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Original Article

Bioindication of heavy metal pollution in the area of Southeastern Serbia by using epiphytic lichen *Flavoparmelia caperata* (L.) Hale

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***Abstract*:**
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The content and distribution of 21 metals in the central and peripheral parts of the foliose epiphytic lichen *Flavoparmelia caperata* (L.) Hale, collected in the area of Southeastern Serbia, were analysed in terms of biological monitoring. Inductively coupled plasma atomic emission spectrometry revealed higher concentrations of As, B, Ba, Cd, Ga, Pb, Se, Cr, Cu, Fe, In, Li and/or Ni in peripheral, younger, parts and Ba, K, Tl, Mg, Na and/or Zn in central, older parts of lichens. Principal component analysis and hierarchical cluster analysis were used to identify the relationship among metals in samples and their possible sources. Significant correlations were found among Ni-Cr, Cd-Ga-In-As-Se, Zn-Ba, Cu-Pb-B, suggesting a common source of pollution. Given the location of sampling, these findings probably reflect airborne metal pollution in relation to the main wind directions and vicinity of the roads and industrial complexes. The importance of this study is the evidence that the Special Nature Reserve Jelašnička Gorge is influenced by pollution sources in the area. *Flavoparmelia caperata* could be effective as an early indicator of environmental changes of the studied area.

Key words: air pollution, bioindication, *Flavoparmelia caperata*, heavy metals, lichens

Introduction

Lichens have been the most widely accepted sensors of enviromental pollution since the 19th century (G r i n d o n , 1859). Lack of waxy cuticle and associated stomatas, parenchymal nature, slow growth and long duration of lichens result in the absorption of pollutants (metals, nonmetals, radionuclides, etc) across entire thallus surface and thus allow long-term biomonitoring of persistent pollutants in the ecosystem. Lichens depend on mineral nutrients in the form of soluble salts and particles from wet atmospheric deposition (precipitation and occult precipitation, like fog and dew) and dry atmospheric deposition (sedimentation, impaction and gaseous absorption). They are very efficient accumulators of heavy metals by: ion exchange at specific cation exchange

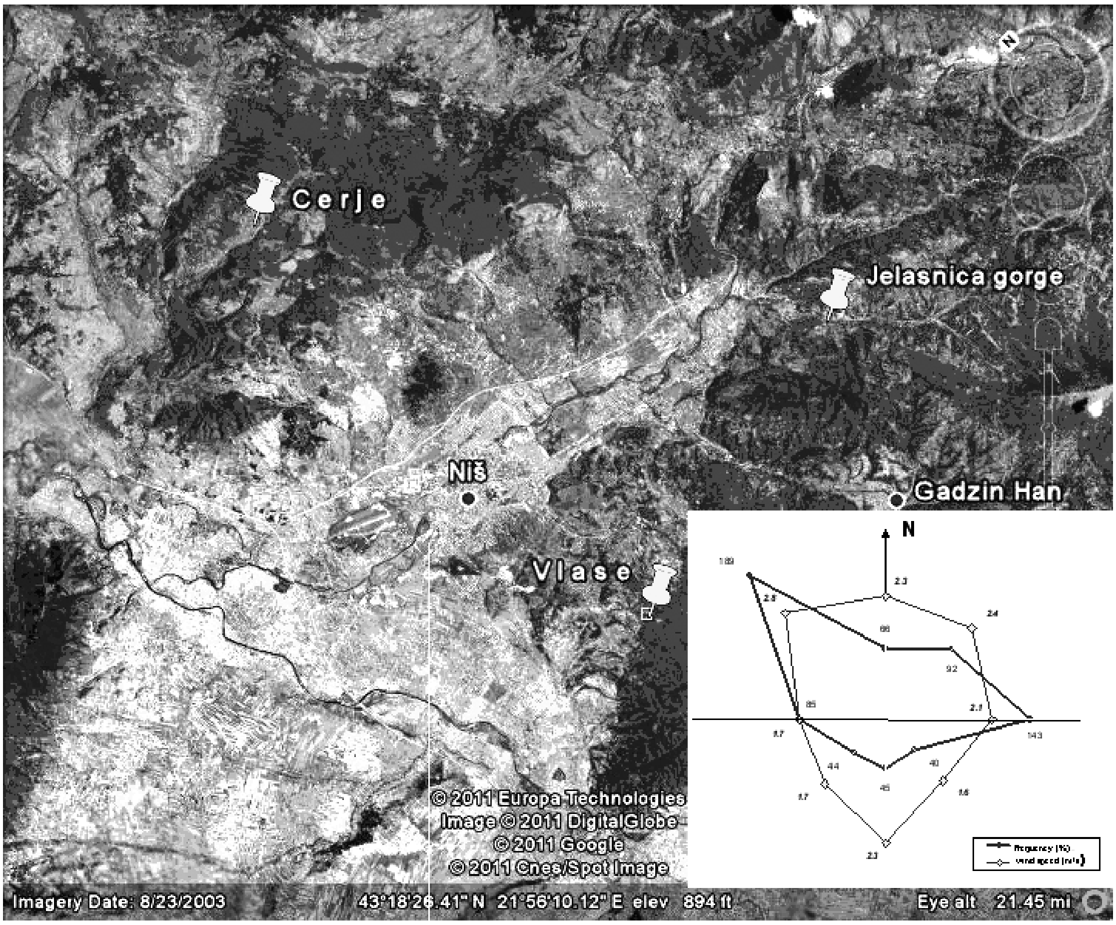

Figure 1. Study area (Jelašnica Gorge, Vlase and Cerje in respect to the city of Niš) withwind roses for the area of Niš (data from Republic Hydrometeorological Service of Serbia)

sites, intracellular uptake of metal solution, and entrapment of airborne, metal-rich particles (size 0.5 to 1.0 μm) (Nash III, 2008). Lichens are capable of accumulating heavy metals to a concentration that vastly exceeds their physiological requirements by sequestering them extracellulary as insoluble oxalates or lichen acid complexes (Beeby, 2001). The presence of particulate materials on the lichen surface which are trapped between the hyphae of medulla is shown by electron microprobe studies (Bargagli, 1998).

Metal accumulation is in correlation with the environmental levels of particulate materials (Bari et al., 2001; Bargagli et al., 2002 ; Loppi et al., 2004) and spatial- and/or temporal- deposition patterns of trace metals are demonstrated (Zschau et al., 2003). The concentration of sequestered trace metals in lichen really reflects ambient level of airborn trace metals in terms that after the withdrawal of the source of contamination from the ecosystem (closures of industries, iron-steel factories, mines, waste treatment plants, roads and railroads, etc) a dramatic decrease of trace metals concentration in lichen thalli is observed (Walther et al., 1990). Nimis et al. (2001) discussed the importance of intra- and inter- specific variability in metal accumulation and relationship between metal content and the age of the thalli.

In this study we investigated heavy metal pollution using *Flavoparmelia caperata* (L.) Hale as bioindicator and bioaccumulator. The study was performed at 3 different locations: northwest (near the village of Cerje), southeast (near the village of Vlase) and northeast (Jelašnička Gorge) of the biggest urban and industrial center of Southeastern Serbia - Niš (approximately 350 000 inhabitants). We would like to emphasize the importance of one location - Jelašnička Gorge. This area, situated 15 km away from Niš and 3 km away from Niška Spa, is a protected natural area having the status of a Special Nature Reserve. One of the reasons to investigate this part of Serbia is the fact that a similar study had never been undertaken previously, although the area is densely populated with large urban-industrial infrastructure, which thus has an important anthropogenic impact on surrounding nature and ecosystems.

Lichen samples were analyzed for metal content. Composition data have been treated with various statistical multivariate techniques, primarily principal component analysis and cluster analysis.

Material and methods

Study area

The study was performed in the subrural and rural area of Southeastern Serbia. The collection sites were: Jelašnička Gorge (330 m altitude), Vlase (350 m altitude) and Cerje (600 m altitude) in the vicinity of roadside (2m, 500 m and 2000 m, respectively) (Figure 1).

The nearest urban and industrial area is the city of Niš (approximately 350 000 inhabitants) (Figure 1). The climate is moderate continental with mean annual rainfall ranging over 543.3 mm, a mean temperature of 11.5 °C, and a mean annual relative humidity of approximately 69 %. Prevailing winds are northwesterly in winter and northeasterly and easterly in summer. Wind roses are shown in Figure 1.

Lichen Material

Foliose lichen *Flavoparmelia caperata* (L.) Hale (syn. *Parmelia caperata* (L.) Ach.; common name: greenshield lichen) was collected in April 2009. Lichen specimens were obtained from a height of 1.5-2 m above the ground, at the side of trunks of *Prunus domestica* and *Salix sp.* not affected by stemflow. The material from each location was sorted into two samples corresponding to peripheral and central parts of lichen. The samples were air-dried, grinded, homogenized and further analyzed. The determination of lichens was performed by using several standard keys (Boqueras, 2000; Dobson, 2005; Wirth, 1995). Lichen samples were deposited in the lichenological herbarium of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš.

Reagents and Chemicals

All chemicals were of analytical grade and were supplied by Merck (Darmstadt, Germany). All glassware was soaked in 4 mol/L HNO_3 for a minimum of 12 hours and rinsed well with distilled water. Ultra-pure water was prepared by passing doubly de-ionized water through Milli-Q system (Millipore Simplicity 185 System incorporating dual UV filters (185 and 254 nm) to remove carbon contamination).

Multi-element stock solution containing 1.000 g/L of each elements was used to prepare intermediate multi-element standard solutions. Sodium borhydride solutions were stabilized with sodium hydroxide (Merck, Germany) for hydride generation.

Table 1. Instrument operating conditions for determination of heavy metals in lichen samples

Spectrometar	ICAP 6500 (Thermo Scientific)
Nebulizer	Concentric
Spray chamber	Cyclonic
Radio frequency power (W)	1150
Principal argon flow rate (L/min)	12
Auxiliary argon flow rate (L/min)	0.5
Nebulizer flow rate (L/min)	0.5
Sample flow rate (ml/min)	1.0
Detector	CID86

Table 2. Selected emission lines

Element	λ [nm]
Ag	328.0
As	193.7
B	249.6
Ba	455.4
Cd	228.8
Co	228.6
Cr	283.5
Cu	324.7
Fe	259.9
Ga	294.3
In	230.6
K	766.4
Li	670.7
Mg	280.2
Mn	257.8
Na	588.9
Ni	231.6
Pb	220.3
Sr	215.0
Tl	276.7
Zn	213.8
Hg	253.6
Se	196.0

Instrumentation

All the measurements were made with a Inductively Coupled Atomic Emission Spectrometer model 6500 Duo (Thermo Scientific, United Kingdom) equipped with a CID86 chip detector. The system is equipped with an integrated unit for

Table 3. Concentration of metals in central and peripheral parts of the thalli of *Flavoparmelia caperata* (μg/g dry wt.)

Elements	*Flavoparmelia caperata* from Jelašnička Gorge		*Flavoparmelia caperata* from Cerje		*Flavoparmelia caperata* from Vlase	
	Center (sample 1)	Perifery (sample 2)	Center (sample 3)	Perifery (sample 4)	Center (sample 5)	Perifery (sample 6)
As	0.0037	0.0038	0.0036	0.0033	0.0031	0.0027
B	19.4303	9.5051	6.1341	6.9682	5.8576	6.7637
Ba	27.2333	21.5575	9.5615	24.5084	13.8145	15.2969
Cd	0.2558	0.1942	0.1681	0.1864	0.1310	0.1546
Co	0.1512	0.0822	1.0894	2.1865	0.0970	0.0856
Cr	1.9119	1.4572	3.7140	1.4670	1.7550	1.4879
Cu	8.3806	5.9573	15.3721	5.7307	6.1412	6.5179
Fe	476.6209	374.5863	645.2168	397.9597	493.2096	399.2086
Ga	0.1069	0.0000	0.0413	0.0000	0.0346	0.0177
In	0.0000	0.0000	0.0802	0.0341	0.0810	0.0000
K	2608.8040	2852.2720	2300.1670	2706.2540	2506.7160	3156.7050
Li	0.6932	0.5035	0.7566	0.4607	0.5617	0.4582
Mg	251.8673	315.6654	274.1127	313.7536	287.8818	373.2304
Mn	14.1271	13.5242	13.6761	12.4049	13.9091	15.6287
Na	71.1085	98.1346	89.5684	103.6953	78.8976	110.4994
Ni	1.2703	1.0977	1.5682	1.1916	1.4227	1.3011
Pb	22.3011	10.0742	12.0318	7.3955	9.8771	7.4223
Se	0.0043	0.0045	0.0042	0.0039	0.0036	0.0031
Sr	31.4610	23.8918	16.8445	11.3996	40.6104	32.6951
Tl	0.0000	0.6093	0.0000	0.9164	0.0000	1.5415
Zn	17.6053	17.0019	18.0832	19.5534	20.7641	24.2578

hydride generation. This instrument operates sequentially with both radial and axial torch configurations. The entire system is controlled by Iteva software. Instrument operating conditions for the determination of heavy metals in lichen samples and selected emission lines are shown in Table 1 and Table 2, respectively.

Microwave digestion was performed in a pressurized microwave (Ethos 1, Advanced Microwave Digestion System, Milestone, Italy) equipped with a rotor holding 10 polytetrafluoroethylene (PTFE) cuvettes.

Procedure for Microwave Digestion

Samples (0.5 g) were transfered into PTFE cuvette, 7 ml of concentrated HNO_3 and 1 ml 30% H_2O_2 were added. Digestion was performed under the following programme: warmed up for 10 mins to 200° C and held for 10 mins at that temperature. After the cool off period samples were quantitatively transferred into a volumetric flask (25 ml).

Statistical Analysis

To identify the relationship among metals in samples and their possible sources, Pearson's correlation coefficient analysis, principal component analysis (PCA) and cluster analysis (CA) were performed using PLS Toolbox version 5.2.2 (Eigenvector Research) for the MATLAB version 7.4.0.287 (R2007a) (MathWorks, Natick, MA, USA). The principal component analysis was performed using Varimax Normalized rotation. Cluster analysis was performed on the data sets using the between-groups linkage based on correlation coefficients (Pearson coefficient) by pair-wise deletion. Normalized data set was analyzed by Ward's method using squared Euclidean distances as a measure of similarity between metal concentrations.

Results and discussion

The concentrations of 21 metals in lichen *Flavoparmelia caperata*, collected at 3 different localities in Southeastern Serbia (Jelašnička Gorge, Cerje and Vlase), are given in Table 3.

The determination of metals was performed on both central and peripheral parts of lichens. Central parts are inner, older parts of thalli and they have therefore been exposed to pollutants longer. Peripheral parts comprise outmost 3-4 mm of the thalli, with highest physiological activity and the maximum age of 1 year (Fisher & Proctor, 1978; Loppi et al., 1997). Thus, peripheral parts represent recent changes of the environment. Our data indicated differences in the metal content of central and peripheral parts of lichens. Central parts tend to give higher concentrations of a number of metals as previously reported in the studies of Nimis et al. (2001).

Pearson's correlation coefficients for metals in lichen samples are shown in Table 4. Determined correlations between metals provided interesting information on the sources and pathways of the metals.

The correlation matrix was created from the values of the variables of all 21 metals in 6 samples. The Pair-Wise method was employed for the missing values. The results showed that these metals were strongly interrelated ($p<0.01$), with correction coefficients ranging from -0.790 to 0.990 at the 99% confidence level. B, Cd, Ga and Pb evidently displayed significant positive correlations with each other (see: Table 3), which indicates their association in analyzed samples. Other metals, like Cr, Cu, Fe, Li and Ni, showed significant correlations, too. The exceptions were elemental pairs As-Zn and Se-Zn with significant negative correlations and As, Mn and Zn without any correlations observed.

In order to better describe the relationship among metals and/or samples, principal component analysis (PCA) was performed. Analytical data were represented in a multidimensional space with variables defining the axes, and projected into a few principal components (PCs) that were linear combinations of the original variables and described the maximum variation within the data (Brereton, 2003). Obtained results clearly showed that the Co, Mn and Sr had no impact. This was in accordance with results of metals determination (Table 3) due to small differences in theirs values in different samples. Therefore, PCA analysis was done without these metals resulting in a three-component model explaining 91.78% of the data variation. The first PC comprised 49.75% of the total data variability, and the cumulative variance explained by the first two components was 81.09%. The addition of more PCs did not significantly change the classification of the analytes described below. Score values for the samples, i.e. their mutual projections, for the first two PCs are shown in Figure 2.

Additionally, score values for the samples and metals, for the first two PCs are shown in Figure 3.

The first PC distinguished two separate groups of samples according to metals content and age of parts of the lichen thalli sampled. Samples 1, 3 and 5 are central (older) parts of the thalli from different areas while 2, 4 and 6 are peripheral (younger) parts of the thalli. The first PC distinguished two separate groups with characteristic patterns of metals accumulation (samples 2, 4 and 6 with Ba, K, Mg, Na, Tl and Zn

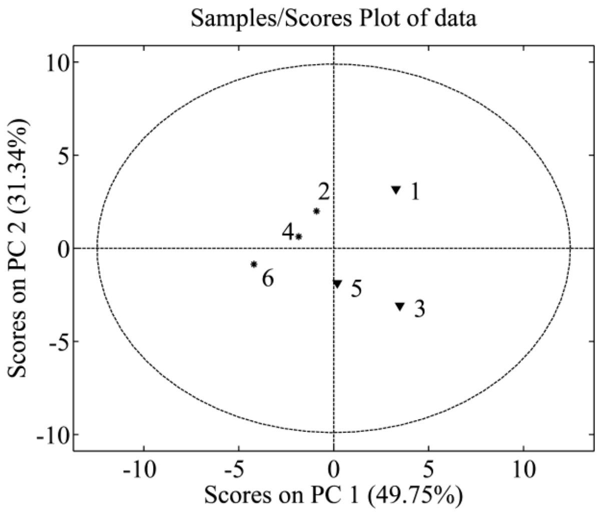

Figure 2. Score values of the first and the second PCs for the samples

Additionally, score values for the samples and metals, for the first two PCs are shown in Figure 3.

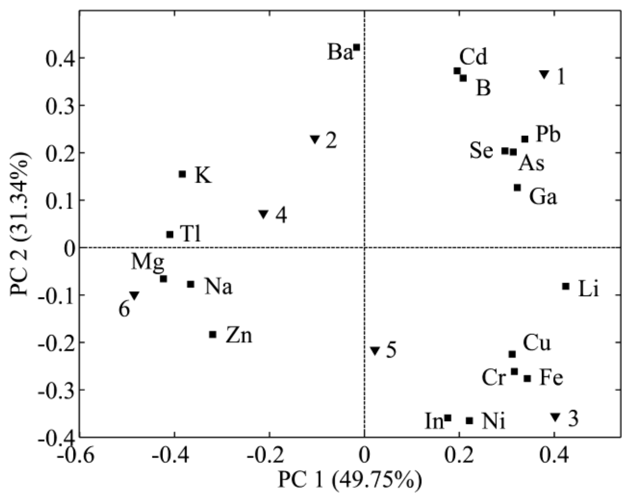

Figure 3. Biplot of the first and the second PCs for the samples and metals

and samples 1, 3 and 5 with As, B, Cd, Cr, Cu, Fe, Ga, In, Li, Ni, Pb and Se). These results clearly defined the highest accumulated metals characteristic for every sample. The following metals were characteristic of sample 1: As, B, Cd, Ga, Pb and Se. Higher concentration of Ba, K and Tl were noticed in samples 2 and 4. Besides, Ba was located on the border that defined a PC and it appeared characteristic of sample 1, too. The accumulation of Cr, Cu, Fe, In, Li and Ni were characteristic for samples 3 and 5. Increased concentrations of Mg, Na and Zn were observed in sample 6. Compared to location of sampling, it

Table 4. Pearson correlations coefficient matrix for the metals concentrations

	B	Ba	Cd	Co	Cr	Cu	Fe	Ga	In	K	Li	Mg	Mn	Na	Ni	Pb	Sr	Tl	Zn	As
Ba	0.717																			
Cd	0.921	0.792																		
Co	-0.303	0.123	0.002																	
Cr	-0.135	-0.605	-0.068	0.186																
Cu	-0.046	-0.542	0.033	0.161	0.988															
Fe	-0.094	-0.615	-0.125	0.099	0.953	0.916														
Ga	0.795	0.232	0.603	-0.329	0.293	0.340	0.432													
In	-0.558	-0.684	-0.596	0.290	0.614	0.489	0.734	-0.048												
K	-0.019	0.271	-0.031	-0.282	-0.735	-0.658	-0.828	-0.390	-0.776											
Li	0.371	-0.246	0.324	-0.076	0.841	0.849	0.881	0.728	0.408	-0.786										
Mg	-0.523	-0.166	-0.479	-0.050	-0.487	-0.457	-0.601	-0.708	-0.376	0.854	-0.809									
Mn	0.073	-0.328	-0.175	-0.726	-0.064	0.001	-0.032	0.233	-0.310	0.511	-0.028	0.432								
Na	-0.591	-0.135	-0.379	0.317	-0.276	-0.243	-0.478	-0.824	-0.288	0.669	-0.706	0.896	0.100							
Ni	-0.311	-0.789	-0.423	0.020	0.819	0.767	0.916	0.313	0.789	-0.641	0.670	-0.332	0.201	-0.328						
Pb	0.915	0.427	0.796	-0.309	0.222	0.282	0.303	0.943	-0.211	-0.363	0.698	-0.760	0.067	-0.807	0.075					
Sr	0.179	-0.147	-0.210	-0.848	-0.299	-0.322	-0.082	0.366	-0.036	0.187	-0.025	0.022	0.663	-0.420	0.130	0.218				
Tl	-0.322	0.089	-0.219	0.109	-0.540	-0.478	-0.678	-0.600	-0.547	0.892	-0.802	0.945	0.341	0.900	-0.465	-0.620	-0.139			
Zn	-0.453	-0.353	-0.600	-0.134	-0.312	-0.311	-0.246	-0.273	-0.034	0.577	-0.522	0.745	0.640	0.494	0.149	-0.526	0.394	0.671		
As	0.504	0.359	0.651	0.062	0.315	0.333	0.227	0.298	-0.063	-0.504	0.533	-0.702	-0.547	-0.461	-0.175	0.561	-0.380	-0.621	-0.990	
Se	0.47	0.373	0.633	0.097	0.284	0.299	0.188	0.240	-0.066	-0.485	0.484	-0.672	-0.587	-0.419	-0.213	0.513	-0.414	-0.591	-0.992	0.998

seems that the Special Nature Reserve Jelašnička Gorge from which sample 1 and 2 originated, is becoming polluted due to vicinity of the road (2m) and towns (Niš and Niška Spa, 15 km and 3 km, respectively). Evidence for this could be found in the increased concentration of Cd, Pb and Se as the levels of these elements are similar to literature values for high-density traffic areas (Mendil et al., 2009). The other two locations of lichen sampling, Cerje and Vlase, with samples 3-6, showed richness in litophile elements and macronutrients such as: Cr, Cu, Fe, In, K, Li, Mg and Na. This could imply a specific composition of geological substrate, as well as the influence of vegetation, i.e. substrate from which lichen samples were collected. Moreover, there is a certain amount of anthropogenic pressure and it is manifested by pollution originating from traffic origin.

Finally, metal concentration data were submitted to cluster analysis (CA) using correlation coefficient as similarity measure and weighted pair group as clustering algorithm. This method is the most appropriate for the validation of the correlation between variables and is often used in environmental studies. The obtained dendrogram is shown in Figure 4.

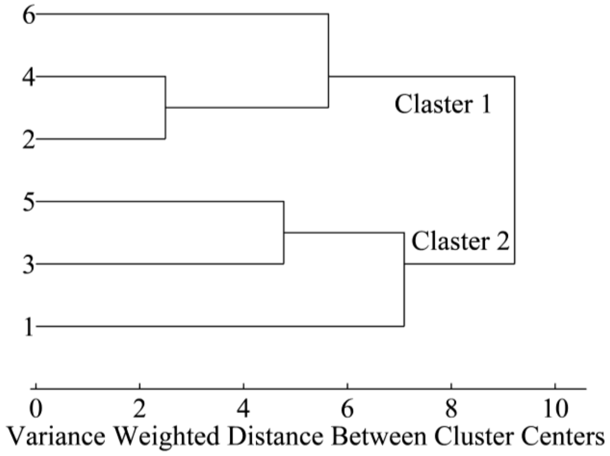

Figure 4. Dendrogram derived from the hierarchical cluster analysis of samples

Nevertheless the cluster analysis organized the metals (without macroconstituents) in such a way that within-group similarity was maximized and among-group similarity was minimized. The CA results for the heavy metals studied are shown in Figure 5 as a dendrogram.

Figure 5 displays four clusters: (1) Ni-Cr; (2) Cd-Ga-In-As-Se; (3) Zn-Ba; (4) Cu-Pb-B in agreement with the PCA results. It is observed, however, that clusters 1 and 2 as well as clusters 3 and 4 join together at a relatively higher level, possibly implying a common source. Fossil fuel emits Ni, Cd, Cr, Cu, Pb, Zn during combustion (Aslan et al., 2011). Besides, the wear of car tires, degradation of parts, peeling paints and greases and metals in catalysts could be sources of pollutants (Pecheyran et al., 2000). Although vehicles with unleaded petrol prevail, the high density traffic of old vehicles using leaded petrol and diesel oil is still present in Serbian roads. Zn, as well as Cd, is well known as an atmophile element subject to long-distance transport (Loppi & Pirintsos, 2003). A part of Zn could be obtained from supporting trees since higher plants are known to release 20 % of total Zn coming from natural sources (Nriagu, 1979). Also, *Flavoparmelia caperata* is known for its higher capacity for Zn and Cd uptake (Nimis et al., 2001).

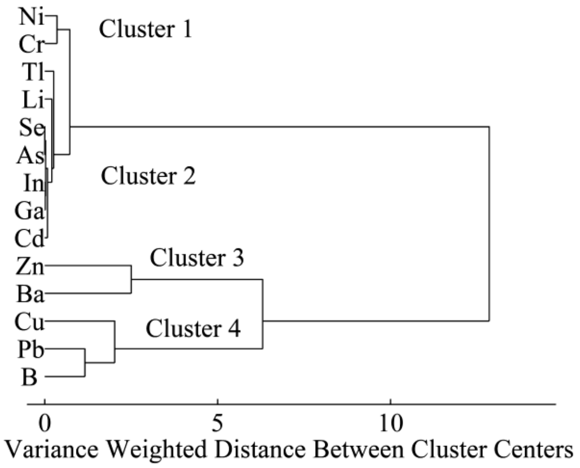

Figure 5. Dendrogram derived from the hierarchical cluster analysis of metals content

Interestingly, cluster 4 indicates pollution caused by exhaust gases due to traffic, but having in mind the poor frequency of traffic at the roadsides in close range of the location of lichen sampling, one could wonder about the origin of Cu, Pb and B detected. This is probably due to the specific wind roses (see Figure 1) which put this area under the indirect impact of metal industry, situated 10-15 km northwesterly (in the outskirt of the city of Niš). Further research and monitoring should be performed in order to obtain the right conclusions.

Conclusion

Our study confirms the metal accumulation capacity of lichen *Flavoparmelia caperata* and its potential for biomonitoring. This lichen species could be effective as an early warning system for detecting changes in the environment. Peripheral parts of lichen samples allow annual changes to be detected. Detected accumulation of heavy metals in lichen samples is probably a result of the frequency of traffic and types of engines on the roads in this area, the activity of industrial complexes in and around the city of Niš, soil and substrate compositions and predominant wind directions. Further research and biomonitoring surveys of air pollution in the studied area should contribute to the amelioration of air quality and environment in Southeastern Serbia.

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