but sensitive to proteolytic enzymes such as trypsin, proteinase K and pronase. It is further heat-stable and the activity is maintained in a wide pH range. The antibacterial activity can be enhanced by treatment with detergents, such as Tween 80 and Tween 20 or by UV irradiation. Acidophilin 801 displays primary metabolite kinetics and a concentration-dependent bactericidal mode of inhibition. It induces ultrastructural changes in the sensitive strains, such as: formation of mesosomes, intracytoplasmic vesicles or transmembrane pores with the loss of the intracellular material and cell death.

Among the EPS-producing LAB strains, ten were studied in detail. *Streptococcus thermophilus* ST111 produces a heteropolysaccharide (HePS) with a high molecular mass (over 4900 kDa), composed of galactose and rhamnose monomers, in a 5/2 ratio. For this purified EPS, the NMR structure was also elucidated. Other nine LAB strains, five *Leuconostoc citreum*, one *Leuconostoc mesenteroides*, one *Leuconostoc pseudomesenteroides*, one *Lactococcus lactis* and one *Weissella confusa/cibaria* were found to produce high molecular mass (over 4900 kDa) homopolysaccharides (HoPS), composed of glucose monomers linked by  $\alpha$ -linkages.

The HePS biosynthesis by *S. thermophilus* ST111 follows a primary metabolite kinetics. Its production can be significantly increased when the producing strain is grown under controlled, optimised conditions (milk supplemented with lactalbumin hydrolysate, 37 °C, constant pH of the medium of 5.8).

Finally, the prebiotic effect of five selected EPS (four HoPS and one HePS) was investigated. None of the five EPS was degraded in *in vitro* experiments for the simulation of the passage through the stomach and small intestine (low pH, addition of pepsin, followed by addition of pancreatin and bile salts). Moreover, the HoPS produced by *Lactococcus lactis* 1.8 was partially metabolised by four *Bifidobacterium* strains, which normally occurs in the human intestine.

In conclusion, traditionally fermented Romanian dairy products are valuable sources for new and diverse LAB strains, with potential applications in food industry and health. The results presented here were obtained during the last years, in the framework of several national (National Research Plan) and international projects (EU-projects, bilateral agreements).



## CYANOBACTERIAL RESPIRATION – THE FASCINATING „STRUGGLE“ BETWEEN PHYSIOLOGY AND GENES

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My research group and I at the University of Vienna have been investigating the bioenergetic processes – focusing on respiration – in blue-green algae (cyanobacteria) since 1971. In the late 1980ies, at the same time as Murata and his group in Okazaki, we started to publish a series of papers on the isolation and biochemical characterization of pure, separated cytoplasmic (plasma) and intracytoplasmic (thylakoid) membranes (CM and ICM) from eventually up to forty different cyanobacterial strains and species (see <http://homepage.univie.ac.at/Guenter.Peschek>). In contrast to Murata et al. we could clearly demonstrate the occurrence in both CM and ICM of a mitochondrial-like respiratory chain leading from NAD-1, NAD-2, and SDH *via* PQ and a chimeric complex-III (cytochrome *bc* complex), somehow similar to that in phototrophic green and non-phototrophic heliobacteria, finally to a proton-pumping and heme-promiscuous *aa<sub>3</sub>*-type cytochrome-*c* oxidase. However, shortly after in 1997 Sugiura, Tabata, et al. had presented the completely sequenced genome of *Synechocystis* PCC6803 at the 9<sup>th</sup> ISPP in Vienna, and a few years later, primarily through the untiring work of the JGI in California, we now have the complete genome structure of almost twenty cyanobacteria. Unfortunately, these genes also comprise – not only the canonical *aa<sub>3</sub>*-type oxidase which we had first discovered and characterized 1979 in *Anacystis nidulans* and found to occur in *all* investigated cyanobacteria (1-3) – but also an amazing variety of other TROs among them heme-copper and *bd*-type (plasto-)quinol, plant-type and other “alternative” oxidases, *etc.* (2), *none* of which has ever been characterized as a functional enzyme protein (2, 3) up to the present day. As it is unlikely that, during aeons of evolution absolutely useless genes might have just been kept in, and not eliminated from, cyanobacteria it is generally assumed that just conditions for expression of these genes have not yet been found – though this is certainly at least likewise unlikely regarding the extreme wealth of experimentation performed on cyanobacteria at least during recent decades. Thus we must assume that the exceedingly tiny quantities of transcripts synthesized on these genes (4) have to do with the (still unknown!) *ontogenetic* reluctance of cyanobacteria to a genetically based adaptation to any changed growth conditions (*cf.* the still basically unresolved reason for the obligate and invariably *phototrophic* growth habit of so many cyanobacteria) which is in strange and marked contrast to the

amazing *phylogenetic* variability and adaptability of cyano- bacteria as a group to very diverse natural habitats all around our world (cyanobacteria as the *pioneering colonists*, cf. the explosion of the volcano Cracatao, Indonesia, in 1883, the hot and hostile remnants of which had been first recolonized by cyanobacteria). On the other hand, the adaptation to slow but sustained growth in the light without much competition by the generally prevailing chemoheterotrophs during the aeons of evolution, when respiratory energy generation has, of course, always been necessary as a supplementary bioenergetic process during inevitable dark periods (in the night!) or when photosynthesis was otherwise environmentally suppresses (5), might have led to “stubborn” (strict) phototrophy which has long made superfluous the complicated rearrangement and adaptation of a whole array of enzymes to streamlined, optimized respiratory energy conversion and thus made superfluous a bunch of TROs as well, each made for a special environmental situation, notably since a single yet fully functional TRO, viz. the canonical *aa<sub>3</sub>*-type COX, has proved absolutely sufficient for all cyanobacterial purposes. The latter is perfectly witnessed by the fact that cyanobacteria are still, and rightly, regarded *the bioenergetic nonplus-ultra* in our terrestrial biosphere and we should not doubt that, basically, they are well capable of coming along without the bunch of alternative TROs that geneticists always want them to have . .

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# RESPIRATION-IN-LIGHT IN CYANOBACTERIA: FUNDAMENTALS, BIOTECHNOLOGICAL POTENTIAL AND ECOLOGICAL IMPLICATIONS

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Cyanobacteria are the largest and most diversified, ecologically most successful and evolutionary most important group of prokaryotes. This group of microorganisms is clearly defined by the ability to carry out both oxygenic photosynthesis within the thylakoid membranes and respiration within plasma (cell) membranes (CM) and thylakoid membranes (ICM) as well (1,2,3), that determines the inhibition of respiration by light. This phenomenon is also found in other oxygen respiring prokaryotic phototrophs (e.g. anoxygenic phototrophic bacteria or bacteriorhodopsin-containing archaea). In this context, our main results (4-11) point out that:

1. Respiration-in-light (RL) is higher in cyanobacteria *Synechocystis* PCC 6803 and *Anacystis nidulans* (= *Synechococcus* PCC 6301) grown at high salinity (0.55 M NaCl) than in cells grown at low salinity (50 mM NaCl);
2. Following our original hypothesis (Ardelean and Pescheck, 2002), we demonstrated that the inhibition of respiration by light is more pronounced in cyanobacteria, where the highest cytochrome *c* oxydase concentration localizes in thylakoidal membranes (e.g. *Synechocystis* PCC 6803), as compared to cyanobacteria, where the highest cytochrome *c* oxydase concentration is at plasma membrane (*Anacystis nidulans* (= *Synechococcus* PCC 6301) (Ardelean and Pescheck, 2010, in press);
3. Respiratory electron transport occurring only at the plasma membrane can be measured *in vivo* by selective disruption of external membrane, thus allowing the horse heart cytochrome *c* to be reduced mainly in the presence of KCN. The rate of cytochrome *c* reduction is higher in cells grown at high salinity (0.55 M NaCl) than that at low salinity (50 mM NaCl), suggesting a higher RL. To our best knowledge, this is the first attempt to measure *in vivo* the cytochrome *c* reduction by cyanobacterial cells having an intact peptidoglycan layer.

RL in cyanobacteria presents important biotechnological applications for the construction of biosensors and of biofuel cells by using the bioelectrochemical properties of the bacterial cells (either intact or without

external membrane) to enhance the electron exchange between these cells and macroscopic electrodes.

The main ecological implication of RL from cyanobacteria is related to the estimation of net photosynthesis and primary production under natural conditions. This is due to the fact that dark respiration is always higher than RL, the degree of inhibition of (dark) respiration by light depending also on the strain and external salt concentrations. Furthermore, the ability of some cyanobacteria (marine or fresh water ones) that live in the presence of gasoline oxidizing heterotrophic bacteria to tolerate this organic pollutant rises the hypothesis of the RL involvement in bioremediation processes (12,13).

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# **FEASIBILITY OF METHODS BASED ON NUCLEIC ACID AMPLIFICATION TECHNIQUES FOR MICROBIOLOGICAL ANALYSIS OF WATER QUALITY: OPPORTUNITIES AND DRAWBACKS**

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Molecular methods -based on nucleic acid recognition and amplification- are offering new opportunities for the detection of microorganisms and can be applied to the field of water microbiology for detecting both pathogens and indicators. However, microbiological water quality assessment has a series of requirements that must be met in order to maximize research efforts. At present, most of these water management decisions are supported by the analysis of microbial indicators measured by traditional methods. These molecular methods are valuable tools to complement and support such decisions.

Nucleic acid based methods show enormous potential for identifying isolates from conventional culture methods, providing data on cultivable and non-cultivable microorganisms, informing on the presence of pathogens in waters, determining the causes of waterborne outbreaks, and, in some cases, detecting emerging pathogens.

However, some features of water microbiology influence the performance of nucleic acid-based molecular techniques and thus challenge their suitability for routine water quality control. These features include the variable composition of target water samples, the generally low numbers of target microorganisms, the variable water quality required for different uses, and the physiological status or condition of such microorganisms. Challenges for these nucleic acid-based methods need to be identified in order to improve their feasibility for routine microbial water monitoring.

## **OPERATIONAL IMPLEMENTATION OF SCIENTIFIC RESEARCH IN LAKE KINNERET (ISRAEL)**

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During the last 80 years the Kinneret ecosystem has undergone natural and man-made modifications. Several of the anthropogenic changes were done before and others after scientific research was initiated: Construction of the South Dam (1933); Construction of the National Water Carrier (operated 1964); Diversion of salty springs (1967); Fish introduction (exotic and native (from early 1950's)). Several of the natural events were followed by research and precise monitoring: Droughts and floods with high fluctuations of water level; Multi-annual decline and increase of water temperature; Cyanophyte bloom (1994); Peridinium decline (1990's); Zooplankton fluctuations; Sardines removal (1994 – 2003); Tilapias crisis. A comprehensive limnological research formulated within one master plan started after the operation of the National Water Carrier in early 1960's. Then it became clear that because Lake Kinneret is proposed to be a national reservoir aimed at multi-annual supply of drinking water their quality must be protected and therefore thoroughly investigated. The comprehensive study as based on routine monitoring program and research started in 1969 in the Kinneret Limnological Laboratory and still continue onwards. This combined program of monitoring and research has three major objectives: to indicate impacts of early operations, to understand the present conditions and to be able to submit predictions for future developments. Significant constraints are those due to water supply. Requirements evolved from increased drought events and increase of domestic demands for water were resulted in by water level decline, and consequently a research of its impact on water quality. The management of the drainage basin gave a push towards changes of nutrient inputs regime which was followed by changes of species composition of the phytoplankton which later stimulated fishery modification. The need for removal of unwanted fish became significant aimed at water quality protection. Sequence of droughts initiated the need for hydro-geological study of the salts flux into the lake in runoffs and underground of sub-lacustrine sources. The salinity of supplied lake water has an impact on soil quality over the whole country. The limnological response of the lake to natural and anthropogenic changes include also fluctuations of salinity, fish reproduction, phytoplankton species composition and biomass density. The

appearance of toxic cyanophytes was predicted long time before the real event occur and the positive effect of removal of unwanted fishes was indicated and implemented appropriately resulted in by zooplankton elevation but nano-planctonic algae were not suppressed due to surplus phosphorus. The present fishery crisis is highly discussed and much effort is invested aimed at implementation of scientific information. Bridging the gap between limnologists, fishery biologists and water managers is essential to prevent deterioration of water quality and multi purpose utilization of the natural freshwater lake Kinneret for water supply, fishery, recreation, and tourism.

## STRUCTURE AND FUNCTION OF THE ZOOPLANKTON IN THE LARGE AND SHALLOW LAKE BALATON, HUNGARY

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Lake Balaton with its 596 km<sup>2</sup> area and mean depth of 3.25 m is the largest shallow lake in Central Europe. The 70 km long lake underwent a strong eutrophication between 1970 and 1985. Then, the water quality turned back to good, or oligo-mesotrophic, but still exist a strong trophity gradient via the longitudinal axis of the lake. The area of the lake is very windy, the annual mean of the wind velocity is 3.2 m sec<sup>-1</sup>. Therefore, and according to the instrumentally measured RMS-turbulence intensity and the estimated energy dissipation rate, Lake Balaton is one of the most kinetic lakes in the world. Taxonomical composition, numerical density, Cladocera/Copepoda ratio, Predator/Total ratio, biomass, production, and biomass turnover time (B/P24h) of the zooplankton were studied in April - October of the years between 1999 and 2009 biweekly in five different basins of the lake, with different trophity. Several physical and chemical background variables i.e. wind velocity, turbulence, turbidity, temperature, water level fluctuation, Chlorophyll-a were measured in parallel. The most common Rotatoria species in Lake Balaton were *Keratella cochlearis*, *Keratella quadrata* and *Polyarthra vulgaris*. Among crustaceans the Calanoida Copepoda *Eudiaptomus gracilis* was the most abundant species which usually amounted to 50-60% and often reached 90% of the total crustacean density, followed by *Cyclops vicinus* and *Mesocyclops leuckarti*. Among Cladocera, the hybrid *Daphnia cucullata* × *galeata*, *Diaphanosoma brachyurum*, *Bosmina longirostris*, *Bosmina coregoni* and *Leptodora kindtii* were usually common. Veliger of zebra mussel (*Dreissena polymorpha*) was usually common member of the zooplankton assemblages between May and October, when water temperature was higher than 11-12 °C. The 11 years mean of the numerical density of the veliger of *D. polymorpha* was 14.0, of Rotatoria 77.6, of Crustacea 163.2 and of the total metazoan zooplankton 254.8 ind lit<sup>-1</sup>. The mean biomass of veliger was 1.30, of Rotatoria 5.68, of Crustacea 788.0 and of the total zooplankton 795 tons dry matter Balaton<sup>-1</sup>. Mean net production of veliger was 0.17, of Rotatoria 1.17, of Crustacea 34.82 and of the total zooplankton 36.16 tons dry matter



24 h<sup>-1</sup> Balaton<sup>-1</sup>. Biomass turnover time of Rotatoria was 6.12, of Cladocera 10.91 and of Copepoda 16.92 days. The zooplankton net production expressed in carbon per unite lake surface was 7.37g C m<sup>-2</sup> year<sup>-1</sup>, which equivalented with 6.15% of the 120g C m<sup>-2</sup> year<sup>-1</sup> planktonic primary production. After consideration of the possible respiratory + excretion + molting carbon loss of the zooplankton, the gross zooplanton production amounted ~25% of the planktonic primary production. Cluster analyses carried out on the time series of the presence-absence of the different species in the five different basins with different trophity, and weighted those with individual density revealed, that rather the seasonal changes than the trophity status of the different basins determined the species composition of the zooplankton assemblages. Abundance conditions were under strong influence of the hydrodynamics of the lake. The usually high turbulence intensity supressed the development of several taxa (i.e. *Daphnia*, *Eudiaptomus*) by physical stress. This impact was even more significant during the low water level periods when the water column was more turbulent. The wind induced waves and turbulent currents also initiated mineral sediment resuspension (up to 600 mg dw l<sup>-1</sup>) which often caused food limitation of the filter-feeder species, especially of *Daphnia*.

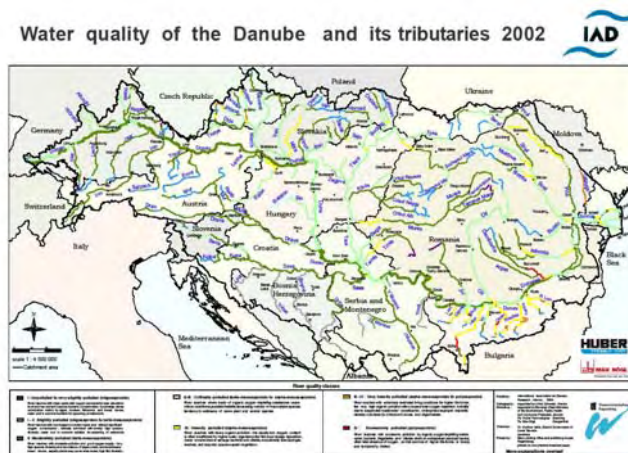
## **BIOLOGICAL ASSESSMENT OF WATER QUALITY FOCUSED ON THE DANUBE RIVER BASIN – A REVIEW**

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Water protection came into political focus after the Second World War, when increasing industrial and domestic pollution was evidenced by algal blooms, fish kills, and poor drinking and bathing water quality. The public concerns and demands for remediation initiated new water protection laws and the installation of waste water treatment plants (WWTPs). The authorities enhanced chemical and biological monitoring of lakes and rivers. While chemical investigations of rivers reflect the momentary concentrations of nutrients and pollutants, biological assessment, in particular macrozoobenthos, provides the integral effect of pollution over time. As benthos communities are known to react in a rapid and differentiated way because they contain sensitive and ubiquitous species, they are good indicator organisms and became one of the key parameters for biological assessment of water quality.

In the 1950s, quite a number of various biological indices were gradually developed, in particular the Saprobic System by V. Sládeček (1973)<sup>1</sup> and others. Despite of different calculation approaches they all based on the same empirical evidence that benthos communities were seriously affected by, and hence indicated, pollution. Theories on saprobity were strongly debated in the scientific community, because sampling was only qualitative or semi-quantitative, reference conditions and autecology of species were not well known and cause-effect relationships a black box; hence, the method was claimed to be not precise and non-scientific (K. Wuhrmann). However, such biological investigations gained value and were useful for applied water management. Water quality maps of whole river systems were developed, integrating site specific classification into five or seven quality (saprobity) classes. The IAD Danube quality map produced by R. Schmid (2002, 2004) provides still the only water quality map of the whole Danube River Basin (DRB) (Figure 1). The map shows that the Upper Danube has “good quality in destroyed channels”, while the Middle and Lower Danube feature “bad quality in intact channels”. Moreover, the inflows of some highly polluted tributaries are diluted by the high discharge of the main river.



**Figure 1** – The IAD map of the Danube shows the general water quality in the DRB. While the Upper Danube is mostly in class 2 (moderately polluted, beta-mesosaprobic), the Lower Danube features often critical pollution (class 2-3, alpha-mesosaprobic). Many Lower Danube tributaries show heavy pollution (class 3-4, polysaprobic). High discharge and strong self-purification of the Lower Danube decrease concentrations; but the load remains high. Schmid, R. (2004): *Water quality of the Danube and its tributaries – 1995 updated and 2002. Explanations to the river quality map.* State Office for Regional Water Management Re-gensburg and IAD General Secretary Vienna.

Scientific competition emerged between the empirical Saprobic System and the predictive English approach RIVPACS using reference sites as the basis of river classification (Wright et al. 1993, 2000)<sup>2</sup>. Later, it was recognized that benthos communities are not only biased by pollution but also by changed flow through dams and water abstraction, and altered habitats by morphological degradation. Hence, community response reflects a complex disturbance and human impact. In consequence of the river continuum concept of Vannote et al. (1980)<sup>3</sup> and the emerging catchment approach (river basin management) the river zonation featuring, e.g., different assemblages in alpine streams as compared to lowland rivers gained more weight in biological quality assessment. In the scientific Danube and IAD community, numerous taxa lists provided the basis for Saprobic river classification.

Nowadays, the EU WFD is the top legislative instrument in DRB management, requiring not only good water quality, but ultimately good ecological status for all surface waters by 2015. This goal is based on integrative water protection where quality, quantity and morphology are equal parameters for healthy ecosystems. The WFD approach prefers the top-down concept based on physical traits, ecoregions (Illies 1978)<sup>4</sup> and river typology. Modified and harmonized saprobity evaluation methods provide the basis for classification by five quality classes that are related to the reference conditions in different stream and river types. Such assessment is included in the Danube River Basin Management Plan elaborated by the ICPDR (2009). A constant problem still is the biological monitoring that

needs a consistent methodology and concept, in particular when it comes to Environmental Impact Assessment (EIA) of large infrastructure projects. We can distinguish between surveillance, compliance, impact, investigative and operational monitoring.

Biological monitoring goes beyond saprobiological assessment reflecting organic pollution. A myriad of chemicals produced by industry end in waters, and hence toxic substances, persistent chemicals, heavy metals, pesticides, hormone active substances, etc increasingly influence the aquatic biota and, ultimately, human health. New approaches include the sub-lethal effects of low concentrations and community response to multiple stress. Also behavioral studies are carried out showing for example that fish migration may be hindered by WWTP input as they “smell” certain chemicals. Ecotoxicology (substance vs. effect monitoring) becomes the focal point of complex pollution, and biological tests (like *Daphnia* and *Lemna*) are used for on-line biomonitoring (e.g., at Bad Abbach, Danube rkm 2400 – <http://www.lfw.bayern.de>). Since bacteria react quickly to environmental changes, their monitoring may function as an early warning system for water contamination. In this context, new molecular technologies in the field of nucleic acids (e.g., PCR = Polymerase Chain Reaction Technique, DNA-Fingerprinting, DNA-Chip Technology) are very useful. Biomonitoring by using biomarkers in fish is also applied in the Danube River Basin. Last but not least, biomonitoring of acute pollution through accidental spills has been repeatedly applied (e.g., in 2000 the Baia Mare and Baia Borsa gold mine accidents in the Tisza catchment).

A look into the future shows some inherent problems of biological assessment. The education of young taxonomists is threatened by “modern” university students programs. Biological methods will be further developed and harmonized. Using traits of macrozoobenthos instead of species lists may improve extrapolation and functional understanding. However, other biota like phytobenthos, phyto- and zooplankton, fish, and macrophytes must be also respected in the sense of ecosystem health and “good ecological status”.

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## **DANUBE STRATEGY - A BALANCE BETWEEN SOCIO-ECONOMIC DEVELOPMENT AND ENVIRONMENTAL PROTECTION**

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The future EU Strategy for the Danube Region represents a step forward in the regional planning, allowing the shift from the sectorial development to an integrative policy interlinking environmental with social and economic aspects.

The “business as usual” approach and sectorial policies led to a marked decline of biodiversity and ecosystem services, threatening the welfare of human society. In order to achieve the EU overarching goal of sustainability (COM 400, 2009)<sup>1</sup> in the Danube River Basin, the Danube Strategy should complement the existent legal framework (e.g. Water Framework Directive, Flood Directive, Birds and Habitats Directives, Bern Convention, Biodiversity Action Plan, etc.) with new policy tools supporting the implementation of environmental friendly measures in projects aiming for social-economic development.

Water enables life on Earth and both quantity and quality of the freshwater resources are vital for our existence – drinking and household consumption, gardening, irrigation, industry, hydropower, transport, tourism – are just few of the water uses we rely on. As a consequence of climate change and overuse, water scarcity became a problem in the Southern part of Europe and, without proper management, these problems will be aggravated in the near future.

The Danube River Basin Management Plan (DRBMP)<sup>2</sup>, published in 2009 by the International Commission for the Protection of Danube River (ICPDR) and elaborated in close cooperation with the riparian countries, represents a first step towards the holistic approach of integrated water management at catchment scale and a good basis to strengthen the environmental pillar of the Danube Strategy. The actions planned in its Joint

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<sup>1</sup> Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions: Mainstreaming sustainable development into EU policies: 2009 Review of the European Union Strategy for Sustainable Development

<sup>2</sup> ICPDR] International Commission for Protection of the Danube River, 2009: Danube River Basin Management Plan, [http://www.icpdr.org/icpdr-pages/danube\\_rbm\\_plan\\_ready.htm](http://www.icpdr.org/icpdr-pages/danube_rbm_plan_ready.htm)

Program of Measures (JPM) should be supported by this strategy and complemented by additional measures following the envisaged trends for social-economic development.

An environmental-friendly policy will have beneficial effects on business development in the long term, as human society ultimately depends on the services provided by the ecosystems. If the current trends of environmental degradation will continue, the human well-being will sharply decrease: *the ecosystem services that are freely available today will cease to be available or become more costly in the near future*<sup>3</sup>. Therefore, investments into further research and development of new materials and „green” technologies are necessary in order to enhance the ecosystem services and reduce the current environmental pressures.

#### Strategic steps for a sustainable development

A sustainable development must be based on a critical evaluation of significant pressures such as ***pollution, land use change, overexploitation of natural resources, hydromorphological alterations, introduction of invasive alien species and climate change*** and their negative impact on ecosystems, environmental health and human well-being. The ecosystem services (i.e. benefits provided by ecosystems) such as: provision of food and raw materials, regulation of atmospheric composition and climate, flood/drought mitigation, water purification, nutrient cycling, pollination, recreation and education areas, etc. must be brought to political and public awareness. In the present social-economic context it is important to address the problem of value and price: some of these services can be transposed in monetary value (such as food provisioning, recreation services, etc.), but others (such as climate regulation, oxygen content in the atmosphere, genetic pool and biodiversity) are priceless and vital for our existence.

To some extent, the natural ecosystems have the ability to cope with different pressures; however, the alarming signals received nowadays (such as massive loss of biodiversity, water scarcity, decrease of fish stock, climate change, etc.) show that the resilience threshold may soon be exceeded; therefore, urgent measures are needed in order to maintain or restore ecosystems functionality and secure our basis of living.

The Danube Strategy must consider also the ***impact of planned measures of future social-economic development on the environment***. A sustainable strategy should address measures to reduce the existing pressures and to develop a new concept of “environmental-friendly”

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<sup>3</sup> Millenium Ecosystem Assessment, 2005 - Ecosystems and Human Well-being: Opportunities and Challenges for Business and Industry.  
<http://www.millenniumassessment.org/en/Synthesis.aspx>

projects that should become a *sine qua non* condition for the future regional development. The following actions are recommended:

- promote environmental-friendly measures to reduce the anthropogenic impact; this includes e.g. favoring of new technologies that reduce dependency on fossil fuel and mitigate CO<sub>2</sub>-production, promoting recycling processes, or supporting green farming practices;
- preserve the natural ecosystems; a sound management of the Nature Parks, the introduction of biosphere reserves and strictly protected areas with buffer zones, and the restoration of the degraded ecosystems should be promoted; in this respect, landscape planning plays a key role; also flood protection strategies and eco-tourism could be integrated into such concepts;
- balance the environmental with economic and social needs for any future project; this means implementation of “true” sustainability by an economic and political paradigm change: the long-term (social) perspectives and benefits would be prioritized over (private) short-term benefits of shareholders;
- improve the involvement of the local stakeholders in the decision making process; this includes the participative process of jointly developing a project to better balance the conflicts of interest (as given by the Aarhus Convention);
- enhance the implementation of the current EU environmental legislation; the Danube Strategy should integrate the requirements of WFD, Natura 2000, Biodiversity Action Plan, etc. with other policies such as: the agriculture, fishery, energy (hydropower), transportation (navigation), human migration, etc. in order to provide a holistic regional strategy.

All these measures can support an EU policy that attempts to foster sustainability and react to environmental problems induced by globalization.

## THE USE OF ARTEMIA IN THE ROMANIAN COASTAL AQUACULTURE

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*Artemia* is the most popular organism used as living food for fish and crustacean larvae.

The present paper research has been done at the NIMRD "Grigore Antipa" Constanta. The experiments designated to zooplanktonic organisms cultivation were focused on:

- The most suitable species selection for cultivation, based on an unlimited number of samples, from marine environment, Sinoe Lagoon, Techirghiol Lake.
- The main criteria for the cultivation technologies establishing, taking into account the biological data, general necessities in environmental and cultivated conditions and stages, methodologies for culture maintaining.
- Experimental studying of the ethological and physiological characteristics with essential importance for cultivation in captivity.
- Assessment and evincing of the valuable qualities (nutritional, biochemical, ecological) of the marine invertebrates for fish larvae nourishment.
- Improving or growing the efficiency of the cultivation technologies for the main groups of small marine invertebrates.

At the Romanian Coast, the main cultivation techniques were the followed:

- incubation and hatching of harvested cysts from natural salty lake,
- intensive culture systems production,
- accommodation and production in salty pond extensive culture.

The main scientific and technologic results have been:

- ⇒ *Artemia* cysts from Techirghiol Lake have biochemical qualities and hatching indices comparable to the ones from the best exploitable populations on the Earth.
- ⇒ The *Artemia* cultivation at sea water salinity conditions ( $18 \pm 2$  g/l), in the controlled environments, permitted:
  - Quality control of the environment and organisms,
  - Accomodation to the cultures conditions,
  - Cultivation during the whole year round,



- Improving the developing stages biochemical qualities, administrating yeasts, dogfish liver oil, soy lecithin,
  - Assessment of the growing ratio of the different developing stages.
- ⇒ Similar conditions created in the two different basins of the coastal area permitted the accommodation of Techirghiol *Artemia* strain to the controlled hyper salty pool from Istria experimental base, obtaining similar productivities with the origin biotope ( $10-15 \times 10^3$  mg/mc); the cultivation parameters and position in trophic chain were established.
- ⇒ Biochemical analyses proved comparatively higher nutritive qualities of the Techirghiol strain and *Artemia franciscana* grew in marine pontic water; the main biochemical parameters, enzymes activity, essential amino acids and fat acids, have been evaluated and compared with other parts of the world analyses for the same species.
- ⇒ Valorization 100-125 kg wet cysts production were registered; 50 kg of viable organisms/year were collected from the 0.3 ha lake's surface and produced minimum 20 kg dried cysts.
- ⇒ The cysts can be used directly in aquaculture or can be commercialized at significant prices. The extending of *Artemia* cultivation depends on market demand, farmers' ability of investing, the developing and the extension of cultures techniques.

At the Romanian Black Sea Coast, *Artemia* have been used as living food for pike-perch, turbot, plaice, pike, aterina, sandy-goby, rainbow-trout, silver, big-head, golden carp and shrimps larval nutrition.

## ROTIFERS CULTURES FOR MARINE FISH LARVAE FEEDING

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The domain of fish larva breeding or larviculture is part of modern aquaculture. The larval nutrition is the most important part of this domain, resolving the critical moments recorded in the earlier larval stages, due to the weak morpho – physiological development of the fish larvae digestive system.

The specific activities, technical aspects and necessary technologies were insured by National Institute for Marine Research and Development “Grigore Antipa” Constanta, the only scientific institution dedicated to marine and coastal aquaculture in Romania.

The necessity to cultivate the best small marine invertebrates for fish larvae comes also from the consumption preferences manifested in natural conditions. Because of this the research has been led to the most important organisms with biological, ecological, adaptative and productive characteristics.

Rotifers are indispensable to feed the fish first larval stages, and are met as common cultivated organisms in worldwide industrial breeding system. The main important species used in aquaculture is *Brachionus plicatilis*

- The main qualities and advantages of the rotifer cultures are: The rotifers small sizes (123 - 292  $\mu$ m); Accommodation capacity for large salinity limits; Survival capacity, till the maximum 2000 ex/ml density; High reproducing rate, 0,7 – 1.4 offspring/female/day; High trophic value (biochemical analyses).
- Directions in which rotifer cultures recorded improvements includes Genetic approach; Cheap nutritional resources (concentrated algae, yeast improved with dogfish liver oil finding); Diseases prevention; Trophic value improving; Eggs Collecting and Preserving, Techniques for harvesting and cultures maintaining improvement, Food diversification, testing and using complex food regimes.
- Main results obtained are linked with biological, productive and economical evaluations, reminding:
  - Inoculation density: 100 ex/ml (extensive culture); 100-1000 ex/ml (intensive culture)

- Maximum production: 300 (obtained) to 3600 mil.rotifers/day (planned for the experimental platform); Minimum production - 2.5 mil. rotifers/day; Average production - 10.0 mil. rotifers/day;
- Maximum productivity - 75 rotifers/ml/day; Average productivity – 25.7 rotifers/ml/day;
- Maintained stock density - 2-5 ex/ml; Harvesting density - 200 ex/ml;
- Food price percentage in total rotifer production - 72%, based on 50% algae and 22% yeasts.

## **THE PROTECTIVE FUNCTION OF THE PROTEIN FAM129B IN APOPTOSIS**

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Inhibition of apoptosis is an essential component of cancer cell progression and metastasis. The protein, FAM129B or Minerva is a poorly characterized protein that has recently been implicated in melanoma cell invasion. Suppression of apoptosis during metastasis is a prerequisite for the survival and spread of cancer cells. During apoptosis, the adherens junctions are disassembled as the dying cell retracts and new contacts are formed between normal neighboring cells. FAM129B is cytosolic in exponentially growing HeLa cells but is translocated to the adherens junctions where it colocalizes with  $\beta$ -catenin whenever contact between two or more cells is established. Silencing the FAM129B gene expression by specific siRNAs does not induce apoptosis or inhibit the growth of HeLa cells. However, when apoptosis is induced by exposure to TNF $\alpha$ /cycloheximide, the onset of apoptosis is accelerated 3-4-fold when FAM129B is depleted. The rapid induction of apoptosis in FAM129B knock down cells is reversed by co-transfection with recombinant FAM129B indicating that its effect on apoptosis is specific. As apoptosis proceeded, FAM129B was degraded and disappeared from the cell membrane. Thus, one crucial facet of the mechanism by which FAM129B promotes cancer cell metastasis is likely to be the suppression of apoptosis.

## **A ROLE FOR TELOMERES IN SKIN CANCER DEVELOPMENT AND PROGRESSION**

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Non melanoma skin cancer is the most common malignancy in man. Furthermore, immunosuppression in organ transplant recipients strongly contributes to an increase in squamous cell carcinomas (SCCs), occurring 65- 250 times more frequently than in the general population. Nevertheless, little is known about the genetic changes contributing to SCC development and progression. Comparing keratoacanthomas (KA) as an early stage and skin SCC as a late stage skin cancer we now demonstrate that, with only few exceptions, similar chromosomal aberrations are present in both KAs and SCCs, including loss of 3p, 4q, and 9p as well as gain of 11q, 7q, 8q, or 7q. However, while KAs only contain one or few of these aberrations, several are generally present in SCCs simultaneously, thus making the latter aberration pattern highly complex and suggesting that KAs are indeed genetic precursor lesions of SCCs. In agreement with these genetic aberrations, we can show two growth promoting factors, cyclinD1 (11q13+) and hTERT (possibly c-myc-induced, 8q+) are up-regulated in KAs and SCCs, thereby substantiating their early role in skin cancer development. The cell cycle inhibitor p16 is highly expressed in KAs but absent in SCCs and this absence often correlates with 9p loss. Accordingly, the anti-angiogenic matrix glycoprotein TSP-1 is expressed in most KA while absent in many SCC. Thus, while gain of growth promoting factors characterizes the early changes, late changes are likely related to loss of growth inhibitors as well as factors favouring the angiogenic switch and with that the ability of the tumor cells to grow invasively. While UVB is well accepted as a factor inducing very specific (C to T transitions and CC to TT double base changes) DNA changes in e.g. the p53 gene, we now provide evidence that also UVA contributes to skin cancer development and progression. UVA irradiation, representing 90% of the solar radiation on earth and being the major radiation source for sun beds, induces indirect damage (oxidative stress) which manifests as DNA strand breaks and in turn can result in chromosomal aberrations. Accordingly, HaCaT keratinocytes can be malignantly transformed by exposure to UVA radiation only. We further propose that the protective caps at the ends of the chromosomes, the telomeres, are responsible for the UVA-dependent chromosomal instability. Stress or damage can lead to accelerated telomere

erosion which in turn can causes critically short dysfunctional telomeres. As a consequence, these “sticky ends” form end-to-end fusions which during mitoses generate anaphase bridges and breakage of the chromosomes - known as fusion-bridge-breakage cycles. We indeed have evidence that this scenario can be induced by UV radiation in skin *in situ* and may be causally involved in the formation of some basal cell carcinomas (BCC) and some SCCs. In addition, we recently proposed another length-independent form of telomere-dependent genomic instability.

Inducing the c-Myc oncogene caused aggregation of telomeres (TAs) and as a consequence formation of new stable translocation chromosomes. We now can show that UV radiation is inducing massive TAs in a similar time- dependent manner as seen for c-Myc, arguing for a similar mechanism. Such TAs are found in all skin tumor types but rarely in premalignant lesions. This suggests that TA formation is a late event that is involved in ongoing genomic instability during tumor progression. Thus both, the telomere length-dependent and length-independent telomere mechanisms seem to play a role in skin cancer development and progression.

## **BASEMENT MEMBRANE – IMPORTANT PLAYER IN EMBRYO DEVELOPMENT AND VARIOUS DISEASES, INCLUDING CANCER DEVELOPMENT AND PROGRESSION**

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Basement membrane (BM) is a very specialized extracellular matrix (ECM) with molecular components that support important functions including proliferation, differentiation, migration, chemotaxis of cells during embryo development. Basement membrane is connecting/isolating cell(s) from the microenvironment, or is connecting/isolating different types of tissues. In order to maintain their stem cell properties, stem cells must preserve contact with the BM. Changes in BM composition play important roles in promoting and sustaining/development of the various diseases. BM is an anhist infrastructure, forming a thin acellular layer of cca. 50-100 nm thickness and is ubiquitous in the body. Some cell types, as is the case of adipocyte or glial cell associated to nervous cells have their individual basement membrane. Mostly, a basement membrane separates and connects two different types of tissues. Simple (intestinal epithelium) or stratifying, keratinizing epithelium (epidermis) is connected/separated *via* basement membrane. Usually, basal cells of epidermis, endo- or exocrine glandular cells, some parenchymatic cells (lung epithelial cells), endothelial cells etc. are faced with the basement membrane by their basal pole. BM determines different tissue compartments in complex organs, playing not only an important structural but also a regulatory role. First, this infrastructure provides a firm support for the adjacent cells/tissues and serves as a selective barrier between different tissue compartments. Taken in consideration the molecular composition and the inside body location of basement membranes, there are different kind of basement membranes (BMs). Common to all BMs are four main molecular species: at least one member of laminin families, type IV collagen, nidogens (ND 1 and ND 2) and the proteoglycan perlecan. A variety of growth factors and cytokines binds to the BMs. A BM is an extracellular complex of informative or inductive/inhibitory signaling molecules. Specific molecules of BMs regulate different (patho)-physiological aspects. Using routine transmission electron microscopic (TEM) and immunogold electron microscopic (IEM) investigations, early during embryo development, specific patterns of vectorial (spatial-temporal) expression of molecules or structured basement membranes are detectable. Cell proliferation, cytodifferentiation (especially, cell polarity maintenance *via* hemidesmosomal junction-cytoskeleton), cell

adhesion and cell migration *via* cell surface receptors (mostly integrins, i.e.  $\alpha_6\beta_4$  dimeric integrin) and non-integrin receptors are strongly under BM regulation. Changes in the qualitative, over- or underexpression as well as ectopic location of some molecular components of the BM are involved in various inborn or acquired diseases, including tumor development and progression. Reparation of injury-induced tissue lesions (mechanical trauma, xenobiotic factors or infectious agents) requires BM contribution. In multicellular organisms, all organs and tissues live and function to the planned, harmonious disciplined cooperation of different types of cells. The interaction with ECM, including basement membrane, is critical for maintenance of tissue homeostasis. Unfortunately, in some circumstances, focal accidents can disrupt this harmony. Totally absence or defective hemidesmosomes for inner plaque (rudimentary hemidesmosomes) associated with loss of basement membrane at the tumor stroma interface or abnormal microvasculature (immature blood capillary) is hallmark of skin tumors (i.e. basalioma and squamous cell carcinoma). Both in basal cell carcinoma and squamous cell carcinoma tumors prelevated from patients, *in situ* TEM and IEM analysis demonstrate that loss of cell-cell and cell-ECM junctions of epithelial cells affronted to the adjacent stroma leads to severely epithelial cell depolarization. High fragility of tumor cell plasma membrane (plasma membrane recombinations, invadopodia formation, numerous shedding membrane vesicles at the tumor-stroma interface and, even focal dissolution of plasma membrane) correlates well with ectopic or absence of some connectors (Plectin/HD1, BPAG 1) or cytoskeleton (keratin K14) molecules. Altogether above mentioned alterations as well as BM absence enhance the invasive ability of tumor cells inside of the peritumoral stroma. Despite genetic alterations preservation inside of malignant cells, under natural circumstances or experimental controlled conditions, tumor phenotype can be converted to the normal behavior (tumor cell repolarization and peritumoral microvasculature maturation). In that case, the basement membrane restoration and maintenance are prerequisite conditions.



# **NANO-BIO-SCIENCES AND TECHNOLOGIES: MOLECULAR NANO-VELCRO SYSTEM RELATED TO SELF-RECOGNITION IN EVOLUTION OF MULTICELLULARITY, CANCER AND VIRAL/RETROVIRAL IMMUNOTHERAPY, AND NANO-BIO DEVICES**

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Physicochemical basis for molecular self-assembly processes reside on intermolecular binding forces. Differences in the degree of binding strengths between diverse types of molecules at the given environmental thermodynamic conditions determine selectivity of their associations. Self-assembly is therefore the bottom-up driven process which defines dynamics, state and physical form of all cohesive structures including the evolution and existence of the most sophisticated cellular life forms.

Molecular self-assembly is fundamental for all life processes related to evolution and maintenance of: i) cell

structures, ii) multicellularity, iii) self-non-self-discrimination and iv) functional anatomical integrity. Better understanding of molecular self-assembly and their potentially extraordinary valuable industrial applications require a truly interdisciplinary approach integrating biology, chemistry, physics and mathematics. Although obvious, such a melange of scientific knowledge is practically very difficult to archive within research teams at the classically structured public and private bodies. Often interdisciplinarity is rather formalized then applied.

Herewith I will present my results and medical and biotechnological applications of experimental and modeling studies on molecular self-assembly related to: 1) *Self-recognition in evolution of multicellularity*, 2) *Cancer and viral-retroviral immunotherapy* and 3) *Nano-bio-devices*

## *1) Self recognition in evolution of multicellularity*

I have developed Atomic Force Microscopy (AFM) measurements of intermolecular binding strength between a single pair of complementary cell adhesion molecules in physiological solutions which have provided the first quantitative evidence for self-assembly recognition and adhesive structure-to function-related properties for biological macromolecules. This study has shown that the intermolecular binding forces as a main quantitative criterion for assessing and defining their functional contribution to evolution, maintenance, and diversity of the biological life

(Science 1995, Nature 1997, J. Biol. Chem. 2004). The presented example of Porifera cell adhesion glyconectin proteoglycans showed that self-assembly molecular nano-velcro system is based on homotypic polyvalent carbohydrate to carbohydrate interactions between two primordial proteoglycans. Binding strength between two molecules can hold the weight of 1,600 cells. Thus, glyconectin type carbohydrates, as the most peripheral cell surface molecules of sponges (today's simplest living Metazoa), are proposed to be the primary cell adhesive molecules essential for the evolution of the multicellularity. Knowledge obtained by AFM measurements of binding forces between individual pairs of different types of cell recognition and adhesion molecules under variable thermodynamic conditions resulted in development of a new modeling and simulation approach, and new software for in silico studies of self-assembly.

### *2) Cancer and viral-retroviral immunotherapy*

The principle of selective molecular self-assembly led me to discover and develop the novel glycan drugs which specifically stimulate proliferation of human natural killer (NK) cells (protected by 5 international and national patents). Definition of natural killer (NK) cells function is to kill cancer and viral/retroviral infected cells. Selective stimulation of different subsets of NK cells proliferation is the prerequisite for specific and effective target killing. The nature of the molecules responsible for such stimulation was not completely established and consequently therapy using NK route could not be valuably achieved. 1) 33 novel fucosylated acidic glycan compounds were obtained. 2) Treatment of PBMC cultures with these compounds resulted in selective stimulation of proliferation of different NK cell subsets from naturally occurring level of 1-5% to 30-80%. Untreated controls remained at level of 1-5 %. No significant stimulation of B or T cells is observed. NK cells of all humans tested, from a variety of ethnic and racial groups could be significantly stimulated. 3) The obtained human NK cells showed massive and continuous killing of target human tumor or viral infected cells during five weeks of co-cultured period under condition of 1000 fold target cells excess. 4) NK "cellular magic bullet" therapeutic effect for treatment of cancer and viral/retroviral infections is suggested to widen and complement existing treatments.

### *3) Nano-bio devices*

My goal was to develop a novel tool for full "Omics-Phenotyping" analysis on a single chip which is in part based on molecular self-assembly. For the first time this tool allows combined quantitative, specific and high sensitivity in vitro measurements of proteins (proteome), nucleic acids (transcriptome), polysaccharides (glycome) and metabolites (metabolome). The "QuantiOmics" tool (Quantitative Omics) has the flexibility to simultaneously measure patients cancer and NK cells, serum, plasma and urine, for diagnostic, prognostic and monitoring purposes (patent pending).

This concept develops breakthroughs nano-bio-sensor chips allowing the direct chemical quantification below 10 molecules per sample with detection limit of a single protein molecule.

Disclosures: This abstract contains novel and mostly unpublished data. For some parts of results disclosed in the abstract Prof. Gradimir Misevic obtained 5 patents. Some other parts of results presented in the abstract are patent pending. Keywords: molecular self-assembly, evolution, multicellularity, atomic force microscopy, glyconectin, carbohydrates, human natural killer (NK) cells, fucosylated acidic glycan drugs, cancer, viral/retroviral infections, immunotherapy, nano-bio-devices, quantiomics, phenotyping, proteome, diagnostic, prognostic and monitoring Gradimir N Misevic, Prof. Dr. and CEO Gimmune GmbH Gimmune GmbH, Poststrasse 24, 6300 Zug, Switzerland, [http: www.gimmune.com](http://www.gimmune.com), E-mail: [gradimir@gradimir.com](mailto:gradimir@gradimir.com), [gradimir@gimmune.com](mailto:gradimir@gimmune.com)

## GLYCAN-GLYCAN INTERACTIONS – A PATHWAY TO MULTICELLULARITY

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The emergence of multicellularity has been tightly joined to the ability of an organism to retain its own anatomical integrity and to distinguish self from non-self. Large cell surface glycoconjugate molecules, such as glyconectins (GNs), might have provided the key recognition and adhesion functions. In order to understand the molecular basis for primordial self-recognition and non-self discrimination, we focused our attention on the role of cell surface GNs in Porifera xenogeneic cellular interactions, as the evolutionary most compatible model system for ancestors of Metazoans. The GNs are different from classical mammalian glycoconjugates and define a novel class of primordial cell adhesion molecules. We have purified GNs from three marine sponge species: *Clathria prolifera* (*Microciona prolifera*) - GN1, *Halichondria panicea* - GN2, and *Cliona celata* - GN3, and performed biochemical and structural analyses of their glycans. Using atomic force microscopy (AFM) we have demonstrated that the strength of GN1-GN1 binding generates essential cell cohesion forces in the sponge *C. prolifera*, as previously implied by functional investigations; the binding force between a single pair of GN1 glycans can hold the weight of 1,600 cells. The mass spectrometric and NMR analyses revealed that each GN presents novel and highly species-specific glycan sequences. All three GN glycans include distinct acid-resistant and acid-labile carbohydrate domains. These differences are sufficient to explain the species-specific separation of glycan-coated beads *in vitro* and the sorting of sponge cells *in vivo*. The molecular mechanism of glycan-mediated homophilic GN interactions in Porifera is based on highly species-specific and  $\text{Ca}^{2+}$ -dependent associations, and approaches the degree of selectivity of the evolutionarily advanced heterophilic immunoglobulin superfamily recognition system. The GN-GN interactions may thus provide a new model for molecular self-recognition. The evolution of GN-like proteoglycan molecules, with the capacity for self-recognition and adhesion, may have been a fundamental condition for the establishment of the first multicellular organisms, as well as for the further divergence of species.

## **MOLECULAR GENETIC APPROACHES TO THE STUDY OF NEURONAL MORPHOLOGY, DEVELOPMENT AND FUNCTION**

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Understanding neuronal morphology and connectivity is crucial to the understanding of the central nervous system. Though neurons have extremely complex dendrite and axon arbors, these arbors are highly stereotyped, allowing their geometry to be used as a major classification criterion for defining neuronal cell types. Moreover, neuronal arbor morphology correlates well with the specific physiological and circuit functions of neuronal cell types.

The mammalian retina contains more than 50 distinct cell types, which can be broadly classified in several classes: photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells. Although many of the developmental mechanisms involved in the differentiation of Retinal Ganglion Cells (RGCs) as a cell class begin to be understood, there is very little information with regard to how the morphologies of the 15 – 20 distinct RGC cell types are formed.

Previously it was appreciated that Brn3b/Pou4f2 plays a major role in the development of RGCs. The role of the closely related family members, Brn3a/Pou4f1 and Brn3c/Pou4f3 in RGC development is largely unknown. We developed a conditional gene targeting strategy in mice, which permits ablation of a target gene, and its replacement with the open reading frame of the Alkaline Phosphatase histochemical reporter, creating a genetic mosaic analysis tool, similar to the one used in *Drosophila*. We applied this strategy to investigate the role of the three Brn3/POU4 domain transcription factors. By crossing the conditional reporter alleles to various Cre expressing lines, we defined the distinct but overlapping populations of RGCs expressing Brn3a, Brn3b and Brn3c, characterized their dendritic arbors and brain projections, and analyzed at the single cell level the consequences of deleting the genes, thus gaining insights into the function of these transcription factors in the development of these neurons. We were also able to make predictions regarding which visual functions would be affected by the loss of particular subsets of RGCs, and confirmed these visual deficits by behavioral assays.

## **IS THE CENTROSOME A CALCIUM REGULATED MECHANOSENSOR IN ANIMAL CELLS?**

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In animal cells, the centrosome is a unique structure that organizes the microtubule system. It is composed of two centrioles embedded in a protein matrix responsible for the microtubule nucleation and an anchorage. In migrating cells, the centrosome integrates all the forces applied to the microtubule network and defines the gravity center of the moving cell. The centrosome duplicates once per cell cycle in coordination with DNA. The preexisting centrosome serves as a template for the formation of the nascent centrosome so that during the cell cycle centrosomic structures at different maturation stages coexist in one cell body. At mitosis, two fully functional centrosomes are present in the cell where they organize the mitotic spindle. They are strongly implicated in the orientation of the mitotic spindle and therefore in the determination of cell polarity. In quiescent cells or differentiated cells that are not motile anymore, the centrosome migrates to the plasmic membrane where it initiates the formation of a primary cilium. Cilia are differentiated organelles that respond to shear stress by deflection and are implicated in numbers of sensitive functions. All these observations strongly suggest that the centrosome structure should be sensitive to applied forces and as such be a mechanical switch triggered by the microtubule network. Centrosomes from animal cells can be isolated, and microtubule nucleation studied in vitro. We used isolated centrosomes to approach their mechanical properties. At first, we tried fractionation using hydrostatic pressure to identify structural elements responsible for given functions. Isolated centrosomes are difficult to manipulate as they are dense micronic structures. Therefore, most of the classical biophysical methods cannot be used to study their mechanics. We built a new device to investigate centrosome mechanical properties and to determine its elasticity. We also investigated the modulation of centrosome mechanics by calcium which is proposed to be a major modulator of its structure.

## NOVEL INSIGHTS INTO THE TRACHEAL INNERVATION AND MICROINNERVATION

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Even if it is hardly believable, the tracheal microinnervation in humans was understudied since 1964 when Fischer offered a comprehensive such study. Recently, the airway intrinsic nervous system (AINS) was defined and the analogy with the enteric nervous system was made and is supported by the tracheal development leaving from the wall of the primitive intestine. In order to fill the temporal gap, of missing specific studies on the human tracheal innervation and microinnervation, we performed the present study on 10 human adult tracheas. Microdissections evaluated the peritracheal pattern of neural distribution. Histological (hematoxylin eosin, toluidine blue, luxol fast blue) stains and immunohistochemistry (IHC) on paraffin embedded specimens were performed. We used primary antibodies for neuronal NOS (nNOS), neurofilament, SMA and the cocktail of citokeratines CK AE1 – AE3 + 8/18. Regarding the microinnervation of trachea, segmental branches of the recurrent laryngeal nerves distributed to the trachea were configuring a lateral peritracheal plexus supplying branches to the antero-lateral and the posterior walls of the trachea. Those posterior branches were deepening into the posterior fibroelastic membrane of the trachea and supplied two symmetrical or a single scalariform longitudinal trunk of the membranous wall. The posterior longitudinal trunks that were previously identified only in dogs, were located on the inner aspects of the posterior ends of the tracheal rings and were ganglionated, with small ganglia distributed and supplied in a segmental fashion, distributing branches of the posterior wall and also branches in the spaces between the rings. We constantly identified an interrecurrent anastomosis crossing the membranous tracheal wall between the left and right peritracheal plexuses, previously undescribed, also linked and linking the posterior longitudinal trunks. Histology and IHC identified the structural plexuses of the tracheal wall. We are the first to confirm in humans the neurons of the trachealis muscle identified by Fischer. Moreover we evaluated these neurons as nNOS positive and this was previously undescribed; as so, the respective neurons must be also considered as components of the AINS.

## **INVERTEBRATE DIVERSITY OF CONACU - NEGREȘTI VALLEY (SOUTHWESTERN OF CONSTANTA COUNTY, ROMANIA)**

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Conacu-Negrești Valley is located southeast of Romania particularly in an area with excessive continental climate. It is characterized by a series of specific habitats, most important being dry steppe meadows, limestone rocks with up to date and lake of the same name. Valley fauna is very interesting, characterized by a number of endemic species, specific to the Dobruja province.

The present paper succinctly presents the data known until now from this area. These data represent the results of the research activities within the project which took place between April-August 2009.



## **CLASS CHILOPODA: THE ENVIRONMENT ADAPTATION AND EVOLUTION**

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The main characteristic traits of the chilopoda great groups were used as practical criteria for different classifications.

The anamorphic larval development of the orders Scutigeromorpha and Lithobiomorpha and the epimorphic development of the orders Scolopendromorpha and Geophilomorpha were proposed by Haase (1880, 1882), as criteria for the subclasses Epimorpha and Anamorpha.

The existence of an original respiratory system in the ord. Scutigeromorpha which was different from the respiratory tracheal system based on the pleural stigmata in all other chilopoda groups, led to their division into subclasses Pleurostigmophora and Notostigmophora (Pocock, 1902).

About the middle of the XX century, the general opinion on the chilopod ancestor was that this one proceeded from a long organism with homonomous segmentation and with little specialization of the sense organs, similarly to the representatives of the order Geophilomorpha.

In 1965 Prunescu proposed a new theory and an evolutionary phylogenetic tree of the class Chilopoda (Fig.1). According to this theory the chilopods evolution departed from an archetype formed of 15 leg-bearing segments with alternation of the long tergites with short tergites having an alternation defect at the level of long tergites 7 and 8, pleural stigmata, etc. This theory was based on the identification of the plesiomorphic and apomorphic characters of the great groups of Chilopoda.

Subsequently, new numerous contributions (1970-2006) were realized on the microanatomy of the genital system of Scutigera and subfam. Anopsobiini, the larval development of the macro and microspermatogenesis in Scutigera, the tracheal lungs of Scutigera, the male genital system in Craterostigma, the tracheal system of Craterostigma, the supplementary rudimentary Malpighian tubuli in Scutigera and Craterostigma, etc. which gave a new insight on the class Chilopoda evolution and classification (Fig. 2). In our opinion the chilopods evolution must be superimposed with the functional adaptations of each order to the existence of particular environments.

According these new data, the last common ancestor of the class Chilopoda presented pleural spiracles on the segments with long tergites, had two pairs of Malpighian tubules like in Scutigera and Craterostigma,

presented anamorphic development and cared the brood, preserving it on the body. From this archetype, the evolution of Chilopoda continued following two different ways:

1). Chilopods which hunted in an environment allowing their rapid displacement. This condition was not favourable for the brood care. The females of this category were selected during evolution to depose their eggs on the soil: Subclass 1. Ovodispersa. This subclass is formed of the orders Scutigeromorpha and Lithobiomorpha.

The separation of the subclass Notostigmophora from the ord. Lithobiomorpha was the effect of the respiratory system adaptation, with the appearance of the tracheal lungs and the median-dorsal stigmata, to avoid the water vapours loss, as an adaptation for the life in the open environment.

The very close relationship of the order Scutigeromorpha with the order Lithobiomorpha was proved by the structure of the male genital system with macro and microspermatogenesis demonstrated in the order Scutigeromorpha and in subfam. Anopsobiini (ord. Lithobiomorpha).

2). Chilopods which were adapted to the life at the deeper levels of the litter and humus. This environment favoured the chilopods slower displacement and the brood preservation on their bodies: Subclass 2. Ovoconecta. This subclass is formed of the orders Craterostigmomorpha, Scolopendromorpha and Geophilomorpha. The life conditions in which these orders differentiated, allowed the body length increase, the attenuation till obliteration of the tergal heteronomy, cephalic capsule diminution, reduction till the disappearance of the visual organs, etc.

The populations of the genus *Craterostigma* which exist only in the most peripheral zones of the earth – Tasmania and New Zealand – presented the second pair of the Malpighian tubules similarly to *Scutigera*.

Another trait which bring near these two types of primitive chilopods referred to the respiratory system with fascicles of thin un-anamostomosed tracheoles from the ord. Craterostigmomorpha which paradoxically were approached to the thin tracheoles without anastomoses concentrated in the tracheal lungs of the order Scutigeromorpha.

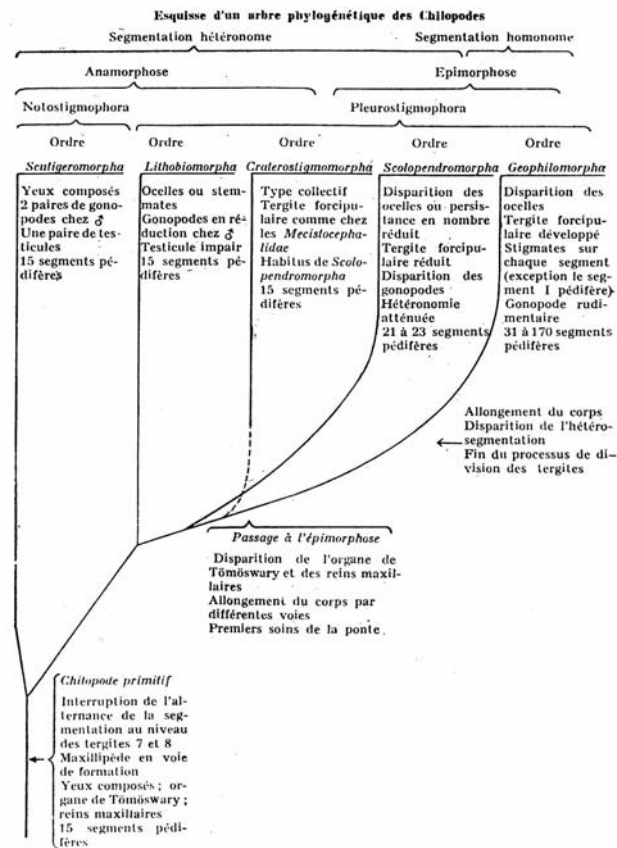


Fig. 1. Phylogenetic tree of Chilopoda Class (facsimile of paper Prunescu, 1965)

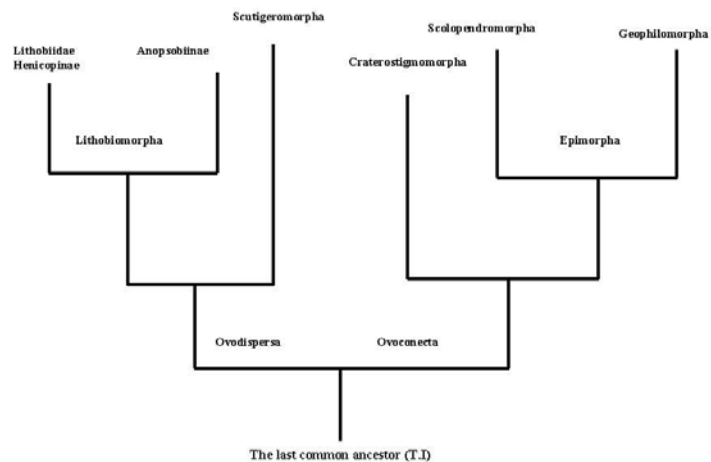


Fig. 2. The new phylogenetic tree of the Chilopoda Class

## VEGETATION DATABASE – FACILITIES AND UTILITIES

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The authors discuss the facilities and utilities of the database program Turboveg for Windows that represents a software package for the input, storage and handling of vegetation and floristic data. The authors point out the possibilities of the usage of Turboveg not only in phytosociology, but also in ecology, taxonomy and in nature conservation, mainly for inventory research, red list set-ups etc. The possibility to state a biotope type (where the relevé or a taxon was sampled) for the input phytosociological relevés opens up the program usage (and hence the data stored in it) also for non-scientific public, particularly for nature conservation institutions.

The database programme Turboveg with its new version for Windows – Turboveg for Windows (Turbowin) – has become an official database programme for input, storage and further processing of phytosociological relevés in more than 25 countries of the world. Turboveg helps to create many national and regional vegetation databases, providing the basis for national and supra-national classification overviews and other scientific studies.

The authors, on the example of structure of the Slovak Vegetation Database point out the necessity of unified national databases that can be linked to any international database, what supports not only the exchange of data, but mainly the possibility of bi-/multi-lateral projects, vegetation overviews of supra-regional areas (e.g. Carpathians) and supra-regional analyses of vegetation data that will enable a better insight into the functioning and distribution of plant communities, as well as evaluating of local and global changes.

The current structure of the database used as a basis of Slovak Vegetation Database reflects the experiences and suggestions of the users after the years of using the programme. The standard fields with the data on floristic composition, cover of individual species, scale, altitude, aspect, locality, etc., were increased recently. It is possible to fill in new coded fields: with orography units – *Orography*, types of geological bedrock – *Petrology*, relief type – *Relief*, biotope type (according to The catalogue of the biotopes of Slovakia) – *Biotope*, *Soil type* and *Soil unit*. The importance of pre-coded fields is the unification of the data inputs, what simplifies the

searching for particular data in the database (within individual fields, such as Orography, Petrology, etc.), as well as the creating of basis for various ecological analyses (Relief, Soil type).

We strongly believe that using the programme Turboveg for Windows on national level in the Romania and using as many functions of this programme as possible will result in an increasing amount of high-quality outputs, such as extensive statistical analyses, national and supra-national (European) syntaxonomical revisions, analyses of ecological affinities of individual plant species, their vertical distribution, changes of vegetation, etc.

## **FLORA AND VEGETATION FROM CONACU - NEGRESTI VALLEY (SOUTHWESTERN OF CONSTANTA COUNTY, ROMANIA)**

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Conacu-Negresti Valley is located in the center of Cobadin Plateau, subunit of Negru Voda Plateau. Cobadin Plateau landscape consists predominantly of Cretaceous and Sarmatian limestone, placed on a Precambrian background and covered by a thick blanket of 40 m of Quaternary loess.

The valley is characterized by an extremely rich and diverse flora with rare or endemic species of wild flora of Dobrogea. Field research results in the period April - August 2009 led to the conclusion that the valley require conservation status.

## **MEADOW RESTORATION AND AGRI-ENVIRONMENT SCHEMES: THE BRITISH EXPERIENCE**

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Throughout Europe, semi-natural grasslands are a key component of high nature value farmland. Following the agricultural revolution of the 19<sup>th</sup> century, and especially during the intensification of farming from 1950 onward, the extent and diversity of grasslands declined in many European countries *e.g.* in England unimproved grasslands had been reduced to 3% of their 1938 extent by 1984. This precipitous reduction in biodiversity led to the targeting of species-rich grasslands within agri-environment schemes from the mid-1980s onward in the member states of the European Union.

In lowland UK, semi-natural meadows are a priority habitat for nature conservation, and also have multifunctional values including biodiversity, amenity and agronomy. British agri-environment schemes have sought to secure the nature conservation value sustainably within the context of viable farming. Ecological and agronomic research has focussed on developing practical management prescriptions for such schemes that can be adapted to the local conditions, but which are also amenable to monitoring, with measurable indicators of success.

This paper describes the development of agri-environment prescriptions for grassland management in the UK since the 1980s, especially in lowland England. The paper reviews the contribution of research to improving this policy instrument for nature conservation, drawing on plant ecological studies, eco-hydrological modelling, agronomic experiments and assessment of the socio-economic aspects, as well as investigations of the contribution that meadow grasslands make to regulation of the carbon budget. The paper will illustrate these themes using a programme of research conducted by the Centre for Ecology and Hydrology (CEH) on behalf of the UK government, and will conclude with some thoughts on the development of agri-environment schemes for grasslands in Romania.

## **NATURA 2000 IN THE CARPATHIANS – LESSONS LEARNT ...**

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The project “Optimization of the use of the resources of the Natura 2000 network for sustainable development in the Carpathians (2007-2011)” is realized within European Economic Area (EEA - Island, Liechtenstein, Norway) Financial Mechanism. The project is coordinated by INC PAS (Kraków) together with 13 local partners and Norwegian Institute of Nature Research (NINA).

The main project purpose is to involve local communities in the process of bringing Natura 2000 concepts into their real life and to implement a number of pilot active conservation, planning and education activities. The project goals are focused on the following questions: (1) where and how we want to maintain biodiversity in the Carpathians; (2) how to protect what shall be maintained and not to impede local development; (3) how to help local people to take advantage of the natural values of their mountains; and (4) how to improve the flow of information between stakeholders, scientists and local people.

The interim project experiences will be discussed, e.g.: data gathering systems, active conservation measures, cooperation with the authorities, stakeholder involvement and how to overcome obstacles at the local level. This project represents a good example and starting point for a wider discussion on the further development of the Natura 2000 network in Europe.



## **NATURE CONSERVATION ACTIVITY IN THE LATEST 50 YEARS IN THE INSTITUTE OF BIOLOGY BUCHAREST**

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The activity in the field of nature conservation from the Institute of Biology has been all the times in good correlation with the activity of the Romanian Academy's Commission for the Protection of Nature Monuments. Several researchers from the institute have been members in this Commission. Most of the results from the Institute's research activities became proposals for protected areas and some of them were transferred into legislation mainly Law no. 5/2000 - section III - protected areas, as well as several Governmental Decisions for new protected areas: H.G. no. 2151/2004; H.G. no. 1581/2005; H.G. no. 1143/2007 or H.G. no. 1284/2007 for protected area. Important natural protected areas were declared Sites of Community Interest (SCIs) as part of European network Nature 2000.

Based on the scientific documentation from the Institute of Biology and its partners (research institutes, universities, museums for natural sciences, botanical gardens, NGOs, etc.), it were proposed as "National Park" the following: Retezat, Piatra Craiului, Munții Măcinului, Domogled-Valea Cernei, Semenic-Cheile Carașului, Cheile Bicazului-Hâșmaș, and as "Natural Park": Bucegi, Comana, Balta Mică a Brăilei, Apuseni, Cozia, Poștile de Fier, Grădiștea Muncelului-Cioclovina, Vânători Neamț. The high quality of the research activity in this area conducted also to preparation of scientific documentation for the "Biosphere Reservation Delta Dunării". Researchers from the Institute of Biology are currently members in the Scientific Councils of the above mentioned Natural and National Parks.

The nature conservation activity in the latest 50 years was permanent developed in the Institute of Biology. The researchers studied a lot of natural reserves from the high mountain areas to the Danube Delta or Black Sea coast. An inventory of this scientific activity shows the presence of researches in protected areas from each of the 40 Romanian counties.

Also, the nature conservation activity was developed by researchers in other European countries, based on the research academic exchange between Romanian Academy and other relevant European academic institutions.

The same scientific studies was develop between Institute of Biology and European Commission were the researchers represents Romania in the scientific body of EC, as: Scientific Habitats Working Group and CITES scientific working group.

## **EX SITU CONSERVATION OF SOME THREATENED *DIANTHUS* TAXA FROM ROMANIAN NATURA 2000 SITES**

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Between 1996-2004, the Global Biodiversity registered a drastically decline, 8321 plant species being introduced in the Red List (IUCN, 2004).

Besides the measures concerning *in situ* conservation, the *ex situ* conservation strategies involving the use of classical cultivation methods combined with the modern alternatives as seeds banks and the establishment of *in vitro* collections, had a major contribution to preservation of a high number of endangered plant species throughout the world (Pritchard, 2004).

Despite the fact that *in situ* conservation field in Romania, strategies and measures concerning both threatened species and habitats has been already implemented, *ex situ* approaches are until now poor developed, only in a few Botanical Gardens being maintained as “living samples” and the number of taxa preserved is quite low.

In the present, in Romania, there are not centers focused on *ex situ* wild plant conservation researches and implementation, only some disparate studies were made by different researchers. Any integrated approach for *ex situ* conservation in the threatened plant species had not made yet. An interesting aspect is that many endemic plant species are preserved in the collection of European Botanical Garden but not in the Romanian ones.

In our project was involved a complex team with people with different areas of concern: botany, ecology, *in vitro* cultures, cryobiology, biochemistry and molecular biology.

The purpose was to initiate complex *ex situ* actions both classical (in the field) and modern ones involving the biotechnological means (*in vitro* tissues cultures, medium-term maintenance, cryostorage and also plant regeneration and acclimatization protocols).

The species studied were chosen taken into account the presence in the Romanian Red lists existed at the date of the beginning of the project and after the advice of several taxonomists.

The plant material in every taxon consisted in several individuals was collected with roots and some substrate from the natural habitats from at least two or three sites, to ensure variability for the *ex situ* preserved accessions.

For the establishing of living *ex situ* collections, in the Botanical Garden, Cluj-Napoca and Forest Research and Management Institute Simeria were created rock areas. The plant material was monitored all the time and the seeds produced collected. Generally, the plants have been adapted well to the new conditions. In the same time, samples for biochemical and molecular analyses were prelevated to compare with the *in vitro* and cryopreserved material.

The taxa taken into study were: *Dianthus nardiformis*, *Dianthus giganteus* ssp. *banaticus*, *Dianthus tenuifolius*, *Dianthus spiculifolius*, *Dianthus glacialis* ssp. *gelidus*, *Dianthus callizonus*, *Dianthus henteri*, *Dianthus pratensis* ssp. *racovitzae*, *Dianthus dobrogensis*.

Individuals from these taxa were cultivated in the Botanical Garden, Cluj-Napoca and Forest Research and Management Institute Simeria, *in vitro* tissues cultures were established in three centers (Institute of Biology, Bucharest, Forest Research and Management Institute Simeria, Biological Research Institute, Cluj-Napoca) and studies concerning the optimization of *in vitro* response, the extension of *in vitro* preservation during medium and long-term were made.

Active tissue collections are now maintained in at least two places (Institute of Biology Bucharest, Biological Research Institute, Cluj-Napoca) and medium-term cultures were established in the Institute of Biology, Bucharest.

Cryopreservation using meristem taken from *in vitro* regenerated plants was made in all taxa using the dehydration-vitrification procedure; regenerated plants after cryostorage were also analyzed.

Seeds have been collected from: *D. giganteus banaticus*, *D. nardiformis*, *D. callizonus*, *D. glacialis gelidus*, *D. tenuifolius* and *D. spiculifolius* and used for inducing the *in vitro* culture or kept at 4°C.

The plant material *ex situ* preserved through modern methods was evaluated to verify if the means used were or not favoured the variability.

All the plant introduced in living collections and *in vitro* collection can be offer for changes, used for different studies and also for the reintroduction in the natural habitats if this is necessary.

**ECOLOGY, TAXONOMY AND NATURE  
CONSERVATION**

**POSTERS**



## **LONG – TERM CHANGES OF ZOOPLANKTON ECOLOGY IN THE DANUBE DELTA**

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Danube Delta is the youngest and most dynamic territory of Romania. Being one of the largest wetlands in the world and also the largest compact area of reed on the planet, the delta is characterized by a high ecosystem diversity.

Due to its special ecological characteristics, it is the only delta in the world that joined the network of biosphere reserves. It was also involved in the "Man and Biosphere" program (MAB), launched by UNESCO and under the Ramsar Convention has been declared a wetland of international importance and was included in the World Cultural and Natural Heritage List. For almost a century, the aquatic ecosystems of the Danube Delta were subject of some research regarding the improvement of ecological conditions and exploitation of natural resources. However, in the last decades of the 20th century, the research of this area underwent a major developmental process.

The long-term research (1975-2010) carried out by the Laboratory of Aquatic Ecology of the Institute of Biology included, among other aspects, the zooplankton ecology in the most representative types of aquatic ecosystems of the Danube Delta (river branches, streams and canals, lakes and shallow lakes, very shallow lakes, marshes with reed beds and sea lagoons). In the frame of this research, new areas have been approached in zooplankton ecology: production, turnover, matter and energy transfer, energy flow, trophic structure and relationships, ecological succession, ecotone structure, eutrophication impact, restoration, resources and services offered to socio-economic systems.

The multiannual analysis of zooplankton in the Danube Delta reveals its high specific richness potential, illustrated by the presence of over 650 species. The largest species richness characterized shallow lakes, followed by very shallow lakes, river arms, streams, reed beds and lagoons.

An entirely different hierarchy was emphasized by the analysis of the multiannual average abundance, the first place belonging to reed bed areas, followed by shallow lakes, streams, very shallow lakes, lagoons and river branches; a similar sequence was revealed for the multiannual average biomass and daily production (Table 1).

After 1981, the extensive modifications of the trophic level of lacustrine ecosystems of the Danube Delta (from mesotrophy to

hypertrophy) under the action of the anthropogenic factors, lead to major structural changes of primary producers consisting in phytoplankton proliferation and disappearance of the submerged macrophytes.

In these conditions, the heterogeneity was significantly reduced, worsening the environmental conditions. Important changes occurred in the structure and trophic relationships that determined also changes in the ecosystem homeostasis.

Since 1992 there was a certain tendency of recovery, evidenced by the dynamics of the ecological parameters, but the process was slow and influenced, to a large extent, by the changes of the thermal and hydrological regimes.

Table 1 Ecological parameters of zooplankton in the main types of ecosystems of the Danube Delta

Parameters	Ecosystem types					
	Shallow lakes	Lakes of very small depth	River branches	Channels	Bogs with reed beds	Lagoons
Species richness (No. species)	546	308	254	242	228	220
Abundance (nr.ind./l)	550	388	299	532	954	352
Biomass ( $\mu\text{g w. w/l}$ )	3764	1786	1242	3512	4657	1060
Production ( $\mu\text{g w. w/l/24h}$ )	443	314		422	676	236

Following the changes of the abiotic environment, between 1975 and 1995 the species richness decreased by 38%, the abundance, biomass and productivity increased by 3.5 times, 5.3 times and respectively 4.5 times, while the turnover was reduced by 1.3 times.

The inverse correlation between the dynamics of the species richness on one hand and the dynamics of density, biomass and daily production on the other shows, according to the principle of Thienemann, the unfavourable influence of ecological changes for an important part of zooplankton species. Meanwhile, these changes represented a benefit for few species, able to proliferate in the new environmental conditions.

The trophic structure of the zooplankton reveals a complex organization in levels, types (micro-and macroconsumers) and trophic groups.

The relationships between the primary and secondary zooplankton consumers are based on the transfer of matter and energy. Among components of the same level, integrated into different types and groups, the access to food is achieved by specialization in capturing a narrow dimensional range of particles; competitive relationships occur between the components of the same groups.

These long-term research data could significantly contribute to an improved management of the rich biodiversity of this protected area.



## PHYTOPLANKTON ASSESSMENT IN DANUBE DELTA: HISTORY, CONSTRAINTS AND PERSPECTIVES

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The phytoplankton community forms the basis of food web in many ecosystems on Earth and is the trophodynamic module most sensitive to developmental responses under the action of natural and anthropogenic factors, including climate change. Consequently, the phytoplankton is the "champion" of trend evaluations at spatial-temporal scale. For these reasons, researchers are very interested to understand the behavior and elucidate the mechanisms underlying the functions of this trophic level.

The assemblage of phytoplankton is part of the structure of ecological systems that sustain themselves and produce a wide range of resources and services by absorbing the solar energy and by nutrient recycling.

In Romania, the study of algae has a relatively long history. First results on algal species in freshwater habitats in Transylvania were reported in mid-nineteenth century by F. Schur. Emanoil C. Teodorescu was the one who published the first comprehensive work in 1907-1908: "Matériaux pour la flore algologique de la Roumanie".

In 1956-1958, I. Tarnavski and M. Oltean published a new synthesis of the algal flora in Romania: „Materials for a summary of algae in Romania”(I-II), in which they listed 1502 freshwater and marine species.

First results on the composition of phytoplankton in the Danube Delta ecosystems were reported by Grimalski (1933, 1938), Leonte (1942) and Teodorescu (1943). Moruzzi (1960), describes even new species of algae, such as *Cymbella deltaica* and *Phacus longicauda* var. *maliuci*.

On the basis of the scientific references such as Caraus (1992), Török (1998, 1999), Porumb (2000), Caraus and Nicolescu (2006), a total of 1098 species have been identified in the Danube Delta, belonging to eight taxonomic groups. *Bacillariophyceae* and *Chlorophyceae* displayed the highest species richness, being represented by 337 species and 418, respectively.

The new qualitative stage in the study of the deltaic phytoplankton was inaugurated in 1975, since much of the research of aquatic ecosystems in the Danube Delta was based on a holistic concept (Botnariuc and Vădineanu, 1982 ).

Oltean (1985) established a threshold of algal bloom (5 mg s. um./l), which is extremely important in the ecological studies of the system. He

also published the first records of statistical approaches using biotic and abiotic factor relationships in Danube Delta. Other researchers from the Institute of Biology (N. Nicolescu, 1993, 2000, Eugenia Stoianovici Stancu, 2000, Renata Mușă, 2005) had important contributions to the study of phytoplankton in Danube Delta.

Mainly during the last decades of the 20<sup>th</sup> century, the Danube Delta has suffered from anthropogenic interventions that led to dramatic changes. Eutrophication was the main pressure that determined a rapid shift of the trophic state. The phytoplankton was the first trophic level which responded to this pressure by changing its ecological parameters, becoming the modulator for restructuring the entire food web.

The seasonal and annual dynamics of phytoplankton is strongly influenced by the hydrological regime and by the nutrient loading. The mean biomass of phytoplankton in the 1970s, before the eutrophication, was 4-7 mg d.w. l<sup>-1</sup>. During hypertrophy period (after 1980), higher biomass values were recorded (15-30 mg d.w. l<sup>-1</sup>), the energy input occurring mainly through phytoplankton communities. The species richness was reduced by 50%, while biomass and primary production increased dramatically. The dominance of *Chlorophyceae* and *Bacillariophyceae* was replaced with that of *Cyanobacteria* with poor nutritional value (Risnoveanu et. al., 2008).

The trends in the evolution of studies on phytoplankton communities were imposed by the gradual shift of both conceptual and developmental methods and techniques presented above. In Romania, the study of phytoplankton has developed, exceeding the level of autecology and sinecology, but gaps still persisted. A critical analysis of the phytoplankton research, according to the concepts of systems ecology, could help complete the current gaps in the ecological studies. In the future approaches it will be possible to assess the carrying capacity of ecosystems based on the study of phytoplankton dynamics in close relationship with the hydro-geomorphological unit, with the aim to avoid over-exploitation and to re-size the anthropogenic pressure.

## EVALUATION OF METABOLIC INTENSITY OF MICROBIAL COMMUNITIES FROM SF. GHEORGHE BRANCH USING ENZYMATIC ACTIVITIES

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Microorganisms are vital for the efficient functioning of any ecosystem due to their role in the organic matter decomposition and nutrient mineralization, representing also a food resource for higher trophic levels. A large variety of these microorganisms densely colonizes waterbodies and sediments and form microbial communities. Nowadays, the recording of enzymatic activities can be used as a method to assess the intensity of metabolic activity of the microbial communities.

This paper presents some characteristics of three enzymatic activities -  $\beta$ -glucosidase, phosphatase and amylase - of bacterioplankton and bacteriobenthos from Sf. Gheorghe branch, Danube Delta. The enzymological studies were performed during April 2008 - October 2009 period in six stations, aiming to assess the differences between the three types of investigated sectors: free-flowing sector, meanders section and the newly built channel.

The results have shown differences between the strength of the enzymatic activities in water and sediment: while in the water column the activity varied in a narrow range in the order  $\beta$ -glucosidase < amylase < phosphatase, in sediment the average values increased by almost 3 fold in the order amylase < phosphatase <<  $\beta$ -glucosidase, emphasizing the intense hydrolysis of glucose by the microbial benthic community.

The seasonal dynamics of amylase activity shows maximum values in summer of both years, while the annual dynamics emphasize higher values in 2009 compared to 2008, both in water and sediment. The annual averages reached 84.22 and respectively 54.72  $\mu\text{g}$  azure-B-chloride/ml/day in the water and 242.16 and 217  $\mu\text{g}$  azure-B-chloride/g w.w./day in the sediment. In both years, in the water and sediment of the investigated stretch, the maximum values were found in the newly built channel and the minimum were in the free flowing sector.

Both phosphatase and  $\beta$ -glucosidase activities recorded higher values in 2008 compared to 2009, but the seasonal dynamics did not show a regular pattern. The annual average values of phosphatase activity in the water column reached 103.41 and 65.46  $\mu\text{g}$  p-nitro-phenol/ml/day in 2008 and 2009 respectively, while in the sediment were found 344.53 and 215.65  $\mu\text{g}$  p-nitro-phenol/g w.w./day. The highest phosphatase activity in water and

sediment was emphasized for the meanders section, except in 2008, when the maximum activity in the water was found in the free flowing sector. The seasonal dynamics differ greatly from the other enzymatic activities: for the water, the highest activity was recorded in summer 2008 and spring 2009, while for the sediment, the maximum values were recorded in autumn 2008 and summer 2009.

The annual average values of  $\beta$ -glucosidase activity in the water column were 69.94 and 61.68  $\mu\text{g p-nitro-aniline/ml/day}$  in 2009 and 2008 respectively, while in sediment were reached 694.14 and 490.29  $\mu\text{g p-nitro-aniline/g w.w./day}$ . The maximum activity fluctuated in the three investigated sectors: for the water, the highest value in 2008 was recorded in the free flowing sector, while in 2009 the maximum was reached in the newly built channel; for the sediment, the highest activity was found in the meanders section in both years. Unlike the other enzymatic activities, which seasonal trend indicated usually the highest activity in summer,  $\beta$ -glucosidase reached maximum mostly in autumn, after the fall of vegetation; the only exception appeared in the benthic microbial activity in 2008, when the maximum was reached in spring.

Bacterial enzymatic activity depends on a multitude of abiotic and biological factors such as temperature, pH, oxygen content, phytoplankton blooms, macrophytes development, etc. that may influence the amount of available substrate as well as the hydrolysis kinetics. Future investigations will be focused on the correlations between the activity of bacterioplankton and bacteriobenthos communities and the dynamics of the other parameters.

## **ECOLOGICAL STUDIES IN BUCHAREST AS THE BASE FOR URBAN PLANNING**

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The giant urban regions represent great challenges for urban planners. For long time planners avoided emphasizing natural systems and ecologists avoided to study urban regions. Urban green areas are very important components of a city's infrastructure. Parks and gardens enrich the lives of citizens, by providing opportunities for resting and reducing stress, anxiety and representing buffers for air and water pollution and also offer places to see wildlife. The loss of natural land in favor of urban areas has resulted in the diminution of the natural habitat and biodiversity of species. Parks management regimes and the variety of urban habitats can affect the abundance and diversity of native species. Wildlife conservation in urban habitats is increasingly important due to current urbanization trends. The green areas from cities have been created mainly for the people. But these scattered patches represent a great habitat potential for wildlife. An effective planning strategy is needed to create corridors for linking these isolated patches for conserving the species that live together with humans in urban areas.

Bucharest is the capital and the largest city in Romania as area and population (2 million citizens, the urban area covering a 228 Km<sup>2</sup>). Air pollution in Bucharest has a complex character because of the multiple source types (traffic, thermal power plants, industry and extensive building construction), conditions and their spatial distribution.

In Bucharest the continuous rate of urban development is leading to an increasing loss of biodiversity. If we cannot recreate the lost natural ecosystems, at least we should try to make urban habitats more suitable for a range of species. By storing chemicals in their bodies, plants (trees, shrubs, herbaceous species) help us, human beings, by cleaning our air. Even if we cannot see the invertebrates beneath our feet in the soil, or hidden in the vegetation, they are there and play their vital role in nature. As long as we keep the vegetation in smaller or bigger (preferably complex) green spaces in the city, these living organisms will live their lives and bring a huge contribution to ours.

In all cities of the planet, air pollution is the major environmental problem due to the industrial plants, power plants, domestic heating and

especially motor traffic. Increased concentrations of air pollutants and the variety of pollutants have negative effects on the increasing population density in the relatively small area of a city. Air pollution harms not only human beings but also plants and animals living together in the city.

The ecological studies have been developed in a pilot area (Cișmigiu, Unirii and Izvor Parks) from the central region of Bucharest; Băneasa and Balotești forests as reference sites, in an urban-rural gradient according to the dilution of the concentration of pollutants.

Based on the shortage of distributional data for plants and animals available for Bucharest, the first step has been to determine the existence of potential bio-indicators. The methodology consisted in visits of the sites, comprising transects using both qualitative and quantitative sampling, methods for estimating species composition, which characterise the presence and occurrence of certain species, combined with biochemical methods. The sampling points have been localised using GPS, and positioned to include samples from the marginal zone of the green space (so as to detect any impact of major roads) and from the core zone of the green space where such impacts should be less pronounced. Chemical analysis of plant and insects samples have been performed in a specialised laboratory and correlated with pollution data supplied by the partners.

The biological material has been conserved, determined and prepared, using specific techniques, for chemical analysis in the laboratories of the Institute of Biology. The resulting species list was used for identifying the main bio-indicator species. The biology team for this project covers a wide range of taxonomic groups.

Passive bioindicator methods have been used, as well as accumulation and reaction indicators. The results allow an overview of general air pollution, manifested by effects upon naturally occurring (and where appropriate cultivated) plants and animals, as well as an overview of regional pollutant patterns, indexed by accumulation in selected species and groups of species.

The samples prepared for chemical analysis have been analysed by an external but well-authenticated specialist laboratory (Toxicology-Chemistry Laboratory, Animal Diagnostic and Health Institute).

Sample mineralization has been performed with a Perkin Elmer model Anton Paar microwave.

Heavy metals (Pb, Cd, Cu, Zn) from soil, plant, lichens, fungi and invertebrate samples have been analysed with Perkin Elmer AAnalyst 800 Atomic Absorption Spectrophotometer incorporating all spectrometer and atomizer components using graphite furnace or flame techniques.

This multidisciplinary and transdisciplinary approach of the air pollution in Bucharest is done for the first time in Romania, having a huge

scientific and practical importance, bringing an important contribution at the European level as well.

The multidisciplinary approach of the studies leads to the awareness about the importance of maintenance of already existent green spaces, increment of their structural complexity and establishment of new ones, the development of methods and ideas, for helping the municipality in planning and realising environmental management.

## **BREBU CLIFFS – BASIC RESEARCH AND POSSIBILITY OF PRACTICAL APPROACHES**

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Only a few cliff researches exist around the world because these were considered for a long time worthless of investigation, lacking life and being hostile. The scarce list of recent publications dealing with this topic specifies that cliffs represent primary habitats for many plant species being among the least disturbed habitats on the Earth and contributing to the biodiversity of a region. Cliffs are unique environments that can support a specialized group of plants, many endemic or rare species and serve as a refuge for native flora and fauna and also are core habitat for the wide variety of birds and other wildlife that are particularly sensitive to disturbance. It is not possible to estimate the full extent of biodiversity in different regions without including in the studies these vertical habitats. Usually, only some ecological studies have been performed on cliffs because they are not viewed as places with their own ecological structure and processes.

The majority of cliff studies in Romania are reduced to general description of the flora and vegetation or are focusing on particular organisms or specific aspects of cliff habitats: distribution and conservation of some saxicolous plant species or phytocoenologic aspects.

Brebu Cliffs (Brebului Gorges) are located in the hilly area of Southern Romanian Subcarpathians, in the Doftana Valley, near to Lunca Mare village (N: 45° 12' 31,1"; E: 25° 44' 23,5") at 535 m above sea level. Brebului Gorges are massif vertical cliffs cut in Inferior Miocene conglomerate.

The rock structure has a small and medium resistance to erosion and is characterized by the dominance of conglomerate intercalate with compact sandstone sandy and clay rocks, quartzite, micaschists, amphibolites, gneisses. Rock fall occurs often because of the weakness of the conglomerate that forms the escarpment. The rates of rockfall and rock particle size have a strong influence over the organisms that occur on the cliffs.

Chemical weathering of the rock is directly controlled by precipitation amount and chemistry, rock temperature and geochemistry. The factors influencing the cliffs are: rock type and strength, climate and the processes of physical and chemical weathering (freeze-thaw activity)



Ecosystems' structure or ecological researches in the catchment area of the Doftana River are only a few, regarding the oak forest (Oromulu et al. 2008), the shrublands from Lunca Mare (Pauca-Comanescu et al. 2008) and the beech forests from Doftana Valley (Pauca-Comănescu et al. 2009).

Our pioneer study on Brebu cliffs was developed in the period 2008-2010.

The main objective of the study is to quantitatively sample the plant communities associated with different cliff expositions, structures and micro-climate.

Study sites are located on vertical cliffs with southern and northern expositions and also one situated in a kind of a chimney (more humid than the other two study sites). On transects set on every cliff we established fix monitoring plots at every 1.5 m till 9 m height. Vertical cliffs are difficult to sample and require special attention for safety. Plant species recordings were performed monthly from May to August on 0.25 m<sup>2</sup> plots. Establishing the exact number of individuals is difficult for clonal plants. In order to analyze the micro-climatic factors acting on the cliffs, we installed data-loggers in all three sites. For mezzo-scale characterization of the climatic factors we used data provided by Câmpina Meteorological Agency.

Multivariate statistics (PCA and DCA), CANOCO program, was employed for data base analysis.

The abundance and distribution of the plant species on the surface of the cliffs is influenced by geological and geomorphological characteristics of the cliffs. All these factors act synergetically with the heterogeneity of cliff surface, the degree of solid rock disintegration and climatic factors.

Cliffs support plant communities amenable to physiological constraints due to limited soil availability, micro-climatic extremes and water stress, and provide unique environment for plant species adapted to these conditions.

Fundamental researches of cliffs might be brought to practice.

In the recent decades, due to climbing development as sport and recreation, more studies are focused on effect/disturbance of rock climbing on vegetation, for conservation of plants and animal species living in this unique habitat and management strategies.

As the stone is extracted from quarry, the rehabilitation of stone quarry slopes should resemble the natural impact as much as possible, the final landform created must be visually, ecologically and structurally acceptable and blend with the surrounding landscape.

Green roofs habitats are characterized by shallow substrate and extreme soil-moisture conditions. This set of characteristics or "habitat template" has natural analogue in rock barren ecosystems such as cliffs, scree slopes and limestone pavement. Cliff's researches have implications of using natural ecosystem as templates for green roofs design.

## PLANT COMMUNITIES WITH *PINUS MUGO* IN THE ROMANIAN CARPATHIANS

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Dwarf pine (*Pinus mugo* s. str.) is a shrub that reaches the optimum of its distribution above the timberline in the Eastern and South-Eastern Alps, the Northern and Central Dinarides, the high mountains of the Balkan Peninsula (the Rhodopes, Rila Mts, Pirin Mts) and the Carpathians. Smaller, isolated populations occur in the Jura Mts, Vosges Mts, Šumava Mts, Jizerské hory Mts and Krkonoše Mts; the most southerly isolated occurrence is in the Abruzzo Mts in the Apennines. In most of these mountains, the dwarf pine shrubs form a coherent, climatically conditioned vegetation belt, mostly known as the subalpine belt. Less often, *Pinus mugo* s. str. occurs on hygrophilous stands on peaty soils in lower altitudes (montane and lower subalpine belt) as azonal vegetation type.

According to altitudinal, edaphic, moisture or geographical gradients the zonal *Pinus mugo*-communities can be divided into four general ecological types: a) dry, rocky type on basiphilous bedrock; b) moist type on nutrient-rich soils on basiphilous, as well as silicate bedrock; c) acidophilous, oligotrophic, species-poor type; d) oligotrophic, windswept type at the transition between the subalpine and alpine belt on silicate bedrock. Based on the occurrence of individual floristic elements, geographical variability was classified either at the association or subassociation level. In the European area, 17 associations are distinguished; six of them occur in the Carpathians. Floristic characteristics, site conditions, distribution and nomenclature are described in detail by Šibík et al. (2008: Phytocoenol. 38: 221-238) and Šibík et al. (2010: Phytocoenol. 40).

In the Romanian part of the Carpathians, several units occur. The *Seslerio haynaldianae-Pinetum mugo* Šibík et al. 2010 presents a floristically medium species-rich plant community found on steep rocky cliffs and screes of carbonate high mountains in the Eastern and Southern Carpathians in the alti-montane to lower subalpine belt. The plant communities of the *Adenostylo alliariae-Pinetum mugo* (Sillinger 1933) Šoltésová 1974 are well developed on the sites with favourable edaphic conditions (with sufficient nutrition supply during all the vegetation period) and with good decomposition of humus. Two subassociations can be found in Romania: *Adenostylo alliariae-Pinetum mugo rumicetosum alpestris*

(Pawłowski et Walas 1949) comb. Šibík et al. 2010 and the *Adenostylo alliariae-Pinetum mugo daronicetosum carpaticae* (Coldea 1991) comb. Šibík et al. 2010. Into the first subassociation we include species-richer dwarf pine shrubs of the Eastern and Southern Carpathians, mainly on silicious bedrock, with the occurrence of several moisture-loving and nitrophilous species. In comparison with the corresponding West-Carpathian subassociation *Aa-Pm athyrietosum distentifolii* (Hadač 1956) Šibík et al. 2010, this is differentiated by the occurrence of several taxa, which are absent in the West-Carpathian phytocoenoses. The second one, the *Adenostylo alliariae-Pinetum mugo daronicetosum carpaticae* (Coldea 1991) comb. Šibík et al. 2010 represents moisture-loving phytocoenoses, which occupy limestone and dolomite screes below the rocky cliffs. In comparison with the corresponding West-Carpathian subassociation – *Aa-Pm cortusetosum matthiolii* Šibík et al. 2010, this is positively differentiated mostly by the occurrence of (sub)species such as *Saxifraga cuneifolia*, *Doronicum carpaticum*, *Soldanella \*major*, *Rhododendron myrtifolium* etc.

The communities of the *Dryopterido dilatatae-Pinetum mugo sphagnetosum nemorei* (Unar in Unar et al. 1985) Šibík et al. 2010 occupy mainly north- and west-facing moister habitats, often formed by stabilized quartzitic and granite boulder screes. The undergrowth is characteristically dominated by bog mosses. These phytocoenoses are well developed in the Gorgany Mts, or in the north of Romania (Maramureş Mts, more rarely the Rodna Mts).

The *Rhododendro myrtifolii-Pinetum mugo* Coldea 1991 is represented by species-poor dwarf pine communities occurring in the subalpine belt of the Eastern and Southern Carpathians. In the Ukrainian and North-Romanian part of the Carpathians, the distributional area of these communities meets that of the corresponding association *Dryopterido dilatatae-Pinetum mugo* Unar in Unar et al. 1985; the phytocoenoses of both associations can form mutual transitions.

The last syntaxon of zonal dwarf pine stands in Romania is the *Cetrario islandicae-Pinetum mugo rhododendretosum myrtifolii* Šibík et al. 2010. Being the correspondent of *Cetrario islandicae-Pinetum mugo typicum* Šibík et al. 2010, this subassociation is distributed in the Southern and Eastern Carpathians. It is differentiated by the occurrence of the Dacian-East Carpathian floristic element *Rhododendron myrtifolium*, or other taxa, which do not occur in the Western Carpathians.

Taking into account the obtained knowledge, the limited vertical distribution (the subalpine belt) of studied phytocoenoses, similar physiognomy, and mutual close syngenetic relationships between individual dwarf pine associations, we confirm their current classification within one alliance *Pinion mugo* Pawłowski in Pawłowski et al. 1928, order *Junipero-*

*Pinetalia mugo* Boşcaiu 1971 and one class *Roso pendulinae-Pinetea mugo* Theurillat in Theurillat et al. 1995.

The azonal vegetation type represents only one type similar for the whole area of the European mountains – *Sphagno magellanici-Pinetum mugo* Hadač, Ježek et Březina 1969 nom. cons. propos (class *Vaccinio uliginosi-Pinetea sylvestris* Passarge 1968) – slightly woody raised bogs and their margins in the (montane) subalpine areas of the European mountains.

## **ECOLOGICAL RESTORATION VERSUS RENATURATION OF TECHNOGENIC AREAS. TIME SCALE MEANING**

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Among the many forms in which industrial activities affect the environment, at terrestrial level, an important category is represented by the waste dumps and tailings ponds. They are accumulations of sterile material from various construction activities and / or residues of or extraction stage processes of various types. While their accumulation in terrestrial natural baseline is practically an interposition in the physical continuity of the natural structures and successional processes, and not only, they represent a pollution source at terrestrial and underground level for medium and long term. Aspects of abiotic and biotic parameters which define dumps (four areas in Retezat Massif) and ponds (two areas from Moldova Noua and two from Ampoiului Valley - Zlatna) were investigated over time (monitorized). Integrated analysis of these elements provides the image of structural differences existing in relation to adjacent natural areas and the rates of succesional processes when ecological restoration strategies are applied on the technogene areas or is about simply natural recovery processes. Informations obtained are important elements of knowledge for ecologists and environmental managers in future environmental and industrial activities in national strategies for sustainable development.

## THE KNOWLEDGE AND MANAGEMENT OF THE BIOLOGICAL DIVERSITY OF THE ROMANIAN FORESTS

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The research program entitled “The Knowledge and Management of the Biological Diversity of Romania” had been unrolled in the Eastern Romanian Plain by the Ecology, Taxonomy and Nature Conservation Department in two projects: in 2002-2004 it targeted the biodiversity knowledge in altitudinal gradient of different ecosystems, while in 2005-2007 the biodiversity of the hilly shrubs and *Quercus* forests of the Muntenia Region. This complex research program was carried on in the different forest types as shown below:

- 2002 - Turkey oak forests (Măgura, Prundu, Ogarca, Teșila localities);
- 2003 - ash forests (Călugăreni, Singureni, Frasinu, Spătaru localities);
- 2004 - alder forests (Călugăreni, Clinceanca, Cumpătu, Azuga localities);
- 2005 - sessile oak forests from the Southern Subcarpathians (Căscioarele, Călugăreni, Cobia, Sărata-Monteoru localities);
- 2006 - durmast and durmast-beech forests (Valea Doftanei);
- 2007 - beech forests (Lunca Mare, Șotriile localities, on Valea Prahovei).

In this program many researchers were involved, few of them retired meanwhile but their contribution is valuable (A. POPESCU, M. FALCĂ, LILIANA VASILIU-OROMULU, V. SANDA, MIHAELA PAUCĂ-COMĂNESCU, VIORICA HONCIUC) and others had left our institution (ANDREEA TATOLE, CRISTINA MUNTEANU, B. MATEI, I. CODRICI).

The methods used to investigate the flora, vegetation and invertebrate fauna from the canopy, herbaceous layer, epigeion and edaphon are mentioned below.

In each studied site the vegetal association (and their biotopes: altitude, soils (pH)) were described (A. POPESCU, V. SANDA).

For the herbaceous synusia the seasonal dynamics of the accumulation of the herbaceous biomass during the summer season (g/m<sup>2</sup>) were determined. The indices of frequency, density and biomass of the herbaceous species were determined on sample areas of 50/50 cm, in 100

replicates. The seasonal dynamics of the herbaceous biomass of the studied areas in the vernal, aestival and autumn seasons - the fresh biomass (g/m<sup>2</sup>) and dry biomass (g/m<sup>2</sup>) respectively was also followed (CLAUDIA BIȚĂ-NICOLAE).

The tree layer was surveyed by species and by age category on circular 500 m<sup>2</sup> areas. Tree diameter was measured indirectly, by calculation, from the length of the circumference, which was determined with a ruler. We used this method in order to obtain average diameters, strongly influenced by the irregular radial growth of the trunk, due to the microrelief- biometric and structure characteristics of the tree layer: average density, tree diameter (cm), tree height (m), tree volume (m<sup>3</sup>), total volume (MIHAELA PAUCĂ-COMĂNESCU, MARILENA ONETE).

The **Bryophyta** species were collected on 50 cm x 50 cm sample areas from May to October and the species were identified (S. ȘTEFĂNUȚ).

The fungal sporocarps (**Macromycetes**) were collected on 50 m x 50 m sample areas from May to October. For the corticolous species, all the sporocarps growing on the same substrate were considered as belonging to a single individual. Because of the growth and replication particularities, further studies are required to establish a convention concerning the number of individuals of the terricolous species. (I. CODRICI, ELENA-DANIELA MOGÂLDEA).

The invertebrate fauna of the canopy and grass layer was collected with the entomologic net of 60 and 30 cm Ø, in 10 samples each; the mobile fauna was captured with Barber traps, 9 samples each; the edaphic fauna, nematode, enchytreids, collembola and acari, was captured with the MacFadyen probe, 10 samples for each groups of organisms; the lumbricides were collected from 10, 25x25 cm areas. The samples were extracted by methods specific to each group. The biomass was calculated on dry weight basis, either with the calculated coefficients mentioned by the literature, or by weighing the individuals of the different species.

In the vertical profile, there is a characteristic structure of the consumers: canopy, grass layer, soil surface and soil, the specificity of each level being determined by the degree of coenotic development. One consequence is the micromosaic structure in the horizontal plane, the fauna being attached to the particular layer where it finds optimal conditions. The surveyed forests have in joint ecological niches species that have quite different requirements (stenobiont, eurybiont, xerophyllous, mesophyllous), due to the particular micro-climatic conditions.

We studied the average numerical density/sq. m. and the biomass density/sq. m. of the invertebrate fauna in relationship with the vertical distribution of the vegetation: **Thysanoptera** (LILIANA VASILIU-OROMULU), **Coleoptera -Chrysomelidae** and **Curculionidae** families (SANDA MAICAN, CRISTINA MUNTEANU), **Carabidae** (DORINA

PURICE), **Araneae** (ANDREEA TATOLE), **Chilopoda** (MIHAELA ION), **Nematoda**, **Enchytraeidae** (LILIANA VASILIU-OROMULU), **Lumbricidae** (M. FALCĂ), **Oribatidae** (VIORICA HONCIUC), **Gamasida** (MINODORA MANU)

The problem of determining the biomass of the animals from the forest ecosystems is much more demanding since the habitats have more animal species than plant species. The difficulty consists in determining the animal species because they are quite mobile and exposed to extreme fluctuations of their numbers throughout the year and from one year to the next.

Certain animals, of considerable ecological signification for the dynamics of the forest ecosystems are present in just some periods of the year. The insufficient data do not allow us to make a picture of the fauna production, but one thing is certain: the total animal biomass is much lower than the total plant biomass. The animal biomass provides insufficient data on the activity and function of the organisms within the ecosystem. The determination of the higher ranking taxa can only be done according to the number of specialists, but in the case of the surveyed ecosystems, groups, conclusive species, characteristic to the vertical structure of the forests have been identified.

The changes in the weight of the zoocoenosis from the surveyed forestry ecosystems appear both throughout the year and on a longer term. Within a quite stable ecosystem, there is a balance between production and decomposition.



## FLORA AND VEGETATION OF BABEȘ MOUNTAIN FROM CIUCAȘ MASSIF

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Ciucăș Massif is part of the Natura 2000 European Ecological Network. It is situated in the alpine biogeographical region, at the intersection of 45°30'26'' with 25°58'37'', in the sector of Round Carpathians from Oriental Carpathians ecoregion, between 795 and 1.954 m altitude.

It is situated between Pasul Bratocea (1267m alt.) and Tabla Buții (1379 m alt.) and is included in a relictar conglomerate region. The Ciucăș Massif, with the highest peak Ciucăș (1954m), is formed of two groups: Ciucăș and Culmea Stâncoasă with 5 subtypes (Ciucăș-Tigăi-Zăganu; Bratocea-Gropșoara-Culmea stâncoasă; Muntele Roșu; Chirușca; Colții Natrii-Dobromir).

The Babeș Mountain is included in Bratocea – Gropșoara – Culmea Stâncoasă subtype.

From the geomorphological point of view, this subtype has a knoll relief and weak fragmented surfaces with eastern inclination and a very abrupt slope on the western side.

The conglomerates of this subtype have rocks with high toughness.

The increased diversity of the environmental factors from Ciucăș Massif and from Babeș Mountain are influencing the richness of flora and the development of a characteristically montane and submontane vegetation.

The cormophyte flora from Babeș Mountain comprises about 210 species. Most of the identified taxons are common for the montane flora, but also species with a high scientific importance. From this last category 19 carpathian endemic species, five carpatho-balcanic elements and nine rare species were identified.

The vegetation from this mountain is grouped in several phytocenological categories, in correlation with altitude, relief and the morphology of the soil. In the investigated area 22 associations were identified, with the following herbaceous phytocenoses: *Campanulo (carpaticae) - Saxifagetum cuneifoliae* (Zolyomi 1939) Sanda et Popescu 1977; *Scorzonero roseae - Festucetum nigricantis* (Pușcaru et al. 1956) Coldea 1978; *Violo declinatae - Nardetum* Simon 1966; *Scirpetum sylvatici* Ralski 1931; *Potentilletum anserinae* Felföldy 1942; *Ranunculetum repentis* Knapp ex. Oberd. 1957; *Junco inflexi - Menthetum longifoliae* Lohmayer

1953; *Chaerophylletum hirsuti* (Soó 1927) Krajina 1933; *Phleooalpini - Deschampsietum caespitosae* (Morariu 1939) Coldea 1983; *Festuco rubrae - Agrostetum capillarae* Horvatič 1951; *Trifolio - Lolietum perennis* Krippelova 1967; *Urtico - Aegopodietum* Tx 1963; *Telekio - Petasitetum hybridi* (Morariu 1967) Resm. et Rațiu 1974; *Digitalo - Calamagrostietum arundinaceae* Oberd. 1957.

The forest vegetation includes the associations: *Fragario - Rubetum* (Pfeiffer 1936) Siss. 1946; *Pulmonario rubrae - Fagetum* (Soó 1964) Täuber 1987; *Hieracio transsilvanico - Fagetum* (Vida 1963) Täuber 1987; *Leucanthemo waldsteini - Fagetum* (Soó 1964) Täuber 1987; *Leucanthemo waldsteini - Piceetum* Krajina 1933; *Hieracio transsilvanico - Abietetum* (Borhidi 1971) Coldea 1991.

On the superior limit of the Babeș Mountain the following associations are developing at high altitudes: *Campanulo abietinae - Juniperetum* Simon 1966; *Campanulo abietinae - Vaccinietum* (Buia et al. 1962) Boșcaiu 1971.

## NEW PHYTOCOENOSIS REPORTED IN MACIN MOUNTAINS NATIONAL PARK

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The Macin Mountains are part of the old Hercynic mountain chain (G. Murgoci 1914) whose height was reduced due to a long process of erosion to 100-450 m; the maximum height is reached by the Tutuiatu (Greci) peak 467m.

In climatic and biogeographic terms, the Macin Mountains are located in the extreme western steppe zone, which includes southern Moldova, eastern Muntenia (Baragan) and Dobrogea.

As a result of its geographical position, the studied territory has a steppe climate characterized by 11 to 11.5 degrees Celsius average annual temperature and low rainfall of only 350-400 mm per year.

Macin Mountains, as generally all Dobrogea, is a very interesting area for botanical research, with a very rich and varied flora and diverse forest vegetation, xerofil grasslands.

The area has been extensively studied by many botanists: I. Prodan (1924), C.C. Georgescu (1928), Al. Borza (1931), G. Dihoru (1962), M. Andrei and A. Popescu (1967), recently Constantina Dinu (1987, 1990), M. Petrescu (2000-2004) and others. However, there are still some lesser known areas in terms of flora, especially regarding phytocoenology.

Our research, together with the previously published ones, highlight the great diversity of phytocoenoses from the Macin Mountains National Park.

130 associations were identified, of which 113 grass phytocenoses, five shrub and 12 forest phytocoenoses.

Of the total 130 phytocoenosis reported, 13 are newly described associations and they are characteristic for different vegetation types as follows:

Saxicolous associations:

1. *Koelerio lobatae* – *Sempervivetum ruthenicae* (zelebori) Popescu et Doniță ass. nova
2. *Moehringio grisebachii* – *Alysetum saxatillae* Popescu et Doniță ass. nova
3. *Diantho nardiformis* – *Campanuletum romanicae* Popescu et Doniță ass. nova

Halophyllous associations

4. *Orlayo grandiflorae* – *Cleistogenetum serotinae* Popescu et Doniță ass.  
nova
5. *Achilleo coarctatae* – *Pöetum versicoloris* Popescu et Doniță ass. nova
6. *Ajugo laxmanni* – *Caricetum intermedi* Popescu et Doniță ass. nova
7. *Minuartio adenotrichae* – *Alysetum muralae* Popescu et Doniță ass.  
nova
8. *Hordeetum jubati* Popescu et Doniță ass. nova  
Forest associations
9. *Doronico orientaliae* - *Fagetum tauricae* Doniță et Popescu ass. nova
10. *Carpino betuli* – *Quercetum robori* – *pedunculiflorae* Doniță et  
Popescu ass. nova  
Shrub associations
11. *Allio taurici* - *Spiraeetum crenatae* Popescu et Doniță ass. nova
12. *Rhamno catharticae* – *Prunetum dasyphyllae* Popescu et Doniță ass.  
nova
13. *Fragario viridis* – *Rubetum canescentis* Popescu et Doniță ass. nova

In this paper saxicolous associations and xerophytic grasslands are described and analyzed.

## CARACTERISATION OF FLORA AND VEGETATION FROM THE “CHEILE CHEII” GORGES (BUILA-VÂNTURARIȚA NATIONAL PARK, ROMANIA)

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The vascular flora of “Cheile Cheii” Gorges (Buila-Vânturarița National Park) is presented according to the provisions of the International Code of Nomenclature. For each taxon the following data are presented: valid scientific denomination, possible synonyms, biological form, floristic element, ecological characterization, dissemination in the massif and by altitude, coenotical affiliation.

The outline of “Cheile Cheii” Gorges flora includes 167 vascular taxa. The flora of the investigated area is reckoned to account for about 30% of the flora of Buila-Vânturarița National Park.

Considering the floristic outline, the high number of taxa belong to plant families as follows: Asteraceae, Poaceae, Caryophyllaceae, Ranunculaceae, Lamiaceae, Cyperaceae, and other families represented by a smaller number of species. It is very important to mention the conifereous (Gymnospermae) species which are present in the area, as: *Abies alba*, *Picea abies*, *Pinus sylvestris*, *Juniperus communis*, *Juniperus sabina*, and especially *Taxus baccata*. This floristic composition is holding the highest proportion and forms the basic fund of flora of the investigated area.

Vegetation was studied according to the method of the Central-European phytosociological school established by Braun-Blanquet and adapted to the conditions of our country's territory by A. Borza. In the area investigated by us in “Cheile Cheii” Gorges, I have identified a number of 18 plant associations. A very important plant subassociation is represented by *Pulmonario rubrae-Fagetum taxetosseum baccatae* which forms the core of the natural reserve in this area.

The areal and geographical analysis of “Cheile Cheii” Gorges revealed several characteristic aspects:

- the large proportion of Eurasian, European, Central-European elements, showing the affiliation to the Central-European area;
- a high proportion of Alps, Carpathian, Carpathian - Balkan species, showing the mountainous character of the vegetation;
- the preservative character of the flora accounted for by the large number of endemic species;
- a significant proportion of arctic-alpine species, ranking among the mountainous area, with some representative subalpine elements;

- lower influence of the southern and oriental elements on the local flora.

Management measures necessary for the protection of the flora and vegetation of “Cheile Cheii” Gorges:

- avoiding the opening of the proposed unsustainable new forest exploitation,

- controlling the occasional illegal rock exploitation by the locals along the main road access in the area of the reserve.

## **ECTOMYCORRHIZAL FUNGI IN ROMANIA: PRESENT KNOWLEGE AND NEW PERSPECTIVE**

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Ectomycorrhizal fungi (ECM) have a key role in the functioning of ecosystems, especially in biogeochemical cycling and ecosystem stability.

In Romania, most of the knowledge concerning specific diversity of ECM fungi is resulting from mycological studies of local mycoflora. In order to identify ectomycorrhizal mycobionts, the main method used was to count the fungal fruiting bodies of known ECM fungal species and, in lesser amount, morphotyping and molecular analysis. For this reason, the species diversity of underground ectomycorrhizal fungi is known in very small extent. Most of the studies concerning the physiology of this type of fungi have been done in laboratory conditions or in experimental fields.

In this paper the studies of ectomycorrhizal fungi in Romania are reviewed. A preliminary list for 60 genera of fungi was compiled, present in Romania and known or assumed to be ectomycorrhizal, using a variety of published sources and mycological herbaria. Also, patterns of ECM distribution in relationship with different biotic and abiotic variables are described. Finally, some new directions for future research concerning ectomycorrhizal fungi are presented.

# **REGIONAL PRELIMINARY STUDY REGARDING THE IMPACT OF LIVESTOCK AND FARMING ACTIVITIES ON LICHEN FLORA WITHIN OLD GROWTH AND YOUNG GROWTH STAND FROM BUCHAREST MUNICIPALITY METROPOLITAN AREA**

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The main objective of this study is to map the gradient composition of epiphytic lichen communities, closely related to environmental quality. On the basis of field data, a positive correlation between livestock, crop farming operations, developed near old growth and young growth stands, and the composition of epiphytic lichen communities has been observed.

From geographical point of view, the studied area stretches along a distance gradient towards the eastern part of Bucharest Municipality, including three stands, namely Pustnicu, Goștilele and Călăreților.

Data regarding lichen species composition in a close relation to environmental quality changes within the study area are investigated and reported for the first time in the Romanian lichenological literature.

The use of lichen species as indicators of eutrophication conditions has focused on the assessment of nitrophyte and acidophyte lichen communities. According to field data, the prevailing of the two main types of lichen communities, Xanthorion and Parmelion communities, was revealed. The Xanthorion community is closely related especially to second-growth stands, for instance Goștilele stand. In a strong contrast with Xanthorion communities, lichen species belonging to Parmelion communities recorded a weak occurrence especially on the trunk of secular oaks within old-growth stands. The results indicate along the distance gradient from Pustnicu Forest to Călăreților Forest a slightly decreasing number of nitrophilous lichen species from 67 % to 56 % simultaneously with the slightly increasing number of acidophilous lichen species from 33 % to 44 %, respectively.

The prevailing of nitrophytic lichens species is positively correlated to the deposition of concentrated animal waste from livestock enclosures near the outskirts of the stand and also with intensive agricultural activities. The occurrence of the lowest number of acidophilous lichen species, threatened therefore with disappearance, is rather positively correlated with the remnant oaks from the old-growth stand and not with the improvement of environmental quality as a function of the distance from polluted industrial and urban areas. The presence of the acidophilous lichen species in the rural areas might indicate that the effect of alkaline pollution indicated by the



abundance of nitrophilous species within sampling units is not entirely distributed on the investigated tree trunks.

During the last two centuries the most old-growth stands from Bucharest Municipality metropolitan area have been converted to second-growth stands. According to field data it was observed how young-growth stands have limited the area of old-growth stands. It is well known that the secular trees are important sources of lichen propagules to young trees. Thus, the lowest number of acidophilous lichens species within sampling units might be correlated to a negative relation between eutrophication stand condition caused by rural activities and a dispersal limitation of lichen propagules from heterogeneous old-growth stands to homogeneous young growth stand.

The age of the stand correlated with the rural activities such as livestock and farming operations is important in determining the spatial distribution of lichen species at regional level.

## THE LIVERWORT RESEARCH IN THE INSTITUTE OF BIOLOGY BUCHAREST

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The liverwort research in Romania has started in the first half of the 19th century when the first papers were published. Although the first paper regarding bryophytes was published posthumously in 1846, J.C.G. Baumgarten had already conducted research in the first two decades of the 19th century.

The bryophyte researches in the Institute of Biology - Bucharest, Romanian Academy, were carried out by G. Dihoru, T. Ștefureac, I. Peicea and S. Ștefănuț. The bryophyte surveys were conducted in the Siriu Mountain (Dihoru 1975), Malul Alb (Dihoru 1983), Bâlbâitoarea Peatbog (Dihoru 1984), Penteleu Mountain (Dihoru 1987), Cozia Mountain (Dihoru 1990), Dobrudja (Dihoru 1997), Rodna Mountains (Ștefănuț 2000, 2010), Făgăraș Mountains (Ștefănuț 1998-2010), Zamoștea Reserve (Dihoru 2002), Cerna Valley (Dihoru & Răduțoiu 2002, 2004), Piatra Craiului Mountains (Dihoru, Ștefănuț, Wallfish & Pop 2003, Dihoru & Pop 2006, Ștefănuț & Pop 2006), Lacul Tătarilor Peatbog (Ștefănuț 2003, 2004), Dâmbovița Gorges (Ștefănuț 2004, 2006), Bucegi Mountains (Ștefănuț 2004, 2007, 2008), Motru Valley (Dihoru & Costache 2004), Tâmpa Mountain, Giumalău Mountain, Rarău Mountain, Căliman Mountains (Ștefănuț 2006-2008) and Retezat Mountains (Ștefănuț 2009, 2010).

Following these efforts many liverwort species were reported as new to Romania: *Scapania paludosa* (Dihoru 1975), *Haplomitrium hookeri* (Ștefănuț 2000), *Palavicinia lyellii* (Ștefănuț 2003), *Lophozia opacifolia* (Ștefănuț 2004), *Marsupella commutata* (Ștefănuț 2004), *Frullania parvistipula* (Ștefănuț 2004), *Scapania gymnostomophila* (and *Marsupella condensata* (Ștefănuț 2010).

In 2008 the first edition of *the Hornwort and Liverwort Atlas of Romania* was published (Ștefănuț 2008), showing the distribution of 4 hornwort and 211 liverwort species reported for Romania. The book covers a subspecies new for science, *Lophozia bantriensis* subsp. *wallfischii* Ștefănuț 2008.

After the publication of the *Atlas*, a new liverwort species, *Marsupella condensata*, was reported for Romania.

Until now, 966 bryophyte species were reported for Romania (4 hornwort species, 212 liverwort species and 750 moss species); Romania being the richest country in bryophyte species from South-East Europe.

## THE TAXONOMY AND CHOROLOGY OF FABACEAE SPECIES IN ROMANIA

S. ȘTEFĂNUȚ, V. SANDA, K. ÖLLERER, C. NICOLAE, I. VICOL, R. ION

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One of the objectives of the *Taxonomy and chorology of macrofungi, lichens, bryophytes and vascular plants from Romania* Project is the mapping of Fabaceae species in Romania. Until now, two volumes of the Fabaceae Atlas of Romania have been published and another one will be published soon. In the first volume the distribution of the *Genistella*, *Genista*, *Laburnum*, *Cytisus*, *Chamaecytisus* and *Sarothamnus* species (S. Ștefănuț and V. Sanda 2008 - *Atlas Florae Romaniae* V) is shown, while the second volume deals with the distribution of *Medicago*, *Melilotus*, *Ononis* and *Trigonella* species (S. Ștefănuț, V. Sanda, K. Öllerer, I. Vicol and R. Ion 2009 - *Atlas Florae Romaniae* VI).

Data accumulated over time, existing literature and Herbarium collections in conjunction with field observations, enabled the development of *Trifolium* species maps which are included in the third volume of the Atlas (*Atlas Florae Romaniae* VII). The fourth volume will include the distribution of *Anthyllis*, *Dorycnium*, *Lotus*, *Tetragonolobus*, *Bituminaria*, *Amorpha*, *Galega* and *Wisteria* species from Romania. The fifth volume will include the distribution of *Colutea*, *Caragana* and *Astragalus* species, the sixth will present the corology of the *Oxytropis*, *Glycyrrhiza*, *Ornithopus*, *Coronilla*, *Hippocrepis* and *Hedysarum*, species the seventh will include the species *Onobrychis*, *Vicia* and *Lens*, while the eighth the species *Lathyrus* and *Pisum*.

The mapping system (Lehrer & Lehrer 1990) uses a map with UTM grid of 10 × 10 km. The distribution data are stored in Microsoft Access database, while the *Corolog* software is used to create the species and subspecies distribution maps.

The software is able to convert the data distribution system with UTM grid of 10 × 10 km in the 50 × 50 km grid system used by *Atlas Florae Europaeae*.

Until now, six volumes of the *Atlas Florae Romaniae* were published:

1. Sanda V. & Ștefănuț S., 2003. *Atlas Florae Romaniae*. I. *Pinophytina*. Edit. Vergiliu. București. 115 p., ISBN 973-85592-2-7.
2. Sanda V. & Ștefănuț S., 2004. *Atlas Florae Romaniae*. II. *Betulaceae*. Edit. Vergiliu. București. 115 p., ISBN 973-85592-6-x.

3. Sanda V., Barabaş N. & Ştefănuţ S., 2004. *Atlas Florae Romaniae*. III. *Quercus*. Edit. „Ion Borcea”. Bacău. 173 p., ISBN 973-86586-4-0.
4. Sanda V., Barabaş N. & Ştefănuţ S., 2005. *Atlas Florae Romaniae*. IV. *Salix*. Edit. „Ion Borcea”. Bacău. 172 p., ISBN 973-86586-5-9.
5. Ştefănuţ S. & Sanda V. 2008. *Atlas Florae Romaniae*. V. Fabaceae: *Genistella*, *Genista*, *Laburnum*, *Cytisus*, *Chamaecytisus* şi *Sarothamnus*. Edit. Ars Docendi - Universitatea din Bucureşti, Bucureşti, 164 p., ISBN 978-973-558-367-5.
6. Ştefănuţ S., Sanda V., Öllerer K., Vicol. I., Ion R., 2009. *Atlas Florae Romaniae VI. Fabaceae: Medicago, Melilotus, Ononis şi Trigonella*, Bucureşti, Edit. Ars Docendi – Universitatea din Bucureşti, 179 p., ISBN 978-973-558-439-9.

# **THE ROLE OF EDAPHICAL ORIBATIDS (ACARI, ORIBATIDA) FROM TROPHICAL LEVELS OF SOME FOREST ECOSYSTEMS (FIR-BEECH, FIR AND BEECH) FROM BUCEGI MOUNTAIN**

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The paper presents the results of a study concerning some functions of oribatid populations from the edaphon of different forest ecosystems from the Bucegi Mountain as: fir-beech forest from Valea Seacă, fir forest from Valea Albă and beech forest from Muchea Lungă.

The trophical structure of biocenoses represents the results of the nutrition relations between species. These three big categories (primary producers, consumers and decomposers) could only be dependent of each other, forming the trophical levels. The first category includes the producers of organic matter (green plants, chemo- and photosynthesizing bacteria).

The second category includes consumers, which can be divided in: primary consumers or rank I (phytophagous) species, secondary consumers or rank II (carnivorous) species and tertiary consumers or rank III (detritophagous) species.

An important role is that of the detritophagous species (animals which feed with organic matter provided by the breaking down and partial decomposition of dead plants and animals). In this category many animals from the edaphical fauna (oligochets, isopods, gastropods, mites, insects, etc.) are included.

Taking account of the ingerated food, mites (Oribatida) are divided in three trophical categories: macrophytophagous, microphytophagous and panphytophagous. The relation how these trophical categories participate in the consumption of vegetal detritus was quantified by their numerical density. Using the analysis of the intestine content of the most representative species (using constancy and dominance), the volume of their consumption was identified, the provenience, the ratio of the nutritional components and their trophical preferences. Taking account this classification and the intestine content analysis of the eudominant- euconstant and of the constant-dominant species, their trophical functions were described, also their trophical levels, as well as the total consumed volume in relation with the time period (month, season, year). In each investigated forest ecosystem the obtained results were extrapolated to the whole population. Data interpretation must consider that each species had a specifical nutritional behaviour and the extrapolation of results to the whole oribatocenosis is

dangerous. This is the reason of selecting out species with the highest values of numerical densities. This fact allows a better appreciation of the role of species, populations and even of the whole oribatocenosis in decomposing processes from the studied forest ecosystems.

## DEVELOPMENT AND PROGRESS IN COLLEMBOLA RESEARCH IN ROMANIA

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The first study of the Romanian springtails was the work of TÖMÖSVÁRY (1883), who recorded three species from the Western part of Romania, followed by VELLAY (1900), who reported 30 species of Collembola from Banat, Crişana, Maramureş and Transylvania – Romanian regions, which belonged to Hungary that time. Since these basic works, numerous taxonomical and faunistic papers on the Collembola from Romania have been published.

From a total of 157 scientific papers on Romanian Collembola, 40 (25%) were published by authors from Institute of Biology, Bucharest. Collembola research done in our institute was started by Prof. Marin Falcă in 1972. He conducted his PhD research on Collembola communities from the Southern Carpathians (Bucegi, Gârbova and Retezat Massifs) (FALCĂ, 1984). One year later, Anca Zamfirescu investigated the litter springtails from three stations of Bucegi Massif (Gruia & Zamfirescu, 1973). Between 1972 and 2009 Falcă published 20 papers about Collembola communities from many types of Romanian forest ecosystems. He made important ecological contributions to the scientific knowledge regarding Collembola in our country (studies regarding numerical structure, biomass and productivity of Collembola from soils, indicating the role of each species in the activity of nutrient cycling and energy flow in an ecosystem; partial and multiple correlations between Collembola and abiotic factors; similarity indices of different communities). He showed that the similarity of Collembola is related, among other factors, to the structure of tree and herbaceous layers as well as to the type of soil (FALCĂ, 1989). Also, he established the negative binomial distribution of *Folsomia quadrioculata* (Tullberg, 1871) (FALCĂ, 1991) and studied the life cycle of *Deuteraphorura inermis* (Tullberg, 1869) related to temperature (FALCĂ, 1978).

Important contributions to the knowledge of Romanian Collembola were made after 2005, when Fiera started her research on Collembola and enlarged the list of species. She described one new species for science (*Protaphorura ionescui* Radwański, Fiera & Weiner, 2006) and identified ten new Collembola records for the Romanian fauna: *Hemisotoma orientalis* (Stach, 1947) - Insula Mare a Brăilei, *Ceratophysella stercoraria* (Stach, 1963) - Cernica forest, *Neotullbergia ramicuspis* (Gisin, 1953) - Câmpina,

Doftanei Valley, *Friesea truncata* Cassagnau, 1958 - Lăptici Peat Bog, Bucegi Massif, *Cryptopygus debilis* (Cassagnau, 1959) – Călugăreni, *Isotoma anglicana* Lubbock, 1862 - Călugăreni, *Deutonura albella* (Stach, 1920) - Sărata-Monteoru, *Neanura minuta* Gisin, 1963 - Sărata-Monteoru, *Protaphorura sakatoi* (Yosii, 1966) - Cobia, *Pseudosinella duodecimpunctata* Denis, 1931 - parks of Bucharest. In 2007, Fiera published the first checklist of Romanian springtails, which contains 388 species. Additional species were added to the list by LÁSZLÓ & TRASER (2008), NIȚU et al. (2009); POPA (2010). Therefore at this moment the Collembola checklist for Romania includes 394 species.

Starting with 2009, modern studies of Collembola were conducted. Ulrich and Fiera (2009) studied the influence of area and environmental variables on the number of Collembola species from 35 European countries and showed that area, winter length and annual temperature differences are major predictors of species richness. They also investigated species body size distributions (SBDs) and spatial distribution of endemic Collembola species across 53 European countries and larger islands, using a unique data set on the spatial distribution of 2102 Collembola species together with associated data on body size, area, climate variables, longitude and latitude (Ulrich & Fiera, 2010, Fiera & Ulrich, in press).

Together with a German team, Fiera investigated trophic niche differentiation of some species of Collembola with respect to food resource, as indicated by dual stable isotope analysis ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) and fatty acids composition. In a feeding experiment, PCR-based approaches have been applied to detect the prey (algae or nematodes) from the gut content of Collembola (*Heteromurus nitidus*) using molecular techniques.



## **CLASS CHILOPODA IN ROMANIA: HISTORY AND DEVELOPMENT IN RESEARCH**

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Chilopoda, generally known as centipedes, are a class of terrestrial predators of the phylum Arthropoda. All over the world, this group includes more than three thousand species, inhabiting various habitats. Five orders are currently recognized, Geophilomorpha order having the largest distribution and diversity. In the temperate region, the lithobiomorphs are the most widespread, while the scutigermorphs, commonly known as house centipedes, are less diverse.

In Romania, the first data regarding the centipede fauna were published in 1861 by V. Sill, who conducted studies in Transylvania. In 1880 Latzel included some new species in his monograph "Die Myriapoden der Osterreichisch-ungarischen Monarchie". In the first summary paper, Daday (1889) presents all known species in the Austro-Hungarian Empire, with a total of 38 species for Transylvania.

Until 1954, the evidence on centipedes from the Romanian territory was further developed by several foreign researchers. The first Romanian miriapodologist, Zachiu Matic started his publishing activity describing eight species (1945), seven centipedes from caves, with one new species for science (1955). In his whole scientific activity he described 204 new taxa for science (from Romania and other countries). Following recent revisions 117 taxa are still valid. The knowledge accumulated about fauna, taxonomy and ecology of 49 species was compiled in 1966 in the first Romanian faunistical tome Chilopoda – "Anamorpha" from the series "Fauna României", and then, in 1972, the second tome "Epimorpha" is added. The second volume brings together the important results of the PhD thesis of Cornelia Dărăbanțu, and of other researchers such as I. Căpușe. Working mainly in speology, Ș. Negrea described 30 new species and subspecies for science, and made important observations regarding the zoogeography of centipedes.

From an open list of 200 scientific publications, about 20% were signed by researchers from the Institute of Biology, Bucharest. Dr. C.C. Prunescu, researcher in the institute, started in 1962 the study of centipedes anatomy and histology, bringing a very important contribution to the knowledge on ventral nervous ganglions, genital system and its evolution. Making a detailed investigation of the plesiomorphic and apomorphic characters of Chilopoda orders, he proposed in 1965 the first phylogenetic

tree of the class. Following his the research activity until 2006, new information about the genital system, tracheal lungs and system, supernumerary Malpighian tubules were published. He also brought his contribution to the taxonomy, describing nine new taxa like *Lithobius decapolitus* Matic, Negrea & Prunescu, 1962; *Lithobius sciticus* Prunescu, 1965; *Lithobius microps ponticus* Prunescu, 1965.

For the PhD research concerning the fauna and ecology of centipedes from Muntenia region, I've started to compile the list of species present in Romania. Taking in account only validated taxa, the list comprises 109 centipedes belonging to 17 genera, 11 families and four orders. In 12 out of the 41 Romanian counties, there is a serious lack of information about the presence and distribution of Chilopoda, these counties having the smallest karstic, as well as forested areas. In the last decade, V. Ilie and László Dányi added new distributional data for Caras-Severin and Maramures counties while in 2009 R. Gava published the first data about the centipede fauna and ecology for Argeş county. Being involved in recent projects, we collected data about Chilopoda presence in four of the counties with previously zero information.

In 2009, we analysed, for the first time in Romania, the structure of the urban centipede fauna from Bucharest, in the framework of a larger LIFE Environment project. Ten species were identified, most of them with large distribution, being present in other European cities as well. At least three species are known to be thermophilic, hence adapted to the meteorological conditions of a large city.

In conclusion, a thorough work in southern and eastern region of the country is needed for completing a database, necessary for further investigation regarding possible influences of environmental factors on the centipede distribution.

## CHRYSOMELIDAE FAMILY (INSECTA: COLEOPTERA) IN THE ROMANIAN FAUNA

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The taxonomic and faunistic studies are the basis for both the biodiversity evaluation and the management of the natural resources. The Chrysomelidae, commonly known as leaf beetles, is one of the most numerous family of Coleoptera Order, including about 40,000 species all over the world.

**Biodiversity.** According to the present state of knowledge, the checklist of Chrysomelidae from Romania includes about 570 species, belonging to 13 subfamilies and 82 genera. Among them, *Cryptocephalus*, *Longitarsus* and *Chrysolina* genera include the most numerous species, comprising about 30% of the total number of leaf beetle species recorded in Romania. Most of the records are known from Transylvania, which is the best investigated Romanian region regarding the Coleoptera fauna. Generally, there is a significant lack of knowledge regarding many chrysomelid species from Romania.

The taxonomic studies about the chrysomelids collected by the author in the last years, from various regions of Romania, or preserved in the collections of natural museums from Romania, led to the recording of 90 species from the Black Sea coastline, 214 species from the Maramureş region, 225 species from Bucharest and surroundings, 185 species from the Carpathian Mountains, 94 species from the Maramureş Mountains Natural Park and neighboring areas etc. Most of these taxa were recorded for the first time in the studied areas.

**Chrysomelids with uncertain status in Romania.** This category includes about thirty species whose occurrence in Romania is questionable, the records of these species requiring confirmation (*Cassida deflorata*, *Hypocassida meridionalis*, *Cryptocephalus cyanipes*, *Lachnaia pubescens*, *Luperus nigripes*, *Phyllotreta paralella*, *P. variipennis* etc.).

**Ecology.** Chrysomelids are phytophagous insects, strictly specialized on certain species, genera or families of plants (monophagous, oligophagous or polyphagous). Many species are important from an economical point of view, being serious pests for forests and agricultural crops (*Oulema melanopus*). The Alticinae (the largest subfamily of Chrysomelidae, with approximately 240 species recorded in Romania) are monophagous or oligophagous, both in the larval and adult stage, being associated mainly with herbaceous plants.

**Alien species.** *Diabrotica virgifera virgifera* (the corn's root western worm), native to the western part of North America, was recorded for the first time in the Romanian fauna in 1996. The host plants of this species are represented especially by *Zea mays*, and also by different plants belonging to families like Brassicaceae, Cucurbitaceae, Chenopodiaceae, Fabaceae, Poaceae. The dispersion rate of *D. virgifera virgifera* in the Romanian territory is high, being present now in many western and southern districts.

**Endemic and rare species.** The Chrysomelidae family includes certain Carpathian endemics, recorded along the entire Carpathian Mountains chain, and also a few restricted only to the Romanian Carpathians. Thus, in the Romanian Carpathians the following endemic species are present: *Pachybrachis carpathicus*, *Chrysolina weisei*, *C. atrovirens*, *C. biharica*. Among the Carpathian endemics, in Romania the following species have been recorded: *Sclerophaedon carpathicus*, *Asiolestia transsilvanica* and *Psylliodes frivaldszkyi*. One can notice the occurrence of some widespread European mountainous endemic species (*Longitarsus pallidicornis*, *L. rubellus*, *Minota carpathica*, *Neocrepidodera melanostoma*, *Orestia aubei*, *Phyllotreta christinae*), Alpine-Carpathian endemic species (*Neocrepidodera cyanescens*), Carpathian-Dinaric endemic species (*Psylliodes subaeneus*). *Aphthona valachica* is known until now only from Romania (described from Comana, southern part of the country). Some species appear to be very rare in the Romanian fauna: *Macrolea mutica* (a very rare halophilous species), *M. appendiculata*, *Cheilotoma musciformis*, *Cryptocephalus laetus* etc.

## THE SIMILARITIES BETWEEN PREDATOR MITE POPULATIONS (ACARI: GAMASINA) FROM SOME NATURAL FORESTS IN BUCEGI MASSIF

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The ecological research was made in 2001-2003, in Bucegi Massif, in three natural forest ecosystems: with *Picea abies* (situated at 1350 m altitude, on Valea cu Brazi, at N = 45°21'09.15"; E = 25°31'10.59"); *Abies alba* (situated on 950-1000 m altitude, on Valea Urlătoarei, at N = 45°23'50.20"; E = 25°32'00.07") and *Fagus sylvatica* (situated on 1200 m altitude, at N = 45°20'56.45"; E = 25°31'24.9").

In order to show the similarities between predator mites populations from these areas, the vegetation and some abiotic parameters on soil level (temperature, humidity and pH) were analyzed and described. Using the Jaccard index (q), similarities between gamasid populations from these investigated ecosystems were made. In total 2016 samples were analyzed, 96 species with 173637 individuals.

Taking account of the bio-edaphic conditions, in the studied forest different numbers of species were identified: in ecosystem with *Abies alba* 78 species, in ecosystem with *Picea abies* 67 species and in ecosystem with *Fagus sylvatica* 71 species.

Among the gamasids identified in ecosystems with *Picea abies* and *Abies alba*, on the soil level, the highest similarity index ( $q = 0.706$ ) was recorded, in comparison with those from *Abies alba* and *Fagus sylvatica* forests ( $q = 0.656$ ). The common species identified in forest ecosystems with *Picea abies* and *Abies alba* were: *Gamasodes spiniger*, *Leioseius lawrencei*, *Dendrolaelaps foveolatus*, *Pachylaelaps pectinifer*, *Zercon peltatus*, *Zercon peltadoides*, *Zercon triangularis*, *Zercon pinicola* and *Zercon carpathicus*. Common species from ecosystems with *Abies alba* and *Fagus sylvatica* were: *Parasitus minimus*, *Paragamasus motasi*, *Paragamasus sp.*, *Veigaia paradoxa*, *Epicriopsis rivus*, *Leitneria granulata*, *Macrocheles decoloratus*, *Pachylaelaps magnus* and *Olopachys vysotskajae*.

At the same time, the lowest similarity index was obtained between gamasid populations from ecosystems with *Picea abies* and *Fagus sylvatica* ( $q = 0.624$ ). Common identified mites were: *Eugamasus monticolus*, *Iphidozercon venustus* and *Pachylaelaps latior*.

Analyzing the values of Jaccard index on gamasids from the litter and fermentation layer, we observed that the biggest level of similarity was obtained between mite populations from ecosystems with *Picea abies* and

*Abies alba* ( $q=0,71$ ), followed by the populations from ecosystems with *Abies alba* and *Fagus sylvatica* ( $q=0,63$ ). The highest difference was recorded between gamasids from forest with *Picea abies* and *Fagus sylvatica* ( $q=0,529$ ).

On the humus layer, the similarity index from the investigated areas showed a decrease. Between mite populations from ecosystems with *Picea abies* and *Abies alba* the highest value ( $q=0,614$ ) was obtained. This index is with 13.52% lower than those obtained in litter-fermentation layer. The biggest difference was recorded between mites from ecosystems with *Picea abies* and *Fagus sylvatica* ( $q=0,514$ ), decreasing with 2.84%, in comparison with the first soil layer. The same phenomenon was investigated on gamasid populations between ecosystems with *Abies alba* and *Fagus sylvatica*. The decreasing of the Jaccard index was 3.97%, in comparison with the first soil layer ( $q=0.605$ ).

We could conclude that there is a significant similarity between gamasid populations from ecosystems with *Picea abies* and *Abies alba*, due to the presence of the same category of primary producers (coniferous trees) and to the same type of soil (brown-eumesobasic). In all three ecosystems, high values of the studied index were recorded between soil layers. These values could be explained by vertical gamasid migration and by their identification in both layers.

Using Jaccard index, we highlighted that the gamasid population structure was influenced by the type of soil, by the specific composition of the herbaceous, shrub and tree layers, by the micro- and macroclimatic factors, characteristic for each studied ecosystem.

## **EIGHT YEARS OF STRUCTURAL AND DYNAMICAL STUDY ON MITE POPULATIONS (ACARI: MESOSTIGMATA) FROM SOME DEGRADED AREAS FROM RETEZAT MOUNTAINS**

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The ecological research was made in 2000-2007, in Retezat mountains, in five waste areas (Bîrlui, Rîuşor, Nucşoara, Ciurila, Netiş). These areas, which resulted from digging the water adduction galleries to the dam lake on Râul Mare, are situated at an altitude of 1000 m to 1400 m.

950 soil samples were taken, 29 species belonging to 22 genera and 9 families being identified. The mite populations had structural and dynamical differences, caused by the various environmental conditions (like vegetation, soil humidity and pH). In order to evidence these modifications the following statistical parameters were analysed: number of species, numerical density and relative abundance. In comparison with the natural ecosystem nearest to the degraded areas, the values of these populational parameters were very low. In Bîrlui waste area 13 species of gamasids were identified, with a numerical density of 5800 ind./sq.m. The highest number of species, as well as numerical density, was recorded at Ciurila (26 species with 26600 ind./sq.m. At Netiş, Nucşoara and Rîuşor these parameters had the most decreased values (2 species with 4200 ind./sq.m.; 11 species with 6600 ind./sq.m. and 13 species with 5000 ind./sq.m.). At Bîrlui meadow, the investigated natural ecosystem, 13 species with 13800 ind./sq.m were recorded.

Analysing the dynamics of predator mite populations, we observed that after eight years of study, the anthropical interventions had a negative impact. If in 2001 and 2002 the most increased values of the numerical densities (11400 ind/sq.m. and 27800 ind./sq.m.) were recorded, in 2007 this parameter had a serious decline (200 ind./sq.m.).

Species *Veigaia nemorensis*, *Rhodacarellus kreuzi*, *Cheroseius nepalensis*, *Cheroseius borealis*, *Hypospis aculeifer* and *Prozercon traegardhi* were dominant. These mites can adapt to the specific environmental conditions of degraded areas (low soil humidity, poor vegetation and lack of organic matter), due to their ecological preferences (some of them being ubiquitous) and small dimensions.

This study showed that the anthropic intervention on degraded areas, together with specific biotic and abiotic factors had a significant influence on structure and dynamics of the predator mite populations.

## GASTROPODS WITHIN THE ROMANIAN SECTOR OF THE BLACK SEA HYDROGRAPHICAL BASIN

O. CIOBOIU

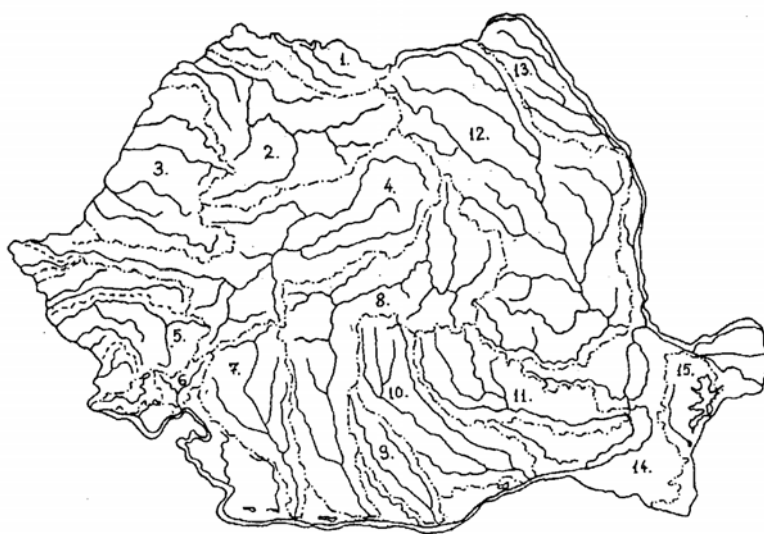
The Oltenia Museum Craiova, Romania

The Romanian territory of the Black Sea hydrographical basin is characterized by the presence of a complex river system made up of plain, hill and mountain rivers, floodplains, torrents and temporary streams that flow directly into the Black Sea.

The surface of this hydrographical basin within Romania reaches almost 900.000 sq km, the continental river system comprising 15 basins with a total length of 60,000 km.

The river system hosts 214 species of gastropods distributed as follows – 132 species identified within the basin of the Danube, which includes 17 smaller basins and 82 species on the sea shelf. Out of the 132 species, 66 were identified in the Danube and the Danube Delta.

The distribution of the gastropods within the river system of Romania:



1. The upper Tisa – 30 species; 2. The Someș – 30 species; 3. The Crișuri rivers – 39 species; 4. The Mureș – 35 species; 5. The Bega, the Timiș, the Caraș – 54 species; 6. The Nera, the Cerna – 51 species; 7. The Jiu – 28 species; 8. The Olt – 31 species; 9. The Vedea – 29 species; 10. The Argeș – 29 species; 11. The Ialomița – 31 species; 12. The Siret – 29 species; 13. The Prut – 31 species; 14. The Danube – 113 species (54 species along the Romanian sector); 15. The shore area – 96 species (82 species on the shelf of the Black Sea, the Romanian sector).



The distribution of the gastropods within the hydrographical basin of the Black Sea is as follows: 30.9 % in rivers; 30.8 % in the Danube and the Danube Delta; 38.3 % on the shelf of the Black Sea.

The freshwater species considered eurytrope represented in the highest number are: *Viviparus acerosus*, *Esperiana esperi*, *Physa fontinalis*, *Physella (Costatella) acuta*, *Lymnaea stagnalis*, *Stagnicola palustris*, *S. corvus*, *S. turricula*, *Radix auricularia*, *R. balthica*, *R. lagotis*, *Galba truncatula*, *Acroloxus lacustris*, *Planorbis (P.) planorbis*, *Anisus (A.) spirorbis*, *A. leucostoma*, *A. (Disculifer) vortex*, *Gyraulus (G.) albus*, *G. (Armiger) crista*, *Hippeutis complanatus*, *Segmentina nitida*, *Planorbarius corneus*.

The species *Viviparus contectus*, *Valvata (Cincinna) piscinalis*, *V. (Tropidina) macrostoma*, *Bithynia (B.) tentaculata*, *Amphimelania holandri*, *Gyraulus (G.) acronicus* display a more limited distribution.

The species *Theodoxus (Th.) euxinus*, *Borysthenia naticina*, *Hydrobia ventrosa*, *Pseudamnicola (P.) razelmiana*, *P. dobrogica*, *Clessiniola variabilis*, *Rissoa (R.) membranacea*, *Cerithium vulgatum*, *Acteocina pontica* play a special role as they have been identified both in freshwater ecosystems, as well as in brackish and salt water ecosystems.

The species identified on the Romanian shelf represent 49 % of the total number of species from the Black Sea.

From the point of view of their origin, *Theodoxus (Th.) transversalis*, *Th. danubialis*, *Viviparus acerosus*, *Lithoglyphus apertus*, *L. pygmaeus*, *Esperiana (Microcolpia) daudebardii acicularis*, *E. esperi*, *Borysthenia naticina* are endemic Ponto-Danubian species; *Microcolpia (Turricaspia) lincta*, *Clessiniola variabilis*, *Melanopsis parreysi*, *Paladilhia (Paladilhops) transsylvanica*, *Amphimelania holandri*, *Hydrobia ventrosa* belong to the relict Ponto-Caspian species.

The diverse types of ecosystems within the Romanian territory of the Black Sea hydrographical basin host 28 % of the European malacofauna.

**DESIGNATION OF NEW SITES OF COMMUNITY IMPORTANCE  
IN ROMANIA FOR THE NATURA 2000 PRIORITY HABITAT:  
\*1530 PANNONIC AND PONTO-SARMATIC  
SALT STEPPES AND SALT MARSHES**

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The objective of the study was to improve the decisions of the European Commission regarding the designation of the Natura 2000 network in Romania, especially for the Habitat Directive 92/43/CEE implementation. The main purpose referred to the study of halophytic habitats and their distribution within the biogeographic regions in Romania and their designation as new Sites of Community Importance (SCIs).

The European Commission has sent the “*draft conclusions*” after the Biogeographical Seminar held in Sibiu, 9-12 June, 2008. Regarding the priority habitat \*1530 *Pannonic and Ponto-Sarmatic salt steppes and salt marshes*, the EC conclusion was IN MOD = Insufficient moderate, for the Pannonic region. In this situation, the following action was required: one or a few additional sites (or maybe extension of existing sites) required.

The description of \*1530 habitat according to the EUR 27 - *Interpretation Manual of European Union Habitats*, are the following: Pannonic and Ponto-Sarmatic salt steppes, salt pans, salt marshes and shallow salt lakes, which are highly influenced by a pannonic climate with extreme temperatures and aridity in summer. The enrichment of salt in the soil is due to high evaporation of ground water during summer. These habitat types are partly of natural origin and partly under a distinct influence of cattle grazing. The halophytic vegetation consists of plant communities on dry saltpans and steppes, humid salt meadows and annual plant communities of periodically flooded salt lakes with typical zonation.

During the field research conducted during 2009 and 2010, we have made the characterisation of species that are present and define the priority habitat, as follow:

Plants species which define the habitat are: *Artemisia santonicum*, *Suaeda maritima*, *Lepidium crassifolium*, *Aster tripolium*, *Salicornia prostata*, *Camphorosma annua*, *Plantago tenuiflora*, *Plantago maritima*, *Juncus gerardii*, *Pholiurus pannonicus*, *Festuca pseudovina*, *Achillea collina*, *Puccinellia limosa*, *Scorzonera cana*, *Petrosimonia triandra*, *Peucedanum officinale*, *Halocnemum strobilaceum*, *Limonium gmelini*, *Carex distans*, *C. divisa*, *Taraxacum bessarabicum*, *Beckmannia eruciformis*, *Trifolium fragiferum*, *Cynodon dactylon*, *Ranunculus sardous*, *Agropyron elongatum*, *Halimione verrucifera* (syn *Obione verrucifera*),

*Lepidium latifolium*, *Leuzea altaica* (syn *L. salina*), *Iris halofila*, *Triglochin maritima*, *Hordeum hystrix*, *Aster sedifolius*, *Scorzonera austriaca* var. *mucronata*, *Kochia laniflora*, *Festuca arundinacea* ssp. *orientalis* etc.

Plant communities that are present in the designated SCIs are the following: *Aeluropo-Puccinellietum limosae* Popescu et Sanda 1975; *Taraxaco bessarabici-Caricetum distantis* Sanda et Popescu 1978; *Caricetum distantis* Rapaics 1927; *Camphorosmetum annuae* (Rapaics 1916) Soó 1933; *Limonio gmelini-Artemisietum monogynae* Țopa 1939 (syn.: *Staticeto-Artemisietum monogynae (santonicum)* Țopa 1939); *Halimionetum (Obionetum) verruciferae* (Keller 1923) Țopa 1939; *Puccinellietum distantis* Soó 1937; *Plantaginetum maritimae* Rapaics 1927; *Hordeetum hystrixis* (Soó 1933) Wendelberger 1943; *Artemisio santonici Festucetum pseudovinae* (Magyar 1920) Soó (1933) 1945; *Achilleo-Festucetum pseudovinae* Soó (1933) corr. Borhidi 1996; *Puccinellio-Salicornietum* Popescu et al. 1987; *Bassietum sedoidis* (Ubrizsy 1948) Soó 1964; *Camphorosmetum monspeliacae* (Țopa 1939) Șerbănescu 1965.

The equivalent Romanian habitats (Donita & al., 2005, 2006) corresponding with plant communities are: R1504, R1507, R1508, R1510, R1514, R1517, R1519, R1521, R1530 and R1531.

Animal species present in the SCIs are:

- Bird species: *Pelecanus onocrotalus*, *Egretta garzetta*, *Ardeola ralloides*, *Chlidonias hibridus*, *Egretta alba*, *Ciconia ciconia*, *Ardea purpurea*, *Nycticorax nycticorax*, *Himantopus himantopus*, *Recurvirostra avosetta*, *Platalea leucorodia*, *Circus aeruginosus*, *Ardea cinerea*, *Motacilla cinerea*, *Vanellus vanellus* etc.

- Amphibians: *Rana ridibunda*, *Bufo bufo* etc.

- Invertebrates: *Sympetrum sanguineum*, *Sympetrum fonscolombii*, *Erythromma viridulum*, *Aeshna affinis*, *Lestes barbarus*, *Plebejus argus* etc.

**Results:** According to the field studies, the following new SCIs were designated for the Pannonic, Continental and Steppe biogeographic regions:

- Pannonic region: Dinaș (Timiș County), Grăniceri-Foieni (Timiș County), Sânmartin (Arad County) and ROSCI0231 Solurile sărăturate Socodor (Arad County) – extension of existing site.

- Steppe region: Ianca-Plopu-Sărat-Comăneasca (Brăila County), Sărăturile de la Gura Ialomiței –Mihai Bravu (Ialomița County and Brăila County) and Lacul Sărat-Brăila (Brăila County).

- Continental region: ROSCI0259 Valea Călmățuiului (Buzău County) – extension of existing site.

**Conclusion:** the designation of a new sites (SCIs) for the Natura 2000 priority habitat \*1530 Pannonic and Ponto-Sarmatic salt steppes and salt marshes, cover about 90% of the European Commission required action for the extension of Natura 2000 network in Romania.

**MICROBIOLOGY**

**POSTERS**



## THE UV EFFECT ON HALOARCHAEA-RESEARCHES IN THE FRAME OF EXPOSE ADAPT AND HALOSPACE PROJECTS

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The resistance of the microorganisms to extreme conditions from extraterrestrial space was proved to the sporogenic bacteria *Bacillus subtilis* many experiments with this one being done in the frame of ground and space experiments (Horneck, 1993; Horneck et al., 2001). Some experiments were done with halophilic archaea like *Haloarcula* sp. By the NASA specialists (Mancinelli et al., 1998). In the frame of the Expose and Adapt and Halospace programs of the European Space Agency and of the Austrian Academy of Science were tested some strain of halophilic archaea for their resistance to UV using a UV lamp Mars simulator which beam have the intensity of the sun UV rays at the level of Mars surface (Rontó et al., 2003). The tests were performed in liquid cultures and to the biomass embedded in salt crystals obtained by the centrifugation of 5 ml of culture with optical density of about 1 and resuspension in 4 M NaCl buffered sterile solution. Aliquots of the mixture was deposited on quartz sterilized disks and allowed to dry at room temperature in plastic containers in sterile bench. The survival was tested by growing in liquid medium dilution plates on appropriate medium, and by fluorescence microscopy with previous staining with BacLight LIVE DEAD kit and numbering of viable against dead microorganisms.

The cells embedded in crystals showed a decrease of viability after exposure at a radiation dose of 21 kJ/m<sup>2</sup>. The liquid cultures growing showed an important delay of time after exposure to much decreased increase in over 148 kJ/m<sup>2</sup>. The D<sub>37</sub> (37 % survival rate) for the strain *Hcc. dombrowskii* H4 was about 400 kJ/m<sup>2</sup> in crystals, for the liquid culture D<sub>37</sub> was about 5 kJ/m<sup>2</sup>.

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## EXTRACELLULAR HYDROLYTIC ENZYMES OF HALOPHILIC MICROORGANISMS FROM SEVERAL ROMANIAN SALINE ENVIRONMENTS

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Recent search for biocatalysts that can cope with conditions of industrial processing is continuously increasing. Despite the large numbers of enzymes that has been used for various biotechnological and industrial applications, the demand for more efficient biocatalysts is still high. The majority of main industrial processes are conducted under specific conditions of pH, ionic strength, temperature, etc., for which some of the enzymes do not present optimal activities. In respect to these requirements, the extremozymes, enzymes produced by microorganisms able to flourish under extreme environments, could help to develop new biotechnologies. Among these, halophilic microorganisms that require high salt concentrations for growth are sources for both thermotolerant and salt adapted enzymes (1).

The aim of this study was to determine the extracellular hydrolytic activities in halophilic microorganisms isolated from several Romanian saline environments such as subterranean rock salt and salt lakes.

The investigated strains showed at least one of the tested extracellular hydrolytic activities, while one strain was hydrolyzing five of the tested substrates. Our investigations showed that hydrolytic activities using Tween 80 and casein were predominant at NaCl concentrations between 0 and 2 mol l<sup>-1</sup>.

Techirghiol Lake, located in the proximity of the Black Sea coast, harbor haloarchaea belonging to *Haloferax* and *Halorubrum* genera. The isolated strains were able to hydrolyze starch, a process that appeared to be influenced by Na<sup>+</sup>/Mg<sup>2+</sup> ratio. The increasing NaCl concentration conducted to a reduced or abolished amylase activity. The different Mg<sup>2+</sup> requirements for cell growth also affect the starch hydrolysis, revealing that an increase in salinity determines the decrease of this activity in the case of the haloarchaeal strains isolated from salt lakes containing low salt concentrations.

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**HIGHLY THERMOSTABLE EXOPOLYSACCHARIDES  
PRODUCED BY HALOPHILIC BACTERIA ISOLATED FROM  
HYPERHALINE HABITATS IN SLANIC, PRAHOVA, ROMANIA**

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Moderately halophilic bacteria represent an excellent model of adaptation to frequent changes in the extracellular osmolarity, being regarded as an interesting group of microorganisms from a biotechnological point of view. They have the advantage that most of the species are able to survive in a wide range of salinities, comparing to the very precise salt content required by the haloarchaea. Thus, their eurihaline response allows them to be used in different processes in which the salt content or the concentration of metal ions are variable and suffer modifications from very low to close to saturation, recommending them for a multitude of actual or potential applications in several fields of biotechnology.

This study reports the isolation and characterization of some moderately halophilic bacterial strains harvested from hypersaline habitats in the Prahova county. The preliminary results revealed that the number of halophilic bacterial strains that could be isolated from different locations and their biochemical characteristics were influenced by the physico-chemical properties of the environments.

Following the Gram-staining, the estimation of the salinity range for growth and the testing with some inhibitors (sodium deoxycholate and chloramphenicol), three representative strains were chosen for further investigations in order to establish the optimal salt concentration required for growth and the effects of the culture medium composition on the ability to synthesize exopolysaccharides.

Our data suggested that all three strains, represented by Gram-negative rods, could be assigned as moderately halophilic bacteria having oxidizing properties and being able to produce indole and reduce nitrates to nitrites. Two of them presented amylolytic activity and none of them had the capacity to produce caseinases and gelatinases.

The investigated strains showed different behaviors in the presence of several salt concentrations. The ability to produce exopolysaccharides under various growth conditions differentiated these bacterial strains. Both the carbon source and growth conditions influenced the dynamics of the culture development and the exopolymers synthesis.

These exopolysaccharides produced by moderately halophilic were isolated and characterized, exhibiting a high thermostability.



## HALOPHILIC ARCHAEA FROM MAN-MADE AND SUBTERRANEAN HYPERSALINE ENVIRONMENTS IN SLĂNIC PRAHOVA, ROMANIA

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Halophilic aerobic archaea (haloarchaea) are able to thrive and grow in hypersaline environments distributed throughout the world, such as salt lakes, salterns, solar salts, and subsurface salt formation. These microorganisms make the object of basic studies in relation to the origin of life on our planet and molecular mechanisms of adaptation to hypersaline conditions. Apart from their evolutionary and ecological significance, haloarchaea have promising biotechnological applications.

Haloarchaea are classified within the order *Halobacteriales*, family *Halobacteriaceae*, which consists of a number of 29 genera (with 113 species) of aerobic extreme halophiles that require high concentration of NaCl for growth, with optimum concentrations of 1.7 - 5.1 M.

Hypersaline environments, such as salt lakes and salt mines, are widely distributed in Romania, mainly in the proximity of the Carpathian Mountains, such as those from Slănic Prahova, Praid, Cacica, Ocna Dej, Târgu Ocna and Ocnele Mari.

Salt exploitation in the Romanian Carpathian area was conducted from antiquity to nowadays, due to the presence of about 200 salt massifs with characteristics that promote continued use, such as surface proximity, superior purities of NaCl, or large reserves. The salt deposit from Slanic (formed in Neogen period) is located underground in Prahova County in the outer Carpathians area, 45 km north of Ploiesti. This salt deposit is 2.8 km long, 0.8 km breadth and between 45.5 m to 499 m thick. The salt is a mixture of crystals of grey and swarthy color, of smaller dimensions than the white ones produced in other salt mines in Romania. This deposit is variegated, as a consequence of turnovers that took place during precipitation process, due to the climatic and sedimentary variation. Salt extraction in Slanic area started in 1685 by using the bell type exploitation technology. After 18th century, some exploitations points were relinquished resulting in the appearance of man-made salt lakes, having various depth and width, known today as Green Bath (Baia Verde), Shepherd Bath (Baia Baciului), Red Bath (Baia Rosie) and Bride Cave (Grota Miresei) lakes.

Some analysis about chemical composition of the water body revealed the presence of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{NH}_4^+$ ,  $\text{Cl}^-$  and other elements.

The other investigated salt lake is Telega (Palada) lake located in Telega village, in the west part of Prahova County. The salt deposit of Telega (Doftana – Telega), formed also in the Neogen period, is a mixture of crystals with color varying from white until to grey and swarthy, having a surface of  $2.1 \text{ km}^2$  and  $0.7 \text{ km}$  thickness. Salt extraction in Telega started before 1685 and was conducted for more than 330 years, until to 1900, by using also the bell type exploitation technology. Before to the year 1872 the salt exploitations point were relinquished resulting in the appearance of various man-made salt lakes, having various depth and width. The Telega (Palada) Lake is situated at the entrance of a saline having a maximum depth around of  $36 \text{ m}$ , a surface of  $1416 \text{ m}^2$ , salinity around  $2.7 \text{ M}$  and pH value around  $8.3$ . The water is also rich in calcium, magnesium and potassium.

The aim of our studies was to characterize by a polyphasic approach using biochemical and molecular methods some haloarchaea strains isolated from various salt lakes (Bride Cave, Shepherd Bath, Red Bath, Green Bath and Telega Lake) and from the salt crystals taken from subterranean salt mine Unirea, located in Slanic Prahova, Romania, at around  $208 \text{ m}$  depth. The obtained data will be useful for comparatively assessment of the strains isolated from salt crystals and those from the hypersaline lakes situated in the nearby area, in order to evaluate the diversity of halophilic archaea present in hypersaline environments from Slanic Prahova.

The number of colony forming units decreased with increasing chloride and sodium concentrations in the investigated salt lakes, but the colonies assigned to be archaea had no apparent correlation with concentrations of these two ions.

The results revealed that all investigated strains grew in the presence of chloramphenicol but were unable to grow on sodium deoxycholate, thus being included into halophilic archaea group. Generally, the haloarchaea strains isolated from salt lakes were characterized by optimal growth at  $2.0 - 4.0 \text{ M NaCl}$ , compared to  $3.0 - 5.0 \text{ M}$  for the strains isolated from salt crystals.

These results showed the predominant presence of *Haloferax* species in the investigated salt lakes. This suggests that members of this genus play an important role in the ecology of salt lakes, even though the largest number of species of the genus *Halorubrum* was identified in hypersaline environments. On the other hand, the microbiota of subterranean rock salt from Slănic area was characterized by the presence of *Halorubrum* (mostly *Hrr. saccharovorum*), *Haloarcula* and *Halobacterium* species. The *Haloferax* members observed in hypersaline lakes located at the surface of Slănic salt deposit were identified also in subterranean rock salt, but in lower number.

## BIOTECHNOLOGICAL APPLICATIONS OF HALOARCHAEA ISOLATED FROM HYPERSALINE LAKES LOCATED IN SLANIC PRAHOVA COUNTY

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For the last years, the microorganisms living under extreme conditions (extremophiles) represent a great interest for scientists, focused on both unraveling the adaptation mechanisms of these microorganisms to extreme environmental conditions, and also on the use of extremophiles as sources of new molecules in biotechnologies. Some extremophiles, particularly *Archaea*, present novel metabolic pathways, and so might serve as a source of metabolites with novel properties and applications.

Haloarchaea, members of the *Halobacteriaceae* family, are a group of extremely halophilic microorganisms which require high salt concentrations for growth, and forming a part of the domain *Archaea*. Haloarchaea are distributed all over the world in hypersaline habitats such as solar salt evaporation ponds, natural salt lakes and salt mines. In the last years there has been considerable interest in exploring the biotechnological applications of this group of microorganisms adapted to live under these extreme environments. Several species of halophilic *Archaea* synthesize different useful products, such as extracellular polysaccharides, poly- $\beta$ -hydroxyalkanoate, bacteriorhodopsin, carotenoid pigments, salt-tolerant enzymes, halocins, biosurfactants (lipids). The ability of haloarchaea to degrade different toxic compounds (hydrocarbons, heavy metals) in the presence of high salt concentrations is also an important applied aspect of this group of extremophilic microorganisms.

Our studies focused on potential biotechnological applications of haloarchaea strains isolated from some hypersaline lakes located in Slanic Prahova County. The aspects investigated were: i) the synthesis of exopolysaccharides by *Haloferax* strains (studying the influence of some physical-chemical parameters on the exopolysaccharides production and chemical characterization of the EPS); ii) the response of the strains to some heavy metals and their capacity to reduce the metal ions concentration from media with high salinity; iii) the isolation and characterization of S-layer from haloarchaeal cell envelope (biochemical characterization, transmission electron microscopic examination, preliminary investigations related to the binding of S-layer to some porous silicon substrates).

The results showed that the optimal conditions for EPS production by *Hfx. prahovense* were the same as those resulting in highest cell growth. The maximum EPS yield (0.475 g %) was obtained in medium with glucose as single carbon source at 2 M NaCl, under stirring conditions, at 37°C. The strain produced EPS also in media with galactose, lactose, maltose, sucrose, or fructose as carbon source. The high salt concentration (5M) and high temperature (45°C) had an inhibitory effect both on growth and EPS synthesis. The polymer of *Hfx. prahovense* was a heteropolysaccharide containing mainly glucose, fructose, galactose and mannose as was observed by TLC. The differential scanning calorimetric analysis revealed that the polymer was stable up to 207°C and the chemical composition observed by TLC was confirmed by FTIR investigations. The FTIR also showed the presence of uronic acids and sulfate in the polymer structure.

The investigated haloarchaea strains were susceptible to Zn and Hg but moderately resistant to Cr and Ni, being classified as tolerant according to criteria proposed by Nieto (1991). The wild strain *Haloferax* sp. TL5 showed a similar behavior to the collection strain *Haloferax mediterranei*; both strains tolerated 5.0 mM Cr and 2.5 mM Ni and Pb. The wild strain *Haloferax* sp. TL5 has a higher susceptibility for Zn ion compared with the collection strain *Haloferax mediterranei*. The *Hfx. prahovense* and *Haloferax* sp. TL5 strains showed the capacity to reduce the concentration of Pb, Cr, Zn and Ni ions from media with high salinity. The two *Haloferax* strains showed the same capacity to reduce the concentration of Pb ion from an initial concentration of 331 mg Pb/L to 5 mg/L. The *Haloferax* sp. TL5 had a higher biosorption capacity of Cr and Ni ions from medium with or without glucose than the collection strain. *Haloferax mediterranei* presented a higher removal activity of Zn ion from media with or without glucose than the wild *Haloferax* TL5 strain. The results revealed that the synthesis of EPS enhanced the reduction activity of Cr, Zn and Ni by the investigated haloarchaeal strains.

The transmission electron microscopic examination of the S-layer isolated from *Haloferax* sp. strain GR 2 (deposited as JCM 13922) showed the existence of the monomolecular crystalline lattice with a highly ordered arrangement in the dense form, while in relaxed form after treatment with 4 M urea. The S-layer proteins attached to all investigated plates of porous silicon, on both hydrophilic and hydrophobic surfaces; but it seemed that the hydrophobic surface was more favorable. Thus, the treatment of silicon plates with hexamethyldisilazane (HMDS), which gives hydrophobic and organic character to the porous silicon surface, increased the amount of attached S-layer protein.

## **TiO<sub>2</sub> and SiO<sub>2</sub> OXIDIC STRUCTURES: ANTIBACTERIAL EFFECT AND THEIR FUNCTIONALIZATION WITH HALOPHILIC PROTEOLYTIC ENZYMES**

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The unique properties of nanomaterials and the various methods available for the preparation of controlled shape and size of nanostructures, provide building blocks for producing nanoscale structures and devices. The convergence of biotechnology and nanotechnology led to the development of hybrid nanomaterials that combine the characteristics of biomaterials (protein, enzyme, DNA) and nanoparticles. Association of nanostructures with biomolecules represents a recent domain of nanobiotechnological research. TiO<sub>2</sub>-based (TNT) and SiO<sub>2</sub> nanotubes can be used as supports for immobilization of biologically active compounds, such as: enzymes, antibodies, microorganisms, drugs, leading to obtain nanocomposites used in biocatalytic processes.

The current work studies the synthesis conditions of silica and TNT nanotubes and their potential antibacterial activity. We also investigated the immobilization of extracellular enzymes with proteolytic activity produced by halophilic microorganisms on TNT nanotubes obtained by hydrothermal procedures and SiO<sub>2</sub> microtubes obtained by sol-gel method. The immobilization capacity was expressed as the amount of enzyme fixed on 1 g of substrate (mg enzyme/ g substrate). The immobilization of proteolytic enzymes on TNT support was more efficient as compared to that obtained in the case of SiO<sub>2</sub> microtubes. The antibacterial activity against *Lysinibacillus sphaericus*, *Bacillus subtilis*, *Escherichia coli* and *Virgibacillus halodenitrificans* was examined using the plate counting method. The viability was determined by measuring the dehydrogenase activity. The results revealed that TNT nanotubes exhibit a higher antibacterial activity as compared with silica.

## ANTIBACTERIAL ACTIVITY OF NANOSTRUCTURED TiO<sub>2</sub> FILMS PREPARED IN DIFFERENT CONDITIONS BY SOL – GEL METHOD

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The use of photocatalysts for organic compounds degradation has been relatively well investigated in the last years. The interest for technological applications of photocatalytic inactivation using TiO<sub>2</sub> under ultraviolet light irradiation increased when Matsunaga *et. al.* reported disinfection of *Escherichia coli* in the presence of TiO<sub>2</sub>/Pt.

The TiO<sub>2</sub> particles act as photocatalysts when are illuminated, generating HO<sup>•</sup> radicals and other reactive oxygen species (ROS) from electrochemical reactions conducted at the surface of photocatalyst. Because of their high level of reactivity, ROS are also characterized by a very short life time. During irradiation, TiO<sub>2</sub> particles are in direct contact or close to microorganisms, the microbial surface being the primary target of the initial oxidative attack. Phospholipids are the integral components of the bacterial cell membrane, and their sensibility to ROS attack is well known.

This study aims to characterize the antibacterial effect of nanostructured TiO<sub>2</sub> films, as treatment of contaminated water with *Escherichia coli*. TiO<sub>2</sub> film was prepared by sol-gel method and the densification of the films was obtained by thermal treatment. These films were doped with Fe and PEG and treated in O<sub>2</sub> and NH<sub>3</sub> at different temperatures.

Under these conditions, bacterial growth is inhibited by the nanostructured TiO<sub>2</sub> films and the photocatalytic activity is correlated with the chemical composition of the coatings.

## THE BACTERIAL AMMONIUM REMOVAL FROM RESIDUAL SOLUTIONS CONSEQUENT ON HYDROTHERMAL SYNTHESIS OF NANOSTRUCTURED $\text{TiO}_2$

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The main pollutants derived from industrial wastewaters are organic and inorganic substances, solved or in suspension, with different harmfulness degree. The microorganisms from these types of environments constitute a diverse microbiota adapted to different physical-chemical conditions, and very important for pollutants biodegradation.

Although  $\text{NH}_3$  is an essential element for life, in high concentrations ammonia nitrogen is extremely toxic to fish and other aquatic life, and also could lead to eutrophication and loss of biodiversity. Removal of the nitrogen compounds, especially ammonia, is one of the main applications in the field of wastewater treatment. Biological removal processes are generally the most efficient and cost-effective solutions. Ammonia removal from wastewater is mainly performed by two microbial conversion steps: nitrification and denitrification. During nitrification, autotrophic nitrifiers aerobically oxidize ammonium (the major form of nitrogen in wastewater) to nitrite, then to nitrate. During denitrification, heterotrophic denitrifiers reduce nitrate to  $\text{N}_2$  gas under absence of oxygen, using organic carbon as electron donor. However, heterotrophic bacteria that can simultaneously perform heterotrophic nitrification and aerobic denitrification would be useful for removing  $\text{NH}_4^+$  and  $\text{NO}_3^-$  aerobically in a wastewater treatment system. Such heterotrophic bacteria could be used to reduce the costs of maintaining anoxic conditions in such treatment system.

The aim of this study was to determine the capacity of heterotrophic bacterial strains isolated from residual solutions with high ammonium content, resulted in hydrothermal synthesis of nanostructured  $\text{TiO}_2$ , to remove or reduce the concentration of these toxic ions. A number of 27 heterotrophic bacterial strains were isolated and characterized considering their morphological and biochemical features, and tested for the ability to reduce the toxic ions concentration. The results showed that the strains were represented by Gram-positive cocci or rods with catalase activity. Some of the strains presented oxidase activity and were able to reduce the nitrate to nitrite, to grow in anaerobic conditions in the presence of nitrate, but did not produce gas from nitrate. The strains isolated from

two samples present differences in their capacity to reduce the concentration of ammonium from residual solutions investigated. Thus, the strains isolated from  $\text{TiO}_2$  – washing water II sample presented a higher capacity to reduce ammonium (78 – 81%) as compared to the strains isolated from  $\text{TiO}_2$  – basic solution sample (18 – 30%). Therefore, these strains represent good candidates for ammonium removal from industrial effluents, in order to decrease the pollution with toxic ions.



## ISOLATION OF SOME BACTERIAL STRAINS WITH OIL DEGRADATIVE POTENTIAL FROM POLLUTED ENVIRONMENTS IN ROMANIA

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The development of decontamination technologies based of accidental or chronical polluted sites by oil derivatives using microorganisms is a current issue of top priority. In view of setting up a collection of microorganisms with degradative potential and proposing solutions for in field applications, this study focuses on the isolation and identification of bacterial strains collected from the oil park Boldesti-Scaieni, Prahova County, an area polluted with oil hydrocarbons. Special attention was paid to hydrocarbon-oxidizing bacteria directly involved in the metabolization of oil contaminants such as crude oil and petroleum derivatives.

Several bacterial strains, mostly Gram negative, were isolated and identified from soil and water samples contaminated with oil and its derivatives from this area.

The growth curve of the strains was performed at 28°C under stirring conditions over a period of time exceeding 48 hours. Some isolates showed degradative activity against crude oil or others petroleum products, present as contaminants of soils or surface waters.

For all these strains, the decontamination percentages obtained of the crude oil ranged between 61.4% and 91.2%, after two weeks incubation. The ability to metabolize various substrates such as glucose, fructose, lactose, and urea was observed of only some of the isolates, when cultivated on the multitest TSI (Triple Sugar Iron) and MIU (Mobility, Indole, Urease) media. The respiratory type of these bacterial strains was determined by measuring the oxidase and catalase activities.

Microscopy analysis and biochemical tests using the BIOLOG system on selective media identified the most active degradative strains as belonging to *Pseudomonas* and *Brevundimonas* genera. Further species identification by PCR amplification and sequencing of their 16S-RNA genes is currently under way.

## MECHANISMS BEHIND RESISTANCE OF *Shewanella putrefaciens* IBB<sub>CT4</sub> AND *Pseudomonas aeruginosa* IBB<sub>CT5</sub> TO TOXIC ORGANIC SOLVENTS

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The high interest for solvent-tolerant bacteria isolated from polluted environments is due to their high biotechnological potential for applications in different remediation technologies of polluted ecosystems. Two bacterial strains, *Shewanella putrefaciens* IBB<sub>CT4</sub> and *Pseudomonas aeruginosa* IBB<sub>CT5</sub> which are tolerant to organic solvents were isolated from Constanta seawater samples. Alkanes (*n*-hexane, *n*-heptane) with logarithm of partition coefficient in a mixture of *n*-octanol and water ( $\log P_{OW}$ ) between 3.86 and 4.39 were less toxic for *Shewanella putrefaciens* IBB<sub>CT4</sub> and *Pseudomonas aeruginosa* IBB<sub>CT5</sub>, as compared to aromatics (toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) with  $\log P_{OW}$  between 2.64 and 3.69. Cell tolerance, cells viability, adhesion and  $\beta$ -galactosidase activity of *Shewanella putrefaciens* IBB<sub>CT4</sub> and *Pseudomonas aeruginosa* IBB<sub>CT5</sub> in the presence of organic solvents differ from one strain to another, and even for the same bacterial strain, depending on the type of the hydrophobic substrate. The mechanisms underlying solvent tolerance in *Shewanella putrefaciens* IBB<sub>CT4</sub> and *Pseudomonas aeruginosa* IBB<sub>CT5</sub> showed a complex response of bacterial cells, including modification of cell hydrophobicity, changes in the membrane's lipid and protein content, to the presence of organic solvents in the culture medium. These results indicated that *Pseudomonas aeruginosa* IBB<sub>CT5</sub> is more tolerant to organic solvents than *Shewanella putrefaciens* IBB<sub>CT4</sub>, although both bacterial strains contain catabolic genes involved in known solvent biodegradative pathways and transporter genes critical for toxic compounds resistance.

## THE BACTERIAL DESULPHURIZATION OF DIFFERENT COAL SAMPLES FROM ROMANIAN MINING SITES

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Pollution from coal combustion is the largest problem raised by the current use of coal and the biggest constraint on its increased use. The main objection to the combustion of high-sulfur fuels is the production of sulfur oxides which may play a major role in the formation of acid deposition with detrimental effects on the environment. These considerations led to renew the interest in studying the basic mechanisms of microbial sulfur transformations and the technology for microbial precombustion desulphurization of fossil fuels. During the past decade, a special attention was paid to the microbial biodiversity that could constitute an ecological source for selecting adequate microorganisms for environment bioremediation. The microbial communities evidenced in such sites include both groups of chemolithotrophic and heterotrophic microorganisms involved in metals solubilization, bioaccumulation or biosorption processes. The role of bacteria in oxidation of insoluble iron sulphide (pyrite) and other metal sulfides to soluble ions is well established. Pyrite solubilization is well documented in the gram-negative chemolithotrophic *Acidithiobacillus ferrooxidans*, which utilizes either ferrous iron or reduced inorganic sulfur compounds as a sole energy source. Best results on coal desulphurization were obtained with mixed cultures of *Acidithiobacillus thiooxidans* and *A. ferrooxidans*. The presence of other species (such as neutrophilic heterotrophic bacteria) in the desulfurizing culture increased the rate of coal desulphurization. Some of these species may also metabolize simple organic sulfur, thus degrading a small percentage of the organic sulfur remaining in coal matrix.

This study focused on: (i) the quantitative determinations of the physiological groups of microorganisms present in the coal samples isolated from the two Romanian coal mines; (ii) isolation of microorganisms as pure cultures and phenotypical characterization of the strains; (iii) the testing of organic acid biosynthesis by the isolated aerobic heterotrophic bacteria; (iv) the coal desulphurization experiments using both the aerobic heterotrophic bacteria and the chemolithotrophic bacteria; (v) the study for efficiency improvement of the coal biodepyritization processes using the acidophilic chemolithotrophic bacteria like *A. ferrooxidans*.

The results revealed that the microorganisms from coal samples belong to the following physiological groups: aerobic heterotrophic bacteria (neutrophilic and acidophilic), strictly anaerobic heterotrophic (sulphur-reducing), nitrifying bacteria, denitrifying bacteria and acidophilic chemolithotrophic bacteria on  $\text{Fe}^{2+}$ , on  $\text{S}^0$  and on  $\text{S}_2\text{O}_3$ . The higher number of heterotrophic neutrophilic bacteria was obtained from raw coal, as compared to non-magnetic and mixed samples. 14 strains or mixed cultures of heterotrophic neutrophilic bacteria have biosynthesized organic acids on selective medium. A decrease of coal mass was obtained during desulphurization with heterotrophic bacteria mixed cultures. The loss of coal mass was more important in the lignite sample (13%) in comparison with the mass reduction observed in pit coal samples (7.6%). The oxidative activity of *A. ferrooxidans* cultures was optimal at pH values close to the acidity of their habitats. The adapting of the P<sub>7</sub> population to higher concentrations of metal ions increased the efficiency of coal biodepyritization to 67.01-86.64%, as compared to those obtained when using P<sub>9</sub> population with the low tolerance to ferrous sulphate (40.34-57.12%).

## STRESS RESPONSES OF SOME LACTIC ACID BACTERIA ISOLATED FROM ROMANIAN FERMENTED DAIRY PRODUCTS

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Lactic acid bacteria (LAB) are widely used as starter cultures for the production of fermented dairy products. The starter strains should resist to adverse conditions encountered in industrial processes (i.e. low and high temperatures, low pH, the presence of salt). On the other hand, LAB have recently found new applications such as probiotics, vaccines, nutraceuticals, and therefore they need to survive in the digestive tract, maybe colonize the digestive mucosa and express their specific functions in this environment with unfavorable conditions (i.e. low pH, the presence of bile salts, starvation). The aim of this study was to characterize the responses to various stresses of some LAB strains isolated from artisan dairy products, one bacteriocin-producing strain, *Lactobacillus acidophilus* IBB801, and four exopolysaccharide-producing strains: *Leuconostoc citreum* 1.11, *Leuconostoc pseudomesenteroides* 20.6, *Leuconostoc mesenteroides* 21.2 and *Weissella confusa/cibaria* 38.2.

Strains were subjected to different stress conditions: temperatures ranging from 10°C to 60°C, pH values from 2.0 to 6.5, addition to the growth medium of NaCl up to 6 % or bile salts up to 0.4 %. Growth or survival was followed by OD<sub>600</sub> and pH measurements and viable cells (cfu/ml) counting, respectively. Protein extracts obtained by sonication were used to study the activity of some intracellular enzymes (lactate dehydrogenase, malate dehydrogenase, alcohol dehydrogenase, superoxide dismutase), and the electrophoretic pattern of proteins and isoenzymes. Phospholipid composition was also investigated by thin-layer chromatography for the strains grown in the presence of bile salts.

All strains grew at temperatures ranging between 24°C and 42°C and they showed a slight growth at 10°C. High temperatures (over 45°C) were lethal to most of them, except for *L. acidophilus* IBB801, which still grew at 47°C, but did not grow at temperatures higher than 50°C. This strain was also able to grow in the presence of 0.2 % of bile salts or in the medium with an initial pH of 4.0. The maximum NaCl concentration which allowed the growth was 6 %, in the case of *Weissella confusa/cibaria* 38.2. Enzymatic activities showed significant differences between strains incubated under different stress conditions. The protein profiles revealed by one dimensional SDS-PAGE showed overexpressed or additional bands for the stressed variants, probably heat shock proteins. Moreover, the

bacteriocin production by the strain *L. acidophilus* IBB801 was stimulated under some mild stress conditions, like the presence of low amounts of salt in the growth medium or incubation at 10°C. Thin layer chromatography showed significant differences of the phospholipid composition of the strains grown in the presence of bile salts comparing with the controls.

As shown here, LAB, like many other bacteria, have evolved specific mechanisms to adapt and survive in less favourable environments, mainly the synthesis of some specific proteins, but also changes in the phospholipid composition.

## THE USE OF QUANTUM DOTS TO VISUALIZE HETEROTROPHIC AND PHOTOSYNTHETIC BACTERIA IN PURE CULTURES AND MICROCOSMS

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Semiconductor quantum dots are nanometer-sized crystals of  $3 \div 5$  nm diameter with unique photochemical and photophysical properties that are not available from either isolated molecules or bulk solids. The aim of this study is to investigate the use of CdSe/ZnS core-shell quantum dots emitting at 490nm, 520nm, 560nm or 600nm for visualizing heterotrophic and photosynthetic bacteria in pure cultures and microcosms. The microcosms consist of water samples from the Black Sea collected at 0.5 m depth as natural sample control (M3), a control supplemented with petroleum hydrocarbons (gasoline-0.25% v/w) (M2), and M3 control supplemented with nutrients (ammonium nitrate/ acetate 0.005% w/w) (M1). Incubation parameters of the microcosm varied with respect to: i) the nature and concentration of nutrients; ii) incubation temperature and light source, iii) use of unfiltered or filtered seawater using 0.45  $\mu$ m pore filters to eliminate bacteriovorous microorganisms. The microscopic investigations using CdSe/ZnS core-shell quantum dots focused on the interactions of QD with pure cultures of cyanobacteria (*Synechocystis* sp. PCC 6803) and alive or dead heterotrophic bacteria (Gram- negative and Gram-positive e.g. *Escherichia coli* and *Staphylococcus hominis*), in order to check the possibility to use different QD for the visualization and quantification of bacterial cells (different types of prokaryotic cells). Cytotoxicity of the QD against heterotrophic bacteria (*E. coli* and *S. hominis*) and cyanobacteria was also investigated, including correlations between the ability of the QD to label the cells and QD's cytotoxicity.

The microphotographs were analyzed by CellC automated image software for processing multiple digital microscope images for count cells (<http://www.cs.tut.fi/sgn/csb/cellc/>). In addition, we used ImageJ and CellProfiler for the cell counting, the size and shape of individual cell measurements, or the fluoresce intensity of cyanobacteria filaments labeled with QD. This software extracts quantitative information from biological images, including step-by-step protocol for automated analysis of the number, colour and size of bacteria/cyanobacteria cells growing on

microcosm, being adapted also to identify and measure any objects in images. The flexibility of the software allows users to tailor pipelines of adjustable modules to fit different biological experiments, to generate accurate measurements from dozens or even hundreds of thousands of digital images.



## **IMPROVED LYSOZYME METHOD TO OBTAIN CYANOBACTERIA AXENIC CULTURES**

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This study presents an improved purification method to obtain cyanobacteria axenic cultures using the lysozyme method supplemented with the second antibiotic tienam. Two axenic cultures were isolated by the newly improved antibiotic method that consists of the classical antibiotic method using lysozyme in which the bacterial growth was sustained before the addition of the second antibiotic, tienam. Basically, the lysozyme-treated bacterial suspension was first supplemented with LB medium to promote the growth of heterotrophic contaminants, followed by addition of tienam, so the heterotrophic bacterial contaminants are in contact with this second antibiotic during their active growth. Under these conditions, axenic cultures were easily obtained. No contaminant colonies appeared after 24 hour of incubation on LB, the culture purity being also verified by epifluorescence. This improved method seems to be suitable for the purification of cyanobacteria from different natural sources and presenting different morphologies.

# THE INFLUENCE OF TEMPERATURE ON EXTRACELLULAR HYDROLASES FROM *Acidiphilium* SPECIES ISOLATED FROM ACIDIC MINING EFFLUENTS

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The ability of microorganisms to adapt to acidic environments is highly exploited in bioremediation of mining effluents containing high concentrations of heavy metals. Among the enzymes involved in metal biosorption, extracellular amylases are one of the most important and widely used in biotechnologies. The enzymatic activity of these extremophiles is influenced by the pH and temperature of the bacterial environment.

Recently, we isolated several strains from mining effluents of two different sulphidic mines from Romania, Rosia Poieni, Alba, and Ilba, Maramures (Cismasiu, 2004). These strains, identified as acidophilic mesophilic heterotrophic bacteria belonging to *Acidiphilium* genus, were highly adapted to these polluted environments.

This current work focused on the study of extracellular hydrolases from two *Acidiphilium* species isolated from acidic mining effluents, considering their putative applications in bioremediation. We investigated the influence of growth temperature on their amylolytic activity, using starch as carbon and energy sources. The temperatures considered were 20°C, 24°C and 28°C, in accordance with the variations of this parameter in the effluents' environment.

Bacterial growth was carried out at different temperatures and pH for up to 21 days, under stirring or static conditions, and in the presence of various concentrations of yeast extract. The amylolytic hydrolyzing activity of the extracellular enzymes in the culture media was measured spectrophotometrically, using Wohlgemuth method (Gupta et al., 2003), by monitoring the starch-iodine complex hydrolysis.

Under these conditions, the highest extracellular amylolytic activity of both *Acidiphilium* strains was obtained at 28°C and pH 3, while at 20°C the microbial hydrolysis was negligible, as expected for a mesophilic species. This enzymatic activity was dependent on the yeast extract concentration, with an optimum at 0.2%, while for values lower than 0.1% the extracellular hydrolases activity was absent. Moreover, the amylolytic activity increased when cells were cultivated under stirring conditions as compared to static conditions.

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## CLONING AND EXPRESSION OF CARBAMOYL PHOSPHATE SYNTHETASE GENES FROM *Methanobrevibacter smithii*

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*Methanobrevibacter smithii*, the major hydrogen-consuming methanogenic archaeon identified in the human gut, possesses two putative carbamoyl phosphate synthetases (CPS) involved in the arginine and pyrimidines biosynthetic pathways. Structure-function characterization of this unique CPS system from a one of the main representative of human gut microbiota aims to analyze its possible important input in host obesity mechanisms.

Screening of *M. smithii* genome revealed the existence of an atypical CPS enzymatic system composed of three genes: *carA* (1080 bp) coding for a glutaminase subunit (GLN - 360 aa), *carB1* (3174 bp) and *carB2* (1101 bp) coding for two different synthetasic subunits (SYN) of 1058 and 367 aminoacids, respectively. An additional putative *carB* gene of SYN 1173 bp encodes a corresponding SYN subunit that lacks essential residues in the active site.

This study focuses on obtaining the recombinant CPSs enzymes from *M. smithii* by cloning and expression in *Escherichia coli* of the genes that correspond to presumed active subunits.

*M. smithii* was cultivated anaerobically at 37°C, under a gas atmosphere of 80% H<sub>2</sub> and 20% CO<sub>2</sub>. The growth medium containing a mineral and a fatty acid mixture at pH 7 was prepared under anaerobic conditions. *M. smithii* *carA*, *carB1* and *carB2* genes were amplified by PCR using specific primers and genomic DNA as template, and ligated in pRSET bacterial expression vectors (Qiagen) after digestion with restriction enzymes. The plasmids obtained, pGLN, pSYN1 and pSYN2, appending a His-tag sequence to the amino end of the recombinant protein to facilitate the purification in one step.

The expression of these recombinant CPS subunits CarA, CarB1 and CarB2 was obtain into BL21(DE3) *E. coli* strain by co-transformation with the constructs and pSJS1240 vector containing the genes for rare codons in *E. coli*.

The transformed cells were cultivated at 25°C, 30°C and 37°C up to 18 hours in LB medium containing 100 µg/ml ampicillin and 50 µg/ml spectinomycin, after induction with 1 mM IPTG. The cells were disrupted by sonication and centrifuged, and the soluble and insoluble fractions were analyzed by SDS-PAGE. Under these conditions, the optimal parameters for

obtaining soluble CPS subunits were (a) GLN – 6 hours at 37°C, (b) SYN1 – 18 hours at 30°C, and (c) SYN2 – 3-6 hours at 37 °C. Purification of these CPS subunits is currently under way using  $\text{Ni}^{2+}$  based affinity chromatography.

Further functional studies of the reconstituted CPS recombinant enzymes from this human archaeon will be carried out for identifying a putative obesity controlling target.

# **MORPHOLOGICAL AND MOLECULAR CHANGES INDUCED BY APOPTOSIS FOLLOWING TREATMENT WITH *P. aeruginosa* BACTERIAL CULTURE AND CELL FREE CULTURE IN ENDOTHELIAL CELLS**

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The aim of this study was to investigate the morphological and molecular changes induced by apoptosis following treatment with *P. aeruginosa* bacterial culture and cell free culture in endothelial cells.

All strains were evaluated for virulence factors expression: haemolysins, other pore forming toxins (lecithinases, lipases), proteases (caseinases, gelatinase), DNA-ses, mucinases. The adherence and invasion capacity of *P. aeruginosa* to endothelia cell was done using Cravioto's adapted method. The presence of *P. aeruginosa* inside the endothelia cells was demonstrated by transmission electron microscopy. For apoptosis detection was used acridine orange/ethidium bromide staining. Expression of apoptotic genes such as caspase 3, caspase 9, Bax, Bcl-2 and MCL-1 was achieved by qRT-PCR.

The qualitative assay of the bacterial adherence to the cellular substrate demonstrated that all tested strains adhered to endothelial cells, exhibiting a diffuse, aggregative or mixed diffuse-aggregative pattern and 20-70% adherence rates. *P. aeruginosa* strains proved also the ability to invade the epithelial, non-phagocytic cells. In case of bacterial culture, acridine orange staining and qRT-PCR proved the capacity to induce apoptosis, presenting an increase expression of caspase 3 and Bax genes, and also a decrease level of anti-apoptotic factor Bcl-2. In case of cell free culture only Bax gene was over expressed, while anti-apoptotic genes (MCL-1 and Bcl-2) are expressed at lower levels than control.

*P. aeruginosa*, regarded until recently as an exclusively extracellular pathogen, expressed capacity to induced apoptosis in endothelial cell. Regarding cell free culture, the present study didn't show their capacity to induce apoptosis by caspase-pathway.

## **THE KINEMATIC AND DYNAMIC PARAMETERS OF THE MAGNETOTACTIC BACTERIUM**

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The magnetotactic bacterium is a prokaryote which contain magnetosome and is very sensitive to magnetic field.

The parameter of motion of the magnetotactic bacterium was studied. The authors present and comment the different kinematic and dynamic parameter fields (by different references ); the microdisplacement, the speed, the acceleration, the microforce, the specific nano and microtorque of the bacterium on magnetic field, The Reynolds hydrodynamics number etc. Also there are presented some kinematic specific experiments of the authors.

In paper is presented an micromechanical model of magnetotactic bacterium.

## **THE ELECTROMECHANICAL ASPECTS ABOUT THE FLAGELLAR ROTARY NANOMOTOR**

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The flagellar rotary nanomotor is one of the interesting microbiological systems.

This pape presents the functional principle with different electromechanical aspects; the main nanocomponents (as stator and rotor),the role of the proton flux to the movement of the nanomotor, the electrostatic nanotorque (with the nanorotor arhitecture of charges), the relation of the steady state of rotation, the nanomotor frequency, the flagellum geometry, nanomechanical properties and motility.

The authors comment the different models of the flagellar rotary nanomotor.

The distinct motion of flagellar rotary nanomotor can be utilized to perform various nanoelectromechanical a ctuators in microfluidic systems, such as pumping.





**PLANT AND ANIMAL CYTOBIOLOGY,  
BIOTECHNOLOGY**

**POSTERS**



## **BONES OF THE HEART: MÖNCKEBERG'S SCLEROSIS LIKE OF THE PAPILLARY MUSCLES IN A DIABETIC PATIENT**

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The complete calcification of the cardiac papillary muscles (PMs) is quite a rare finding and was previously observed within the left postero-medial papillary muscles and correlated with myocardial infarction. There are no existing evidences on the pathologic changes within such calcified PMs. We report here such a rare case of PMs calcification, discovered incidentally at autopsy in an human adult male, known as positive for type II diabetes. The left postero-medial PMs were extensively/completely calcified at the macroscopic inspection. Microscopy revealed on the HE stains different histological alterations: (1) focal calcifications; (2) osseous metaplasia, appearing as foci of Mönckeberg's sclerosis, with peripheral post necrotic calcifications of these foci; (3) aspects of chronic myocardial inflammatory disease, overridden by elements of acute myocardial infarction; (4) diffuse hyaline fibrosis; (5) particular aspects of longitudinal calcification of the PM core, with deposits of osteoid on a preserved cytoskeleton, with persisting intercalar discs, of the cardiac muscle. Our findings appear important as time as it is known that Mönckeberg's sclerosis presence predicts risk of cardiovascular events and leg amputation in diabetic patients.

# **THE IMMUNOHISTOCHEMICAL DETECTION OF SUBSTANCE P (SP) IN THE PANCREAS AND INTESTINE OF THREE SPECIES OF LOWER VERTEBRATES; LIGHT MICROSCOPIC OBSERVATIONS**

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The substance P was the first regulatory peptide with a dual localization (intestine and brain) proved since 1931 (von Euler and Gaddum, 1931). Isolated, chemically characterized and much later synthesized (Chang et al., 1971; Tregear et al., 1971), this neuropeptide, with a sequence of 11 amino acid residues, represents the prototype of a peptides family entitled tachikins (Eledoisin, Physalemin, Uperolein a.o) involved in the prompt stimulation of the smooth musculature.

The greatest amount of SP was identified in various brain formations such as substance nigra, hypothalamus, limbic system, dorsal rachidian bulb and dorsal pons of Varolli, where was detected immunohistochemically both in perikarya as well as in their neuronal extensions.

Extraneuroaxial SP appeared co-localized with somatostatin (SOM) in the perikaryons of paravertebral and perivertebral ganglia, including adrenal medulla, but also in the nonmyelinated nerve fibers (Bertaccini, 1980; Konturek et al., 1981; Trümper et al., 1982).

At the level of alimentary tract substance P was immunohistochemically identified alone or co-localized with serotonin (5HT) or with SOM in some endocrine cells disseminated from the esophagus to the colon, as well as in the intrinsic nervous plexuses (Bertaccini, 1976; 1980). Finally, the occurrence of neuropeptide was revealed not only in the mucous cells of the gallbladder, in the respiratory and genito-urinary tracts, in the nervous fibers of the salivary glands and of the exocrine pancreas, but also in the hypophysis and pancreatic islets (Powell and Skrabanek, 1970; 1981). Taking into account the above results regarding the various SP localizations in the mammalian and human organs, the purpose of this study was to detect immunohistochemically in light-microscopy, by using peroxidase anti-peroxidase (PAP) procedure, the occurrence and distribution of this neuropeptide in the pancreas and intestine of two amphibians and a reptile species.

The results revealed a relative rich SP innervation consisting of nerve fibers with a peri-acinar distribution both in frogs and turtle pancreas. In turtles, thick bundles of nerve fibers, localized in the

pancreatic connective tissue, have been also observed. In addition to the neural structures, the pancreas of frogs and turtles displayed often singular immunostained cells of endocrine nature. Finally, both varieties of “closed” and “open” enteroendocrine cells have been immunodetected in the villi epithelium of these animals.

This paper is one of the few immunohistochemical demonstrations dedicated to the occurrence and topographic distribution of SP in the pancreas and intestine of poikilotherm vertebrates. Its purpose is to enrich our knowledge on the presumed phylogenetic perenity of this multifunctional neuropeptide in the above glandular organs, considering that its multiple functional involvements are still debated and even contradictory.

## METABOLIC AND REGULATORY FEATURES OF THE TOMATO CHROMOPLAST REVEALED BY PROTEOMIC ANALYSIS

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During evolution higher plants have adopted strategies to attract insects and mammals so as to facilitate flower pollination and seed dispersal. One of these strategies has been the development of bright colors most often within a type of plastids named chromoplasts. Chromoplasts are responsible for yellow, orange or red colours of many flowers and fruits. They are also present in some roots, such as carrot, or tubers such as sweet potatoes. Plastids are typical organelles unique to lower and higher plants that originate from the endosymbiotic integration of a photosynthetic prokaryote, cyanobacterium, into a eukaryotic ancestor of algae. The ancestors of plastids, chloroplasts, have diversified into a variety of other plastid types, including chromoplasts to carry-out specialized functions in non-photosynthetic organs (Pyke 2007).

Chromoplast differentiation proceeds from pre-existing plastids, most often chloroplasts. One of the most prominent changes is the remodelling of the internal membrane system associated with the formation of carotenoid-accumulating structures. Chromoplasts that accumulate pigments during fruit ripening and flower development are functionally different from senescence-derived plastids. The yellow colour of senescent plastids is due to the disappearance of chlorophyll and retention of carotenoids in the absence of *de novo* carotenoid biosynthesis. In addition, contrary to chromoplasts, they undergo an extensive loss of plastidic DNA and are designed as gerontoplasts (Matile 2000).

During the differentiation process the plastid genome is essentially stable and transcriptional activity is restricted. The build-up of the

chromoplast for giving its specific metabolic characteristics is essentially dependent upon transcriptional activity of the nucleus.

The chloroplast-chromoplast transition is one of the most important changes occurring during ripening of tomato fruit (*Solanum lycopersicum*). Analysis of the proteome of red fruit chromoplasts revealed the presence of 989 proteins corresponding to 803 Arabidopsis unigenes, among which 210 had not been listed so far in plastidial data banks. These data allowed revealing several specific metabolic and regulatory features of the chromoplast. Proteins of lipid metabolism and trafficking were well represented, including all the proteins of the lipoxygenase pathway for the synthesis of lipid-derived aroma volatiles. Proteins involved in starch synthesis co-existed with several starch-degrading proteins and starch excess proteins. Chromoplasts lost all proteins of the chlorophyll biosynthesis branch and, similarly to senescent chloroplasts, they harboured proteins involved in chlorophyll degradation. Surprisingly, they kept the whole set of proteins of the Calvin cycle, including Rubisco, and the oxidative pentose phosphate pathway (OxPPP). The absence of thylakoid transport machinery reflected a structural disintegration of thylakoids. Therefore, the major feature of the chromoplast proteome, besides losing proteins of the chlorophyll synthesizing pathway and most of the thylakoid transport proteins, is the persistence of basal plastidial proteins of the Calvin and OxPPP. The present proteome analysis suggests that tomato chromoplast could use the chloroplastic pre-existing machinery (Calvin cycle and OxPPP), for re-orientating the metabolism towards the accumulation of carotenoids.



## THE *IN VITRO* BIOSYNTHETIC POTENTIAL IMPROVEMENT OF *Vitis vinifera* L. CALLUS EXTRACTS UNDER THE INFLUENCE OF SOME ELICITORS

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Plant cell culture has been shown as a feasible experimental system, successfully exploited for secondary metabolite production. This provides an opportunity for the extensive manipulation to enhance production of these natural products over levels found in intact plants and to identify parameters for improving productivity. Because of their biological activities, some plant natural products have long been exploited by human beings as pharmaceuticals, stimulants, and poisons. In recent years much attention has been devoted to the natural antioxidants and their association with health benefits, plants being an important potential source of its.

Plant phenolics (anthocyanins and resveratrol) have been reported to have multiple biological effects, including antioxidant activity, mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. Based on large applicability of secondary metabolites resveratrol and anthocyanin pigments in pharmaceutical, cosmetic and food industry, the study of their biosynthesis constitutes a real challenge for the all scientific world.

The experiments demonstrate that anthocyanins occur in a large number of callus and suspension cultures but excellent results on anthocyanin and resveratrol biosynthesis were obtained by us using *V. vinifera* cultivars. The elicitation with biotic or abiotic stimuli represents the most efficient strategy for increasing the production of those metabolites in *in vitro* tissue culture.

In this context, we developed a multidisciplinary research in order to elaborate an original and efficient technology for obtaining the above mentioned secondary metabolites of interest based on modern biotechnologies. We have followed during the research to stimulate the biosynthesis of these two secondary metabolites in a *long-term* non-morfogen callus culture initiated from the pericarp of *Vitis vinifera* L. cvs. Isabelle grape berries and in the two primary callus culture of *Vitis vinifera* L. cvs. Negru Vartos and Grasa de Cotnari initiated from the leaf fragments. This process was achieved using some biotic (fungal extracts of *Fusarium oxysporum* and *Botrytis cinerea*) and abiotic elicitors (salycilic, abscisic, jasmonic acids, ethephon and mannitol) for the modulation of the

proliferative and biosynthetic capacity of this plant system. Growth medium for initiation, subcultivation and treatment with elicitors consisted in a variant of basal Gamborg-B5 (1968) medium, supplemented with 0.1 mg/l NAA ( $\alpha$ -naphthalene acetic acid), 0.2 mg/l kinetine, 2 mg/l casein hydrolisate, 30g/l zaharose, 8g/l agar (Difco).

The subsequent experiments accomplished during the study have been pursued studies concerning the antioxidant and antitumoral properties of this plant system correlated with the quantitative determination of total phenols, anthocyanins and resveratrol present to the callus extracts level. It has been tested a large scale of concentrations and combinations of elicitors for identifying the optimum culture system capable of improving the biosynthetic capacity of *Vitis vinifera* L. calus extracts.

The results of the experiments revealed that the highest concentration of anthocyanins in the callus extracts of *Vitis vinifera* cvs. Isabelle could be achieved using a two-stage culture system which involved the presence of 2mM mannitol for the proliferative and 0.1 mM ethephon for the biosynthetic stages. A screening of the anthocyanins varieties at the level of the callus extracts using a HPLC method demonstrated the presence of compounds such as: malvidin, cyanidin and delphinidin chloride.

The culture system containing in the first stage mannitol (2mM) and in the second stage jasmonic acid (40  $\mu$ M) proved to be the most efficient regarding the resveratrol and total phenols accumulation at the level of the *Vitis vinifera* cvs. Isabelle callus extracts. A DPPH test for the antioxidant activity indicated that the callus extract under the influence of the two-stage system using mannitol (2mM) - jasmonic acid (40  $\mu$ M) offered the highest antioxidant character. The data is sustained by the high concentration of the total phenols and resveratrol, compounds which are in general responsible for the antioxidant and antitumoral activities. Regarding the antitumoral activity a MTT test for demonstrating the cellular viability of a hepatocellular carcinoma Hep G2 cell line under the influence of the callus extracts were also achieved. The data obtained denote that the callus extract of the *Vitis vinifera* cvs. Isabelle under the effect of the elicitors mannitol (2mM) – ethephon (0.1mM) proved to have the highest antitumoral activity. Analyzing the data obtained by comparing the callus extracts from the three *Vitis vinifera* varieties regarding the biosynthetic capacity we can conclude that the *long-term* callus of cvs. Isabelle and the primary callus of cvs. Negru Vartos provide a convenient experimental system for the study of anthocyanin and resveratrol pathways and eliciting factors that could optimize them.

The original data obtained by us permitted to design new system for the modulation of callus proliferative and secondary metabolites (anthocyanins and resveratrol) biosynthetic potential. The study represents a novelty regarding the presence of these two important secondary

metabolites in the same system and the modulation of the biosynthetic capacity of the system using these concentrations and combination of elicitors.

## THE EFFECTS OF SOME BACTERIAL STRAINS ON FUNGAL PATHOGENS AND PLANT GROWTH PROMOTION

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In their natural environment, plants interact with various pathogenic and non-pathogenic microorganisms. These are crucial for plant's life, on the one hand acting as plant pathogens, and on the other hand as plant growth promoters, activators of systemic resistance, or antagonists for plant pathogens. Except for plant pathogens, these microorganisms are very useful for plant development and health, being involved in biocontrol of plant pests.

Plant Growth Promoting Bacteria (PGPB) is the most abundant group of microorganisms involved in biocontrol, and within them, the root-colonizing bacteria, also known as Plant Growth Promoting Rhizobacteria (PGPR) is the most important group. PGPRs as biocontrol agents can have indirect and direct modes of action. Direct modes of action are defined as the mechanisms by which PGPRs influence directly the plant, while indirect modes of action are those by which the pathogen is directly affected, and the plant is influenced only in an indirect manner.

The current study focused on some aspects of the interaction between some antagonistic bacterial strains, fungal phytopathogens and plants. In order to understand the mechanisms of these complex interactions, the cellular and biochemical modifications induced by antagonistic bacteria on both fungal phytopathogen and plant were evaluated. The effects of bacterial strains on fungi were observed primarily using dual cultures method, by which the inhibitory activity of phytopathogenic fungi was quantified. Further, cellular-level modifications, like plasmalemmal detachment and cytoplasm vacuolization, were evidenced using transmission electron microscopy. Also, the lytic enzymes production was tested on specific media; however, bacterial strains were positive only for protease production.

The direct modes of bacteria action on plants were also analyzed, by the effect of treatments with bacteria on plant growth. First, we observed that the seeds treated with bacterial suspension present an initial delay in the germination process, delay that afterwards is recovered. Second, colonization with bacterial strains induces in both vegetative, but mostly in radicular system, modifications in activity of antioxidant enzymes, like peroxidases, superoxid dismutases and catalases. The same as in the case of

treatment with pathogens, an increase in enzymatic activity was identified, but in a lower degree. Third, transmission electron micrographs showed that bacteria colonize root surface, and also root epidermis. Also, the excretion of vesicles and electron-opaque material from the cell walls was observed.

We concluded that at least two of the bacterial strains inhibit significantly the fungal growth of the four phytopathogenic species and induce cellular-level modifications on fungal cells. Also, the enhanced enzymatic activities of the samples treated with bacteria indicate the activation of the antioxidant system.

## **IN VITRO CONSERVATION OF *Campanula polymorpha* WITAS. – AN ENDEMIC PLANT OF CARPATHIAN MOUNTAINS**

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The loss of plant genetic resources has made necessary the development of new *ex-situ* conservation methods. Among the tools of modern biotechnologies tissue culture is being increasingly applied to complement other *in-situ* or *ex-situ* methods. Within the Institute of Biology from Bucharest, several *in vitro* techniques have been developed, with recent establishment of endangered plant tissue culture collections.

*Campanula polymorpha* is an endemic plant of Carpathian Mountains, included in the Carpathian List of Endangered Species. An efficient micropropagation system was developed for regeneration and *in vitro* conservation of *Campanula polymorpha*. Single-node aerial stem segments from mature flowering plants collected from natural growing populations, was sterile inoculated on regeneration media. For an efficient regeneration a range of growth regulation combinations was tested within 12 Murashige and Skoog (MS) culture media formulation. The best rate of shoot production (27 shoot/explant) was achieved after 30 days of culture on media supplemented with 0.1mg/l NAA (1-naphthylacetic acid) and 1mg/l BAP (6-benzylaminopurine). Shoots of about 5-7 cm high, developed roots after 12-14 days on media without growth regulators.

The established protocols offer a valuable alternative *ex situ* conservation method for this threatened plant and could be useful also for *ex-situ* conservation of other endangered *Campanula* species.

## **IN VITRO CONSERVATION OF SOME SPECIES FROM THE ROMANIAN COAST OF THE BLACK SEA**

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Nowadays, *in situ* conservation strategies applied for some plant species in such global conditions (habitat destruction, transfrontier pollution, dramatic climate change, intensive harvesting for commercial purposes), prove to be insufficient or inapplicable. Therefore other conservation methods must be enforced, such as *in vitro* conservation (tissue and cell cultures) and *ex vitro* (botanical gardens). The *in vitro* culture represents today one of the most important strategy for plant germoplasm conservation.

Various methodologies have been used with the purpose of including in the collection some endangered taxons such as: *Ecballium elaterium* L., *Cucurbitaceae* family (vulnerable), *Plumbago europaea* L., *Plumbaginaceae* family (critical endangered) and *Crocus pallasii* Goldb., *Iridaceae* family (endangered), by using *in vitro* culture system and clonal multiplication (variants of the basal Murashige & Skoog 1962 (MS) culture medium was used as cell culture media). Thus, MS basal cell culture medium was used, having as inocula sources seedlings resulted from aseptic germination of mature seeds. In case of *Ecballium elaterium* L. species, the explants consisted in caulinary apex and single node segments, and were cultivated on MS culture medium, with the addition of 1mg/l indolil butyric acid (IBA). In the attempt to induce rooting, these were cultivated on different variants of MS medium, enriched with various combinations of auxins and cytokinins. 0,5- 2mg/l IBA has considerably stimulate the rhizogenesis; the roots have been regenerated at a high rate especially after using the variant containing 1mg/l of IBA. *Ecbalium elaterium* roots and cucumber-shaped fruit have been used in folk medicine since antiquity. The alleged use of the fruit juice is known as a potent cathartic, analgesic, and anti inflammatory agent. Cucurbitacin B, a triterpene derivative being the active anti inflammatory agent.

*Plumbago europaea* L. species was added to the endangered fitotaxons collection using as inocula source axillary and apical buds, grown on MS medium, supplemented with 0.18-2 mg/l indolil acetic acid (IAA) in combination with a concentration between 0.05 and 0.02 mg/l kinetine. *Plumbago europaea* L contains a bitter substance called plumbagina presents to the root cortex level. Both the roots and the fresh leaves, mashed and applied topically, produced a reddening and swelling of the skin

instantly, to the point of blistering fast. This property is known as rubefacient and vesicant. Poultices prepared from this plant represent remedy for many ills, like pain from sciatica, lumbago and persistent headaches.

The growth rate and the speed growth of the regenerants depended of each species. The most reactive of all-given *in vitro* conditions was *Ecballium elaterium* L., *Crocus pallasii* Goldb. registered a very slow rhythm of seeds germination and seedlings growth, on all of the nutritive culture media tested so far. Our attention is concentrated now on the optimization of the experimental conditions and trials using larger variety of hormones.

Studies in progress concern two aspects: regeneration and plant multiplication for a conservation strategy and callus culture initiation in order to obtain cellular mass for the biosynthesis of secondary metabolites with biotechnological and pharmaceutical properties.



## **IN VITRO COLLECTIONS - AS TOOL FOR EX SITU PRESERVATION OF THREATENED VASCULAR PLANTS**

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According to Convention on Biological Diversity, *in situ* conservation of the natural habitats and ecosystems is complemented with *ex situ* approach that means the removal of plant genetic resources outside of their natural habitats and the preservation through different ways as: field collections, botanical gardens, seeds gene banks and also using unconventional methods as DNA storage, pollen storage, *in vitro* collections and cryopreservation of plant germplasm.

Our works concerning the application in this field of biotechnological methods have been developed for about 7 years being focused on several species belonging to different family.

In some of them, only the methodology for micropropagation and short-term maintenance had been established: *Artemisia tschernieviana* Besser (*Asteraceae*); *Astragalus pseudopurpureus* Gusul. (*Fabaceae*), *Doronicum carpaticum* (Griseb. et Schenk) Nyman (*Asteraceae*); *Hieracium pojoritense* Wol. (*Asteraceae*), *Campanula carpatica* Jacq. (*Campanulaceae*); *Erygeron nanus* Schur (*Asteraceae*), *Armeria maritima* (Mill.) Willd. ssp. *alpina* Willd. (*Plumbaginaceae*), *Primula halleri* J.F. Gmelin (*Primulaceae*), *Papaver corona sancti stephani* Zapal. (*Papaveraceae*), *Crambe maritima* L. (*Brassicaceae*).

For the extension of the duration of maintenance in the collections, medium-term protocols were established in several plant species such as: *Veronica multifida* L. ssp. *capsellicarpa* Jelen (*Scrophulariaceae*), *Gentiana lutea* L. (*Gentianaceae*) and several taxa from *Caryophyllaceae* like *Dianthus callizonus* Schott et Kotschy, *Dianthus glacialis* Haencke subsp. *gelidus* (Schott, Nyman et Kotschy) Tutin, *Dianthus spiculifolius* Schur, *Dianthus tenuifolius* Schur., *Cerastium transsilvanicum* Schur., *Dianthus superbus* L. ssp. *alpestris* Kablik ex Čelak., *Dianthus henteri* Heuff., *D. pratensis* M. Bieb. ssp. *racovitzae* (Prodan) Tutin, *D. giganteus* D'Urv. ssp. *banaticus* (Heuff.) Tutin, *D. nardiformis* Janka, *D. dobrogensis* Prod., *Gypsophyla petraea* (Baumg.) Reichenb., *Silene supina*.

In the present time, based on the established protocols using growth retardants there are maintained in medium-term collection plant materials from *Gentiana lutea* L. (*Gentianaceae*) and some taxa belonging from *Caryophyllaceae*: *Dianthus callizonus* - 5 accessions from 3 populations, *Dianthus glacialis* ssp. *gelidus* - 6 accessions from 3 populations, *Dianthus*

*spiculifolius* - 6 accessions from 4 populations, *Dianthus tenuifolius* - 3 accessions from 3 populations, *Dianthus henteri* - 3 accessions from 3 populations, *D. pratensis* ssp. *racovitzae* - 2 accessions from 2 populations, *D. giganteus* ssp. *banaticus* - 3 accessions from 3 populations, *D. nardiformis* - 7 accessions from 3 populations, *D. dobrogensis* - 1 accession, *Gypsophyla petraea* - 5 accessions from 1 population, *Silene supina* - 2 accessions from 1 population.

Taking into account that *in vitro* cell and tissues cultures can generate variability, for species biodiversity conservation, it is suitable the direct way to regenerate propagules such as mini-shoots and somatic embryos, because this developmental ways don't tolerate alterations in the genetic material.

We succeeded in *Gentiana lutea* and *Dianthus* sp. to induce somatic embryogenesis as a continuous process during several months to produce plant material stored *in vitro* collection. This way of regeneration undergoes as continuous process, during several months on synthetic culture media without growth regulators. The plant material also produced was used for synthetic seeds production and cryopreservation trials.

Synthetic or artificial seeds are considered as somatic embryos engineered for the use in the commercial propagation, having the particular advantage of to allow a rapid clonal multiplication. These hydrated encapsulated embryos could only be stored using low temperatures for a few weeks. The capability of prolonged storage can be achieved when the somatic embryos are dried to moisture contents less than 20%.

For medium-preservation purpose, synthetic seed technology was successfully applied in some *Dianthus* species.

Preservation during long-term storage in liquid nitrogen is based on *in vitro* regeneration protocols and production of propagules such as shoot apices, somatic embryos, involving the establishment of optimal dehydration and/or vitrification treatment followed by a recovering procedure. Every step needs a particular protocol adapted to species and material used.

Studies of the behavior of the plant material to pre-storage treatments represented by somatic embryos produced in long-term cultures were made in *Gentiana* and *Dianthus*. Viability tests and the survival rates determination connected to the regeneration of the treated material were used to evaluate the best procedure to ensure the survival before and after the cryostorage.

Different dehydration solution were tested to establish the optimal treatment to perform cryopreservation.

In the present, experiments concerning cryopreservation of *Dianthus* sp. was successfully, these experiments will be extended in the future in other species.

## **IN VITRO CONSERVATION OF *Gentiana lutea* L. BY STORING DEHYDRATED EMBRYOS AT LOW TEMPERATURES**

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Taking into account the rapid decline of the biodiversity, the preservation of plant genetic resources has become extremely important in the last decades. The *ex situ* conservation by mean of tissue cultures represent an alternative way to overcome the loss of plant species. *In vitro* cultures can be used for the multiplication and for storage of plant germplasm. Plant germplasm can be preserved on short- term, medium-term and long- term.

In the case of long-term storage, the cryoconservation requires some pretreatments, which are crucial to the survival of the plant tissues by conditioning the cells to withstand the stresses imposed by liquid nitrogen through the elimination of water and prevent ice crystals formation and/or vitrification. These methods involve the use of cryoprotective substances which may affect the biological material. Therefore, the identification of those techniques that can eliminate these disadvantages is required.

The dehydration of somatic embryos was tested as an alternative to cryoconservation and was successfully applied to conservation without affecting the viability of somatic embryos for some species. Plantlets kept in cold storage at low temperature can be maintained without subculture for several years.

Compared with other *ex situ* conservation methods, such as cryopreservation, the protocols using low temperatures of dehydrated somatic embryos proved to be an inexpensive technique for the conservation micropropagated plants.

Our aim was to identify an inexpensive and efficient *ex situ* conservation protocol using the storage of dehydrated somatic embryos at low temperatures (4° and -20° C).

The plant material used was represented by somatic embryos of *Gentiana lutea* L. obtained through a recurrent embryogenesis process.

The embryos were dehydrated in sterile air flow hood for different time intervals until they reached around of 45% - 71% of their initial weight. The dehydrated embryos were stored at 4° and -20°C.

The evaluation of the viability was expressed as the percent of green explants cultured on the medium used for long term cultures after 28 days. The water loss was calculated by difference between initial and final weight of 1g of embryos at different sizes.

Our results showed that a dehydration in a sterile air flow until they reached 45% of their initial weight assure 100% of embryo viability. If the time of dehydration in a sterile air flow is increased, the embryos viability is decreasing.

The dehydrated embryos (until they reached at 45% of initial weight) are able to survive at tested low temperature (4° and -20° C) with a good viability rate (around 50%). If the embryos are more dehydrated are not able to survive at low temperatures.

## **EX SITU CONSERVATION OF SOME ENDANGERED PLANT SPECIES FROM COMANA NATURAL PARK**

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Ex situ conservation of endangered plant species, represents part of the general issues of concern at national, European and global efforts on natural biodiversity conservation. Comana Natural Park is one of the few protected areas in the plain area, where the vegetation has been severely affected by the systematic use of land for intensive agriculture. Lowland species have also suffered over time from land desertification and drying of native habitats.

Comana Forest is part of the old Vlăsia forests, former forming a green belt around Bucharest. Today remains small fragments, the Comana being one of them that contain a variety of species, many of them found to the limit of their area of distribution.

In this context, in our experiments a total number of three species from different habitats were analyzed. As far as we know the species were not approached under conservative aspect in our country. Thus *Ruscus aculeatus* is a monocotyledonous perennial feature forest habitats, with special pharmacological and ornamental interest, protected at the national and European level. *Marsilea quadrifolia*, a wetland fern that habituates in the Neajlov meadow is also protected at national and European level. The third species, a Liliaceae, *Scilla autumnalis* is preferring salty soils of Neajlov meadow, an area commonly used in agriculture and fisheries. The plant is critically endangered and also important as a medicinal plant.

For the *ex situ* conservation, the *in vitro* culture protocol was used in order to develop a proper experimental method for short-term preservation. The use of synthetic culture media allowed us to obtain a large number of regenerants as a source of plant material to create an active germplasm collection of protected species. In order to morphogenesis induction, multiply and rooting for each species a specific conditions for *in vitro* culture demanding a particular synthetic type of medium is necessary. For example *Marsilea quadrifolia* propagation media has multiple characteristics both rich in mineral salts such as MS (Murashige and Skoog, 1962) or poor in minerals (Knopp) have been used. The addition of the hormones was not necessary. Also for *Ruscus aculeatus* and *Scilla autumnalis* the basal MS medium supplemented with 5 mg/l BAP (**benzyl amino purine**) and 1,0 mg/l NAA (**naphthyl acetic acid**) was used. Physical state of culture media and the type of explants used for primary starting

culture may vary with species. For instance *M. quadrifolia* prefers a semi liquid culture medium such as Knopp or MS with half of mineral salt level. Nodal areas of the rhizome presented a high reactivity. *R. aculeatus* species prefers a solid culture media and the best explant source for primary culture starting consisted in rhizomes (with buds). Shoots regeneration was performed by indirect morphogenesis via callus. In the case of *S. autumnallis* seedlings obtained by *in vitro* germination of seeds manifested a high reactivity. Regenerants were obtained by direct morphogenesis.

The regenerants obtained were analyzed at cytological, biochemical and molecular level for morpho-physiological and genetic characterization. By analyzing the activity that main enzyme isoforms species are sensitive to changes in environmental conditions, we made comparative estimation of the *in vitro* plant regenerated with those from natural habitat. We can observe that the intensity of metabolism of regenerants obtained on synthetic culture medium is superior to the plants from the natural habitat. In the same time, changes in the isoenzymatic spectra were determined only between individuals heterozygous. Across molecular biology (RAPD analysis) performed on the *Marsilea quadrifolia* species were concerned intrapopulational variability and also the genetic uniformity in *in vitro* culture. By using RAPD method for amplification of restriction fragments, a series of genetic polymorphism data existing differences between individuals belonging to the individuals of their natural habitat were shown. Regenerants from *in vitro* culture from the same individual did not revealed evidence of polymorphism. Rooting and acclimatization of plantlets obtained by *in vitro* culture necessitates specific substrates close to those from the natural habitat.

The cytological (electronmicroscopy), biochemical and molecular analysis applied to the studied species, did not revealed severe alterations at the cellular, biochemical and genetical level, proving that the conservation protocols described by us did not affect the normal pattern of plant development and can be used as experimental system for *ex situ* conservation of these species.

The experiments regarding a long term conservation of the germplasm from these species by crioconservation are in progress.

## **IN VITRO CULTURE OF SOME PTERIDOPHYTES SPECIES OF BIOTECHNOLOGICAL INTEREST**

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Pteridophytes represent a very important group of vascular plants. They have been known for more than 300 million years, present an enormous diversity of forms and grow in many different habitats around the world. During the Carboniferous Period, they dominated the vegetation. While most of them became extinct, some evolved and their currently number is now around 12000 species all over the world (Fernandez and Revilla 2003).

In time, the ferns stimulated the interest of many research teams. After *in vitro* techniques developed, numerous approaches interesting from scientific as well as from biotechnological point of view appeared.

Thus it was experimental demonstrated that by *in vitro* culture system the fern's life cycle was in detail analyzed and in this way represents a powerful tool for studying the mechanisms underlying plant development generally. In the same time many fern species present a great biotechnological interest because of their economical value for ornamental purposes (*Nephrolepis exaltata*, *Cyrtomium falcatum*, *Athyrium filix-femina*) as well as for pharmacology, because of their rich content in some secondary metabolites: ecdysteroids, phloroglucine derivatives, volatile oils, tannins, isolated from *Polypodium vulgare* rhizome and two phenol derivatives, 4-vinylphenol etc. extracted from *Asplenium trichomanes*. On the other hand some species such as *Botrychium matricariifolium*, *Asplenium adulterinum*, *Dryopteris submontana* etc. are threatened species and for this reason they present a conservative interest.

In this context our investigations were focused on the possibility to elaborate an efficient protocol for *in vitro* culture of some ferns of biotechnological interest such as, *Asplenium trichomanes*, *Athyrium filix-femina*, *Polypodium vulgare* from different habitats of Valsan Valley. These species were not studied under this aspect in our country, so far.

For primary culture starting, inocula represented by gametophyte and sporophyte fragments as well as homogenates of them and spores were used.

As culture media different variants of MS (Murashige and Skoog, 1962) liquid and solid, Knudson (1946) and Knop (1865) medium were tested. The addition of the hormones was not necessary. All species

preferred temperatures of  $22\pm 2^{\circ}\text{C}$  and a photoperiod of 16 hours of light and 8 hours of darkness.

Our *in vitro* experiments primarily revealed a specie-specific reactivity of the species under study. *Polypodium vulgare* for example manifested a high reactivity followed by *Athyrium filix-femina*. Generally a long period of time of incubation was necessary for *in vitro* culture induction. Thus in case of *Polypodium vulgare* sporophytes appeared after 21-30 days of culture and after a longer period of time (seven month) in case of *Asplenium trichomanes*.

Regarding the culture media, liquid MS with half of mineral salt level and solid Knudson medium stimulated gametophyte culture proliferation in all cases.

Homogenates from juvenile sprophytes represented an interesting experimental system. In liquid nutritive media (especially) this permitted to regenerate in a shorter period of time both gametophytes and sporophytes but the amount of sporophytes was lower. In the same time this system in some cases stimulated culture proliferation. For instance in case of *Polypodium vulgare*, growth on liquid MS with half of mineral salt level, after 60 days the weight of biomass culture was 7 times higher and for *Athyrium filix-femina* ten times higher. The growth of *Asplenium trichomanes* biomass was not stimulated.

In this way this system could improve the propagation rates of these species and maybe extended to others. Moreover, after Fernandez et al. (2003) opinion, the culture of homogenates in liquid medium is amenable to automation and to establishing large-scale cultures in bioreactors, reducing handling and also improving plant production.

The experimental data obtained by us so far as well as the special significance of *in vitro* culture of ferns to investigate on plant development and for biotechnological applications, justify our present preoccupation to optimize the experimental protocols and to extend *in vitro* culture experiments and on other species of interest.



## DEVELOPMENT OF *IN VITRO* SYSTEMS FOR BRYOPHYTE FUNDAMENTAL AND APPLIED RESEARCH

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The present international interest in halting the loss of biological diversity has resulted in global, regional and local strategies of conservation. Among other measures, *ex-situ* conservation had gained an increased importance under the pressure of unpredictable climate events. The first Romanian initiative for bryophyte conservation using biotechnological techniques started as part of a project financed by the Romanian Academy devoted to conservation of endangered plant.

For Romania, from the 950 species of the bryophytes checklist, 108 (11%) are considered threatened, according to Ștefănuț (2004). Only 10 bryophytes species are included in the national legislation as threatened or requiring protection. No Romanian *ex-situ* conservation programme for endangered plant species includes bryophytes.

In this context, we have started a bryophyte *in vitro* collection for conservation, research and biotechnological purposes. Our objective is to develop reproducible and efficient protocols for collecting, micro-propagation and storage for short, medium and long term. As a working principle, the methodologies were tested previously on common species. The advantages of this approach are threefold: (i) collecting of common species for culture initiation poses no threat to their populations, (ii) developing protocols for species that are not presently under threat represents an excellent preventive approach in case the target species become endangered, and (iii) allows comparative studies on threatened/non-threatened species. Bryophyte samples were collected mostly from protected areas, with little human impact.

The most difficult problem we faced in introducing bryophytes in *in vitro* culture was their sterilization. Due to their structural and functional characteristics (including the presence of fungal and bacterial endophytes), the methodology used in cormophyta for sterilization is not adequate for bryophytes. The most efficient sterilizing agent proved to be sodium dichloroisocyanurate (NaDCC), used in concentration of 0.5% - 1% (w/v) for 1-6 min. We have started the cultures either from spores or from gametophyte thalli fragments, grown on solid minimal nutritive medium Knop (1865), solidified with 2 g/l gelrite or 7 g/l agar, but never with sucrose or other carbohydrate source, bryophytes being *in vitro*

photoautotrophs. Another specific bryophyte culture demand is the lower temperature requirements (18-20°C).

In living plant collections, a problem we are confronted with is the persistence of specimens for medium and long term, without contamination and/or spontaneous genetic alteration (somaclonal variations) and with limited consumptions. We developed a method of medium term conservation of bryophytes by growing cell cultures at low temperatures (10°C) and low illumination (1500 lux) on minimal nutritive medium.

The ongoing researches focus on the cryopreservation of bryophyte germplasm. Cryopreservation has obvious advantages over *in vitro* storage in terms of space saving, improved phytosanitation and diminished genetic instability. The suitability of bryophytes for cryopreservation is sustained by their capacity for revival from anabiosis, and their extreme tolerance to desiccation.

Our *in vitro* bryophyte collection holds presently a number of twelve species, belonging to both liverworts and mosses: *Bucegia romanica*, *Athalamia hyalina*, *Reboulia hemisphaerica*, *Asterella gracilis*, *Bartramia halleriana*, *Marchantia polymorpha*, *Conocephalum conicum*, *Atrichum undulatum*, *Funaria hygrometrica*, *Polytrichum longisetum*, *Tortula muralis*, *Mnium* sp..

On the base of their morphological, physiological, genetic and life history characteristics, bryophytes can be used as simple model systems for studying the evolution of the more complex features in higher plants. In this respect, we found that the plasmodesmata peculiarities in *Sphagnum magellanicum* support a cellular specialization in symplastic transport. The hyaline cells formation and secondary wall patterning, the orientation of microtubules associated with this process resembled ultrastructural development in tracheary elements of the higher plants.

Bryophyte contain numerous potentially useful compounds, including oligosaccharides, polysaccharides, sugar alcohols, amino acids, fatty acids, aliphatic compounds, phenylquinones, aromatic and phenolic substances. Herbal medicines of China, India and Native Americans include bryophytes. A direction for further research in our lab is the investigation of bryophyte potential for pharmaceutical, cosmetic or agricultural use. Species that we have already introduced in *in vitro* cultures are cited in scientific literature for: antimicrobial (*Marchantia*, *Polytrichum*, *Dicranum*, *Conocephalum*, *Reboulia*), antileukemic (*Marchantia*), antitumor (*Conocephalum*, *Marchantia*, *Porella*), anti-inflammatory (*Bryum*, *Polytrichum*), diuretic, laxative and haemostatic (*Polytrichum*) activity.

## ARTIFICIAL RESYNTHESIS OF SOME LICHENS SYMBIOTROPHIC FORMATIONS

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This kind of approaches have a secular history and they were started shortly (Bonnier, 1880 - 1890) after Schwendener (1868) hypothesized lichen symbiosis. Such research has been perpetuated throughout the twentieth century (Thomas, 1939; Quespel, 1945; Ahmadjian, 1959-1993, Honegger, 1988, Werner (1990), indicating the lichen body duality, elucidating the processes which contribute to progress in the sequencing ontogenesis and therefore the successive developmental of this unique type of symbiosis.

Lichens stimulated the interest of many researchers because of their rich content in secondary metabolites (as usnic acid, salazinic acid, stictic acid, difractaic acid) of great biotechnological importance in pharmacology. A considerable number of lichen species seem to be endemic and that is why there is of a great interest their conservation for repopulating strategies.

Investigations on resynthesis of lichens from isolated symbionts (mycobionts and photobionts) are usually difficult, that's why in our country this kind of approaches are very scare.

Experimental lichens thalli resynthesis started therefore from aposymbiotic axenic cultures of mycobionts and photobionts obtained by the method of Yamamoto (1990) Yoshimura & co. (2002) and was achieved using protocols promoted by Ahmadjian (1993), Yamamoto & al. (1998, 2002) and Yoshimura & co. (2002).

Regardless of the protocol applied for lichens resynthesis the following conditions are necessary: *in vitro* aposymbiotic, axenic cultures, of mycobiont and phycobiont obtaining; no organic additive to culture medium; the presence of the cytokinines (kinetin respectively) in nutritive media; physical state of this and the type of explants: generally the liquid culture medium and the inocula represented by a suspension of grinding thallus achieved good results. A long periods of incubation was necessary.

Our experimental data revealed that the first interactions mycobiont-photobiont appear after about one month incubation for species *Xanthoria parietina* and *Cetraria islandica* and a month and half for the species *Usnea barbata*.

Therefore the periods necessary to recognize simbiotrophic partners varies according to the species. The process also depends on the density of photobionts and composition of culture medium used. Recognition speed is

directly proportional to photobiont density. Nutrient-poor culture media or lack of cytokinines shortened recognition and amplifies the sites of interaction.

Only after about 90 or 120 days, interactions are sufficiently numerous to generate a quite dense tissue, comparable to that of gonidial layer of native and that of soredial thallus, for which it was known as stage-like sordial.

Our investigations allowed us to decipher ontogenetic stages of lichens thalli redifferentiation and thus to develop an alternative methodologies to obtain lichen biomass of biotechnological interest.

Given the biotechnological value namely secondary metabolites synthesized by the lichen species, the methodology used by us presents real socioeconomic resonance.



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