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LICHENS AS THE BIOLOGICAL INDICATORS OF AIR POLLUTION IN THE BIO-MONITORING SYSTEM USED ON ICP SAMPLE PLOTS LEVEL II IN SERBIA

SUMMARY

Lichens, a symbiotic association of fungi and algae, are suitable to serve as a bio-indicator of air pollution due to their ability to absorb toxic materials into their thallus. The photobiont performs photosynthesis; it belongs to blue-green algae, i.e. cyanobacteria and/or green algae; a mycobiont is a fungus from the Ascomycetes, Basidiomycetes or Deuteromycetes group.

Biodiversity Index (LBI) which was used to determine the effect of phytotoxic gases released from pollutants on the diversity of lichens, especially on Level II Sample Plots with conifers. One of the goals of the study is to determine which species of lichen are the most tolerant and the most sensitive to air pollution. This is one of several different methods that have been developed to monitor the environmental quality. One of them, which was used as basis for obtaining the basic statistical parameters, with the subsequently used T-test that confirmed some of the basic initial assumptions, is the significance of sample surface coverage by lichens. The percentages of lichen coverage of the recordings were obtained with basic photo processing programs, most simply viewed in black and white technique.

The results are represented graphically through maps. Different aspects of the current biomonitoring should be compared to the results obtained in practice to date, in order to increase the number of the related studies in the years to come. Lastly, the current studies, progress, and challenges of biomonitoring using lichen as a biological indicator were discussed, and relevant recommendations were formulated.

Keywords: biomonitoring, lichen, air pollution, conifers, LBI, Serbia

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INTRODUCTION

The need for control and monitoring of the air quality ensued from the vital necessity of clean air for survival of the entire living world, where any disruption in the proportion between its basic components directly disrupts the sustainability of the whole ecosystem with all its aerobic denizens.

The goal of control of the air quality and the quantity of its elements is to register and monitor the changes in the amount of these substances in the air, identify the sources and levels of pollution, and assess the degree of congestion at certain locations in order to identify the most critical ones. As some of the cities in the Balkans have lately been ranked high on the 'most polluted' list, even in comparison with some of the world's major metropolises, this type of information has become not only a vital issue of national importance, but also a safety issue that jeopardizes the basic existence itself.

Testing the air quality using lichens as bioindicators is quickly gaining contemporary significance and popularity worldwide (Abas, 2021, then according Ajtic *et al*, 2018; yet it is far less present in Serbia). Lichens are particularly sensitive to increased concentrations of SO_2, which suggests that lichen air-quality index maps may be created once their quality and quantity is registered (Besermenji, 2007).

Further, Bozkurt, 2017; Boonpeng *et al.*, 2018; Diamantopoulus *et al.* 1993; Kunze, 1972; Leblanc and Rao, 1973; Vokou *et al.* 1999 had also made progress with studies still in very early stages and their results certainly an insufficient starting point for drawing any conclusions. Registering the air pollution through use of indicator organisms is highly significant as it represents a relatively simple, quick and inexpensive, but above all harmless and environmentally-friendly way to conduct the monitoring of the quality of the environment. The organisms most frequently used for this purpose include lichens, mosses and vascular plants, where lichens stand out as the most relevant. They feed non-selectively and absorb nutrients exclusively from the air, thus taking in harmful substances along with the nutrients. Since their thallus has no protective surface layer, pollutants readily diffuse into the tissue. There are three basic types of thallus distinguished according to appearance - crusted, leafy and bushy, but their groupings of growth forms are more complicated: fruticose, or flattened; foliose or flat, leaf-like lobes; crustose – crust-like; squamulose – formed of small leaf-like scales crustose; leprous – powdery; gelatinous – jelly-like; filamentous – stringy or like matted hair and busied. Some are completely structureless.

This study aimed to assess the air pollution status in Serbia based on Lichen Another very important property of lichens is that they belong to organisms with continuous vegetative period, which means that they remain active throughout the year. However, not all lichen species are equally sensitive to different levels of air pollution, which provides the opportunity to use them for detecting the presence and activity of pollutants or changes in the air quality.

The notable success of this, provisionally speaking, symbiotic relationship between lichens and conifers (in the case at hand) on our ICP Level II sample plots is directly linked to the balance and parity of metabolisms of these two types of organisms, and may be formed only in a pollution-free environment (first zones of protection of the natural assets, etc.). Such “sterile” environments are extremely difficult to find in the modern world. For this reason, the studies conducted within the framework of the ICP Forests Project, referenced in part in this paper, additionally rely on cross-border pollution, i.e. pollution coming in from distant zones. The effects of pollution on lichens are manifested in a very obvious way: through the decrease of their abundance and coverage, even at lower concentration of pollutants, while they may completely disappear from severely polluted areas, thus creating so-called “lichen deserts”, which are a serious cause for alarm.

Two main biomonitoring techniques using lichen were identified, with varying research scope and types of parameters researched in the last two years. Previous annual studies on ICP Sample Plots Level II in Serbia from the last decade could help compare and analyse not only the methods for gaining insight into the current practices, but also the progress and challenges that may be expected (Conti, 2008).

This is also the fact that we emphasized, which relates to the two currently most used methodologies worldwide for air quality monitoring through lichen diversity. High naturalness, or wilderness, meaning the complete absence of anthropogenic influence, greatly favors the development, abundance, and coverage of lichens, so such surfaces can be called "deserts," but in other extremes – where pollution and human impact are entirely absent – they are, in fact, a positive factor and ensure survival.

MATERIAL AND METHODS

Due to the innovative approach used and presented in this paper (use of the Lichen Biodiversity Index – LBI), it is necessary to provide a brief overview of the approach which has so far yielded notable and acceptable results, but from another aspect in terms of methodology.

The method used to date is the lichen air quality indication method, performed through calculation of the value of IAP (Index of Atmospheric Purity) according to the formula: Eq. (1)

$$IAP = \frac{1}{10} \sum_{n=1}^{30} (Q * f)$$

Where:

IAP–Index of Atmospheric Purity, Q–ecological index of each lichen species, f–coefficient representing the coverage of each registered lichen species on every researched site (expressed in the 1-5 value range).

Interestingly enough, the methodological approach of the IAP method is inversely proportional to the LBI method, not only according to the results expressed numerically, but also in procedure. This is the drawback of the IAP method, over which the LBI method has an advantage because the parameter value (concentration of SO_2) need not be considered. This parameter is obtained through the LBI testing itself, which later on facilitates measurements of SO_2 concentration in places where its elevated value is assumed and marked in color on maps, while further analyses can be requested as needed.

Experiences gained in this paper demonstrate that the results of both methods are partially complementary, i.e., that the old and the new method have both identified the same issue, but in two different ways. Due to the factors that have previously not been considered (presence and distance of pollutants, rather than the substance concentration alone), the new method is now more acceptable as the previous one has sometimes led to confusing guidelines. This has been confirmed by our control measuring stations near or within the urban core, where the concentration of SO_2 is inherently higher. However, this neither indicates nor points to altered biodiversity of the species observed in the study.

The lichen air quality indication method comprises detection, determination of species, collecting and locating lichens, based on which the indication zones of various air quality degrees are formed and presented in maps.

Monitoring of the air pollution in the researched area of Kopaonik and Mokra Gora was tested by means of the Lichen Biodiversity Index (LBI), which was calculated and analyzed through the prevalence of lichens, i.e., their biodiversity was calculated against the presence and proximity of industrial plants. The other 18 control sites were located near Belgrade, in the Arboretum of the Faculty of Forestry, or in the city center, in the urban core. The research was carried out in 2022 and 2023 on 30 measuring stations for sampling (6 so-called measuring stations or sites, with 5 sample plots each). The data on the distribution of lichens were recorded and finally analyzed by means of the T-test. Sampling was done by the transect method, on each of five sites located on the ground or in the tree bark (in all 4 cardinal directions), using a 90x90 cm wooden frame (Figures 1 and 2).

Transect represents a simple method where the abundance of an organism is determined based on smaller samples ("sample areas"). These sample areas can take the form of strips or points of varying widths (areas), and are called transects. Transect methods fall into the category of "intermediate" methods, through which absolute indicators of the size of the observed population can be obtained. However, relative indicators can also be derived, much like the index sought here as a result.

Figure 1. Sampling of lichen species and coverage using a wooden frame (Sample Plot Kopaonik)

Figure 2. Used wooden frame and dimensions

Within each site, lichen species were identified and their abundance was recorded.

In addition, the coverage of the surface beneath the lichen tissue is very important. After capturing and reviewing the images, the percentage of coverage (black color) was digitally calculated, which is possible and accessible in any photo editing software (Figures 3 and 3a).

Figure 3. Example, Original captured photograph and coverage values within the frame, and lichen with natural coloring

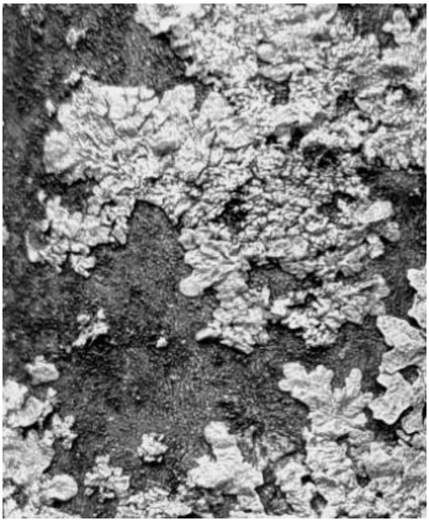


Figure 3a. In the black-and-white (binary) version (technique), the coverage of the lichen on the surface can be easily expressed in %

These values were obtained for all surfaces where measurements were conducted; these are the fundamental data based on which basic statistical values were first obtained (Table 2).

The research on trees was carried out predominantly at breast height - 1.30 m (with the note that the false tissue - plectenchyma of certain lichen species was also found on branches and tree bark, as is the case with the species *Usnea barbata* in spruce stands).

By means of the T-test, a comparison was made to determine whether there was a significant difference between the values obtained from natural diversity on our sample fields (ICP Forest, BIT Level II) and the values obtained around industrial facilities (Table 3).

RESULTS AND DISCUSSION

Clean air and humid environment favor the growth of lichens, while high concentrations of SO_2 confine their growth, which particularly applies to the sensitive type lichens.

The most common lichen species found in the researched area are classified as highly tolerant, such as *Physcia tenella*, *Parmelia sulcata* and *Hiperphiscia adglutinata*, but also as sensitive species, e.g. *Usnea barbata*, *Parmotrema tinctorum*, *Parmotrema praesorediosum*, *Phiscia atrostriata* and *Phiscia pulverulenta*. Knowledge on identification of lichen species and calculation of the LBI results enables determination of the air quality in a certain area, which was the original goal of the research.

Findings are given from each of the measuring stations (total 5 each) located in the vicinity of the above-mentioned plants, due to the different levels of danger to the environment primarily based on the SO_2, emission, as well as from all Level II Sample Plots (Table 1).

Table 1. LBI (diversity) based on sampling according to the transect method, in the vicinity of measuring stations – sample plots 1-6

Sample plots	**LBI results shown on the map**	**LBI in the zone 11-20**	**LBI in the zone 22-30**	**LBI in the zone 33-40**	**Total plots inspected**
1	Sample plot 2 Mokra Gora	4			5
2	Sample plot 2 Kopaonik	2			5
3	Pollutant I – Water bottling plant Kopaonik		17		5
4	Pollutant II – Dairy		13		5
5	Pollutant III – Furniture manufacture “Tomic”		12		5
6	Pollutant IV – Steelworks			35	5
		6	**32**		Total **30**

The obtained results demonstrate that the diversity of lichen species found in a single place enables their use in measuring and quantifying the air pollution levels, both in industrialized areas and in natural reserves. Moreover, within the context of industrial products – air pollutants in cities, it was concluded that motor vehicles have no significant impact on lichen biodiversity, due to the presence of other sources and pollutants that are very easily airborne (Abas and Awang, 2017), which was additionally proven on control samples (Table 2).

Table 2. Basic statistical parameters for measuring stations on 6 sample plots (coverage)

Statistics (in %)	**All sites on 6 habitats (6 sites with 5 sample plots each)**	**Total for all habitats (plus, control)**
Minimum (min)	6%	1%
Maximum (max)	64%	64%
Mean value ($\bar{y}$)	41.12%	29.73%
Standard deviation (σ)	15.6859%	17.5086%
Sample size (n)	30	48

This confirmed the comparison on the potentially significant difference between the values obtained from natural diversity on our sample fields (ICP Forest, BIT Level II) and the values obtained around industrial facilities (Table 3).

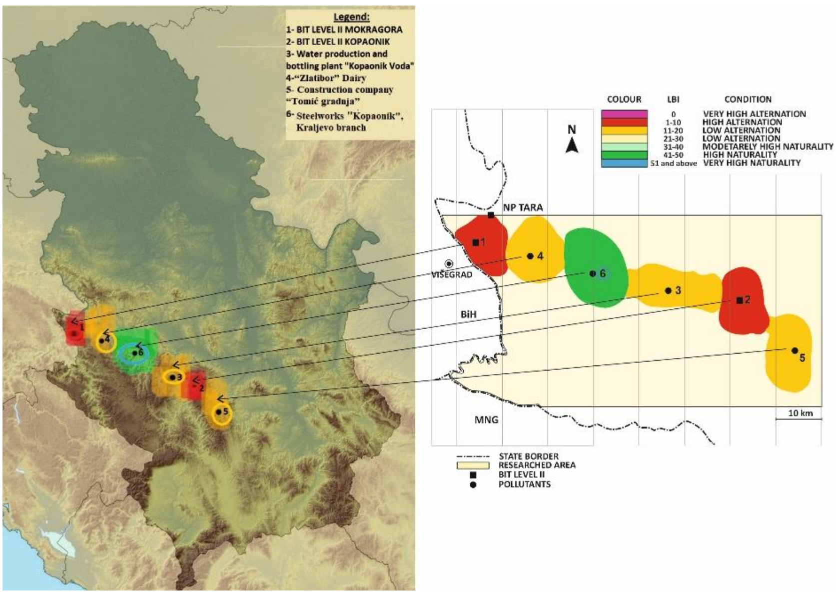
The T-test was calculated on the reliability level $\alpha=0.05$ and the absolute value of t-statistics was calculated at $|-7.992|$, which is associated with t-statistics critical value of 4.0067709. It was therefore concluded that the null hypothesis should be rejected with a 5% risk (Table 3). The alternative H_1 hypothesis was accepted, suggesting that there is a significant statistical difference between mean values expressed in LBI on our samples (there is a possibility that LBI might be higher with the increased presence of pollutants – as presented in maps 1 and 2). Based on the results from 30 control stations (Table 1), high biodiversity was proven on 10 stations, moderate biodiversity on 15 stations, while 5 measuring stations had low biodiversity throughout the researched area.

Statistical analysis through T - test provides a result where P - value between the LBI value and the presence of basic pollutants such as SO_2 amounts to -0.7992 (level of significance $1>r<-1$), and the P value is 0.0009 (level of significance $P<0.05$). This demonstrates that there is an inverted significant relationship (inverse correlation) between the LBI results and the frequency of presence of basic pollutants such as SO_2 (Table 3).

Table 3. Testing of mean values of LBI (coverage) values within 30 (6 study areas X 5 sample plots) (T test)

T test	LBI
Mean value	1.430606061
Variance	9.055871212
Observations (samples pcs.)	30
Pearson correlation	0.000957941
Hypothetical difference between means	0
df (Degrees of freedom)	28
T statistics	-7.992427869
P(T<=t) /One tailed test – distribution	2.0033909
T Critical value for one tailed test	1.693888748
P(T<=t) two tailed	4.0067709
T Critical value for two tailed distribution	2.036933343

Areas with high naturalness – in terms of a consistent lichen response to pollution, or rather – the most commonly used term for the complete absence of primarily anthropogenic influence on the environment where these organisms thrive is lichen desert (0.0%) – were not found at any of the researched sites. Furthermore, naturalness with values exceeding 51 was not observed anywhere (Maps 1 and 2).



Maps 1 and 2. Air pollution zones according to the Lichen Indication Method (LBI) in the researched area in Serbia

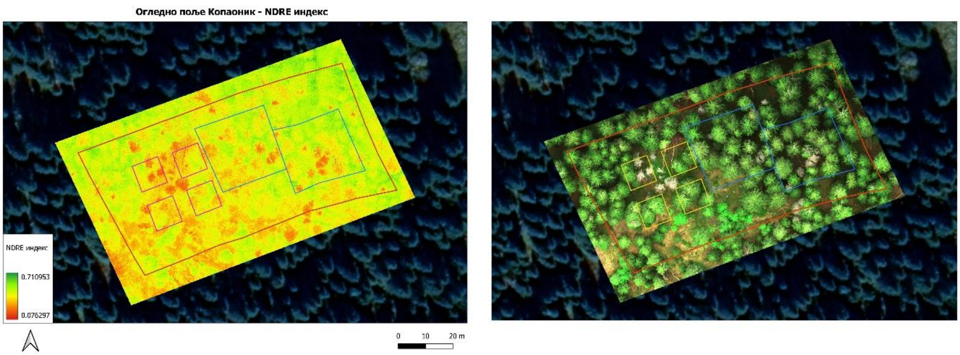
Figure 6. Old-growth forests, or so-called "Virgin" forests, Obed.Bara-Kup.Grede- ravni Srem

Figure 7. *Didymosporina aceris* (Lib.) Mont.)

Declining could be found decline on individual trees or larger forest areas in a locality (Lukyanets *et al.* 2022). Among in general resistant are maples. Only several pests could seriously damage those, such as maple tar spots (*Rhytisma acerinum* Schwein., 1832., Figure 8 and 9) and Maple Leaf Marks (*Didymosporina aceris* (Lib.) Mont.) on *Acer pseudoplatanus* (Figure 7). This is so common in Balkans countries, in the region in general, (Vokou, *et al*. 1999), precisely in or so-called "Virgin" forests (Perućica, Kupinske Grede), (too many sensitive tree species), and so weak to invasive organisms. This Acer is very common in the floor of the bushes, which represent state of air on level, where actually man is usually co-beneficiary as forest user, which means that, according mentioned pathogens, air has not a positive attribute on this height.

Figure 8 and Figure 9. *Rhytisma acerinum* Schwein., 1832.

The cited research indicates that the study should be expanded through more detailed investigation, e.g. for each individual lichen species, based on their sensitivity to the presence of pollutants (Diamantopoulos *et al.* 1993). This would reveal the current pollution status and open up the possibility of predicting an increasingly urgent need for environmental protection in the future. The NDRE index - Normalized Difference Red Edge index was used, which represents a combination of near-infrared and edge-red light. The index is sensitive to subtle changes, showing aberration (Maps 3 and 4). Its basic techniques, such as content analysis or observation of documents and the use of existing statistics, documents and their secondary analysis, were used to assess the impact of miserable alternation extremes (high and low temperatures) on forest ecosystems and identify possible changes over the years (Češljar *et al.* 2021). This is usually are brought about where changes and improvements of environment, in general are strong and vicious (high and mature old forest in South east Asia, where impact of winds or see currents are strong in forest canopies) (Abas, 2021; Češljar *et al.* 2021).



Maps 3 and 4. The NDRE index - Normalized Difference Red Edge index was used, which represents a combination of near-infrared and edge-red light. The index is sensitive to subtle changes even during late vegetation phases - Kopaonik, Sample Plot Level II.

Mentioned researches indicate that the work should be expanded with a more detailed investigation for each individual species of lichen, based on its sensitivity to the presence of pollutants. Those factors that have occurred in the past to which some other factors have been added (Češljar *et al.* 2021). This would show the current state of pollution and open up the possibility of forecasts of increasingly urgent environmental protection needs in the future (Češljar *et al.* 2021), find decline of individual trees or larger forest areas in a locality (Lukyanets *et al*. 2022.) and all synchronized with previous and so by findings of Ajtic *et al*., 2018 - (RTB Bor in Eastern Serbia); followed also by Bozkurt *et al.* 2017).

CONCLUSIONS

For the development of impact assessment studies during the reconstruction of the existing and construction of new facilities, as well as for the execution of works that can generally affect and even jeopardize the quality of the environment, it is necessary to establish a monitoring system for parameters that reflect that quality, in this case, air quality. This entails general spatial planning and resource utilization measures by introducing measuring stations for air quality control (primarily in areas identified as already threatened or where naturalness is complete, i.e., with no indicators of anthropogenic influence – deserted by lichen), creating an inventory of pollution sources, and maintaining records of the pollution status from these sources. It involves designating and establishing sanitary protection zones around larger industrial and communal facilities, proper maintenance and preservation of as much "naturalness" as possible forest resource management.

By implementing measures of systematic air quality monitoring using the Lichen Biodiversity Index (LBI) method within the investigated area, while simultaneously advocating for reduction in air pollution caused by harmful substances to values below the prescribed limits, as well as taking necessary actions to mitigate their emissions, the necessary changes for returning to a safe and sufficiently healthy environment will occur – a restoration of natural areas with minimal anthropogenic impact. Through intensive monitoring of airborne pollution due to the presence of pollutants in the environment, both natural assets (such as old-growth forests, or so-called "Virgin" forests) and the overall younger but environmental more plastique and more quality will be protected, which would eventually lead to achievement of the ultimate goal - ensuring clean air as a resource essential for human life and health. By comparing and approximating this method, which is more adapted to the countries and forests every day. Eengaging agriculture and fishery, and only conditionally ensure a healthy existence - how is at the crossroads of air and water mass currents, in another sense, from the aspect of economics or management, the situation that is currently unfortunately places our cities and they are often declared as the most polluted "black spots" compared to similar ones in the world and the environment and removed from the list of the most polluted areas. Research like this offers hope and provides a scientific basis to change, improve and bring these and more important existential circumstances under control.

An environment with such a "rating" of air quality (moving active during all the time) would also result in a setting conducive to safe forest management, agricultural activities, and ensuring a healthy existence.

Unfortunately, the current situation places our cities is in a position where they are often labelled as the most polluted, even considered as "black dots" when compared to similar areas globally and in the region. This trial should help to remove them from the list of the most polluted areas. Research like this offers hope and provides a scientific foundation to address and control these crucial aspects of the existence. By continuous monitoring for a longer period of time, it is possible to determine the cause of disease on a certain tree - and bring it into connection with a certain environmental factor.

The prerequisites would also be obtained, which is characterized by such an environment with an air "grade" of sufficient quality to safely manage.

The advantage of this research, in relation to other researches that are performed only after the appearance of decline as the final cause of the influence of this factor, can be seen when these researches, the cause is that moment is very difficult to determine, because it can be initiated by various aspects. Therefore, this type of continuous monitoring can accurately determine the initial stages of the impact of a single, but important in its way, every specific factor.

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