**“ALEXANDRU IOAN CUZA” UNIVERSITY OF IASI**

**FACULTY OF BIOLOGY**

# LICHENIZED FUNGI IN AIR QUALITY BIOMONITORING

*ABSTRACT*

*PhD THESIS*

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Lichenized fungi investigations nowadays represent an attractive area of great concern of interest for many reasearchers. An interdisciplinary approach in biology, chemistry, ecology and statistics fields, greatly contribute to the understanding and elucidation of the aspects related to the behavior of these living organisms from the cellular level up to their ability to act as environmental quality biomonitoring systems. Lichenized fungi, ectohidric organisms without specialised structure for water and gases exchanges, allow various chemicals (such as organic pollutants, heavy metals) to be adsorbed on the lichenized fungi thallus surface. Their morphological characteristics (branching rich thallus, large contact surface, favourable intercellular spaces of the thallus, roots absence), globally abundances (currently dominating 6-8% of the Earth surface), availability in all seasons (resistance to extreme conditions), are properties that confer to the lichenized fungi species a special living organisms status.

Currently, it is generally admitted that information obtained from the analysis of various lichenized fungi species corroborated with results obtained by different instrumental techniques for air quality monitoring can lead to an accurate assessment of the pollution level existent in an area. Lichenized fungi, in a similar manner as mosses or bark, absorb in high concentration substances including trace elements through the atmospheric wet and dry deposition. Often these living organisms are widely used as biomonitors. Although in the understanding of interactions between lichenized fungi species and heavy metals abundances or in emphasizing the importance of the lichenized fungi species as extremely interesting systems both from ecological and biotechnological point of view, significant contributions are reported, many researchers still emphasize the need to extend the studies involving these living organisms. It is considered essential accurately to assess how lichenized fungi species reflect their current chemical levels or those resulting from the processes of accumulation. Although it is generally acknowledged that lichenized fungi can not be used as instruments of direct measurements is believed that they can accurately reflect the abundance of elements in the environment, which makes lichenized fungi excellent candidates for important biomonitoring studies.

In Romania, air quality biomonitoring studies using lichenized fungi are relatively few. The work "Lichenized fungi in air quality biomonitoring" upon our knowledge represents a first study of interdisciplinary research, involving areas of biology and chemistry, which through its theoretical and practical aspects sought to highlight the potential of *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi collected from Iasi urban area, north-eastern Romania, to be used as a biomonitors for the characterization of air quality in the area of interest. Assessment of the current state of knowledge at both national and international

levels has led to the identification of interesting issues to be explored which in the case of a positive laboratory investigations response would bring significant contributions in the biomonitoring field by using various lichenized fungi species. The major objectives achieved through the theoretical and experimental approaches of the present work are as in the following:

- **i.** identification, selection and adequate sampling, in agreement with the classification of *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi species, for biomonitoring of air quality in Iasi urban area (intensive sampling in the period May 2011-December 2011 for a selected number of sampling points in areas ranging from polluted to less polluted);
- **ii.** morphological and structural characterization using photonic and electronic microscopy techniques of freshly collected material from the lichenized fungi of interest;
- **iii.** identification of haplotype lichenized fungi species through genetic variability analysis concerning the existence of one or more populations of lichenized fungi;
- **iv.** investigation of the stability of chemical parameters of interest in order to determine the optimal conditions for experimental measurements by *state of art* instrumental techniques;
- **v.** investigation by ion chromatograpy technique of the water-soluble components of *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi species;
- **vi.** investigation by atomic absorption spectrometry technique with flame and graphite furnace of selected heavy metals (Al, Co, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) bioaccumulated in *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi species.

The work "Lichenized fungi in air quality biomonitoring" is structured into two distinct parts. The first part reflects aspects referred in the literature while the second part highlights the results brought by the personal contributions. Experimental investigations were undertaken using *state of art* analysis techniques (Ion chromatography *3000 Dionex*, atomic absorption spectrometer with flame and graphite furnace *ContraA 700*, scanning electron microscopy *Quanta 250*, UV-VIS spectrophotometer *Cintra 10e*) existing in laboratories subordinated to the "Alexandru Ioan Cuza" University of Iasi such as CERNESIM Research Centre, Laboratory of Analytical Chemistry, Faculty of Chemistry, Laboratory of Molecular Genetics and Laboratory of Biology, Faculty of Biology.

The first part of the work, generically called "*Theoretical Considerations,*" presents an overview of scientific publications and research in the investigated area. This first part deals with the following three chapters:

- *Lichenized fungi biology*, presenting morphological and structural organization of lichenized fungi;
- *The chemical composition of lichenized fungi*, surprising mechanisms of lichenized fungi absorption;
- *Lichenized fungi ecology*, highlighting the role of lichenized fungi in biomonitoring processes with the identification of the environmental factors influencing all those processes.

The second part of the work consists of four chapters organized according to the obtained results and presents the personal and original contributions. This part is structured as in the following:

- *Morpho-structural and genetic characterization of the lichenized fungi species*, presents the results obtained using photonics and scanning electron microscopy techniques, and genetic study of populations of *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi;
- *Investigation of chemical and biochemical parameters in the lichenized fungi*, presents the results of laboratory studies performed in order to identify optimal conditions for investigation and analysis (extraction, stability over time, degradation) of interest parameters (pH, conductivity, water-soluble ionic components) from *Xanthoria parietina* and *Phaeophyscia orbicularis* species;
- *Analysis of ionic species in Xanthoria parietina and Phaoephyscia orbicularis lichenized fungi species*, presents results related to the study of the ionic composition of lichenized fungi thallus by ion chromatography technique in order to identify the ionic components behaviour at extra-and intracellular level;
- *Investigation of heavy metal content in Xanthoria parietina and Phaoephyscia orbicularis lichenized fungi*, presents aspects related to the determination by atomic absorption spectrometry with flame and graphite furnace of heavy metals concentrations accumulated in lichenized fungi species.

In line with the objectives defined for the present work, following the field and laboratory investigations conducted in the research laboratories of the "Alexandru Ioan Cuza" University of Iasi, the following relevant conclusions are withdrawn:

i) identification, selection and adequate sampling, in agreement with the classification of *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi species, for biomonitoring of air quality in Iasi urban area (intensive sampling in the period May 2011-December 2011 for a selected number of sampling points in areas ranging from polluted to less polluted):

- field investigations carried out in Iasi urban area, north-eastern Romania, have revealed the presence of *Phaeophyscia orbicularis* and *Xanthoria parietina* lichenized fungi species, with a relatively high abundance of the populations in the interest area especially for *Xanthoria parietina*;
- out of the total of 12 sampling locations from Iasi urban region, Tudor Vladimirescu, Red Bridge and Bucium locations were identified as relevant for passive biomonitoring studies over a long period.

ii) morphological and structural characterization using photonic and electronic microscopy techniques of freshly collected material from the lichenized fungi of interest:

*Xanthoria parietina*:

- photon microscopy technique highlighted evidences for the existence of narrow clave asce with 8 spores with colorless ascospore and polocelate-bi-loculars;
- *Trebouxia* vegetative cells with spherical green algae, ranging in size from 1 to 10 μm, with a unique central stellate chloroplast, have been identified. These cells are showing ongoing internal divisions (autospores formation, asexual reproduction);
- by using scanning electron microscopy techniques fructification bodies or cup-shaped apotecium existing on the surface thallus (vegetation part) were highlighted;
- micromorphological studies of the cortex (after splitting) have shown the presence of a layer filled with air or details of the periphery of the cross-section of thallus. Periphery hyphae agglomeration and conglutinated areas in the microaerobic central areas were also observed.

*Phaeophyscia orbicularis*:

- by using scanning electron microscopy techniques orbicular, linear and discreet lobes were highlighted;
- thallus surface micromorphology revealed the existence of well-defined parafize with dimensions less than 200 μm.

For both lichenized fungi species, EDAX detector investigations revealed the presence of elements that probably are directly related to the chemical composition of the thallus (K, Mg, Ca, P, Fe) or to a possible influence of the atmospheric constituents (Si and Al often are associated with tracers of dust particles in the atmosphere).

iii) identification of haplotype lichenized fungi species through genetic variability analysis concerning the existence of one or more populations of lichenized fungi:

- RAPD method (Random Amplified Polymorphic DNA) has been successfully used in genetic and dynamics characterization of the populations of *Xanthoria parietina* collected in Iasi;
- genetic study performed using *OPA-09*, *OPB-10* and *OPD-03* primers for *Xanthoria parietina* species revealed the existence of high genetic variability for populations collected from the chosen sampling points;
- similarity matrix obtained from the present work revealed the presence of the same populations at Base 3 and Red Bridge, Tudor Vladimirescu and Botanical Garden; Copou-Sorogari and Bucium locations;
- the observed genetic variability was considered to be largely due to physiological and morphological differences of the investigated species or likely due to the existence of a relatively large number of haplotypes (a great capacity for dispersal) at the investigated sampling locations.

iv) investigation of the stability of chemical parameters of interest in order to determine the optimal conditions for experimental measurements by *state of art* instrumental techniques:

- in *Xanthoria parietina* species the pH was 6.409 ± 0.141 while in *Phaeophyscia orbicularis* this had a value of 6.030 ± 0.492 (average accompanied by standard deviation of the measurements undertaken at the 12 sampling locations of interest);
- in *Xanthoria parietina* species measured conductivity had a value of 118.4 ± 36.5 μS/cm while in *Phaeophyscia orbicularis* this was of 98.5 ± 28.6 μS/cm (average accompanied by standard deviation of the measurements undertaken at the 12 sampling locations of interest);
- performed experimental investigations have shown that the measurement of the chemical parameters pH can be accomplished using ultrapure water and potassium chloride solution as extraction agent. Conductivity is recommended to be investigated in extracts made in ultrapure water and not in KCl solution

(measurements are strongly affected by the influence of the matrix used for extraction);

- it has been shown that conductivity measurements should be performed immediately after samples extraction because the values of this chemical parameter showed significant variation from day to day. It was also highlighted that the pH measurements can be achieved after a longer time interval, the values measured for the extracts in ultrapure water showing time stability;
- significant differences were observed between the mean pH specific for the substrate and that of the investigated lichenized fungi samples. This observation highlights significant differences between the two types of matrices which led us to the conclusion that lichenized fungi samples were mainly affected by the atmospheric deposition rather than the nature of the substrate;
- investigation of the chlorophyll content in the interest lichenized fungi samples showed that samples extraction is more efficient and relevant than ultrapure water if dimethyl-sulphoxide (DMSO) is used as extraction solvent;
- the values for the phaeophytinization coefficient (OD435/OD415), expressed as chlorophyll a/ phaeophytin a, of the DMSO and ultrapure water extracts for the two lichenized fungi species, had generally values lower than 1.4 ("chlorophyll appears unchanged at a report value of 1.4"). This observation suggests chlorophyll degradation or substantial conversion of phaeophytin, indicating actually a possible toxic effect of gaseous and non-gaseous chemical combinations.

v) investigation by ion chromatograpy technique of the water-soluble components of *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi species:

- the present work revealed that potassium ions are among the most abundant water soluble cations both in *Xanthoria parietina* and *Phaeophyscia orbicularis* species, an observation which is in agreement with the suggestions from the literature that this ions in such systems would mainly come from the soluble fraction located between the cell walls. The abundance of potasium is immediately followed by that of ammonium and sodium cations;
- degradation of the cell membranes (as indications were obtained by the loss of potassium ions) is believed that most likely was favoured by the accumulation of increased concentrations of the ammonium ion over a threshold level, from which

this ion become toxic and could not be anymore metabolized. This is supported by the fact that in the present study it was observed that the effect is more pronounced in *Xanthoria parietina* species than *Phaeophyscia orbicularis, Xanthoria parietina* being known as a more tolerant species to high concentrations of N;

- significant correlation was observed for the pair involving potassium and magnesium ions in both *Xanthoria parietina* and *Phaeophyscia orbicularis* species, an observation which could indicate either the presence of the two cations at the same location or a common physiological role;
- data obtained in the present study indicate that inorganic anions as sulphate, phosphate and chloride are among the most abundant water soluble anions present in both *Xanthoria parietina* and *Phaeophyscia orbicularis* species while acetate is the most abundant organic anion;
- indications were obtained that *Xanthoria parietina* is more tolerant and resistant to air containing sulphur compounds than *Phaeophyscia orbicularis*. Higher sulphur storage capacity in *Xanthoria parietina*, a relatively more resistant lichenized fungi species, suggests that detoxification mechanisms related to sulphur metabolisation and/or its storage in toxi-tolerant compartments could play an important role in this lichenized fungi;
- the concentrations of nitrite and nitrate anions, although at higher levels in *Xanthoria parietina* compared with *Phaeophyscia orbicularis*, appear to be actually at unexpected low levels in comparison with other anions;
- indications were obtained for the existence of a higher affinity for the ammonium ion rather than for nitrite and nitrate both in *Xanthoria parietina* and *Phaeophyscia orbicularis* species. Taking into account the very low concentration of the N containing species, such as those identified in the present work, it was not possible to propose/support a possible mechanism by which the excess ammonia to be oxidised to nitrate, which is a non-toxic form of N storage;
- principal component analysis revealed the existence, beside other four, of a factor explaining 12.9% of the total variance. This factor included as prevalent calcium and oxalate ions, which actually would suggest a possible role of calcium oxalate on the outer surface of the lichen thallus hyphae or on the superior cortex;
- in the present work evidences were obtained that acetate ion is the most abundant. Its abundance may actually be a result induced by its importance at the cell

membranes levels, acetate-malonate reaction pathway being responsible for the formation of secondary metabolites such as depside, depsidone and dibenzofurans usually occurring in lichenized fungi medulla or formation of anthraquinones, cromone and xanthones occurring in cortex.

vi) investigation by atomic absorption spectrometry technique with graphite furnace of selected heavy metals (Al, Co, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) bioaccumulated in *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi species:

- in the present work for both investigated lichenized fungi species evidences were obtained for the existence of lower concentrations of elements in Bucium (408 m altitude) when compared to Tudor Vladimirescu (83 m) and Red Bridge (81 m) locations. This is probably due to the involved transport mechanisms and the deposition fluxes specific for the elements of interest;
- at all sampling locations *Phaeophyscia orbicularis* showed a higher accumulation potential than *Xanthoria parietina* for most of the elements of interest;
- significant correlations have been observed between heavy metal and chlorophyll contents. Chlorophyll b showed significant correlation with Co (0.735) and the ratio chlorophyll a/b presented significant correlations with Cu (0.943) and Ni (0.835). Also, the ratio carotenoids/chlorophyll showed significant correlations with Cu (0.785) and Ni (0.978). Observed correlations might suggest that the accumulation of heavy metals in lichenized fungi thallus are closely related to chlorophyll degradation, most often a low chlorophyll content of lichenized fungi being observed at high levels of heavy metals;
- it is believed that the variability of the heavy metal concentrations obtained from one month to another and from one species to another may be due to substrate diversity and due to the mechanisms of deposition and accumulation of elements in the lichenized fungi thallus.

The issues mentioned above clearly shows that the present work through the obtained results brings important contributions in the field of lichenized fungi with potential role in air quality biomonitoring studies.

**Dissemination of research – ISI Quoted Articles:**

- PINDARU D.M., TANASE C., ARSENE C., OLARIU R.O, 2012. Micromorphological and chemical aspects of some lichenized fungi species. *Journal of Plant Development*, **19**: 73-82.

- PINDARU D.M., TANASE C., OLARIU R.I., ARSENE C., 2013a. Extra and intercellular concentrations of water soluble cations from *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenised fungi species. *Revista de Chimie Bucharest*, **7**: 715-719.
- PINDARU D.M., TANASE C., OLARIU R.I., ARSENE C., 2013b. Chemical composition and concentrations of ions in *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi species in Iasi, north-eastern Romania. *Revista de Chimie Bucharest*, **8**: 807-814.