

## CANONICAL VARIATE ANALYSIS OF CHLOROPHYLL CONTENT IN PLANTS EXPOSED TO DIFFERENT LEAD CONCENTRATIONS IN AMBIENT AIR CONDITIONS

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### Abstract

This paper presents the results of biomonitoring of Pb in ambient air. For this purpose Italian ryegrass was used to evaluate Pb level. Additionally chlorophyll forms (a+b, a and b) were measured in leaves. Plants were exposed at 5 sites varying in environmental characteristics in the 2011 growing season. A similar set of plants was conducted in control conditions. Three 28-day long exposure series were performed. The aim of this study was to analyse the relation between Pb level and chlorophyll content in various exposure sites and series using multivariate analysis of variance. The analysis revealed variability of Pb and all chlorophyll forms contents in different exposure sites and series. The lowest level of Pb concentrations was noted at a sub-urban site together with the highest level of all chlorophyll forms contents. Canonical variate analysis could be a proper tool for a graphical data presentation of Pb level in plants exposed to various environmental conditions.

Keywords: lead, chlorophyll, Italian ryegrass, canonical variate analysis

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## 1. INTRODUCTION

Lead is an amphoteric element with strong toxic activity. In natural conditions its accumulation in rocks and soils is relatively low. It was widely used in petrol production, nowadays it can be emitted during industrial activities of many products, such as batteries, paints and lacquers. In the past the main source was emission during combustion in car engines, now diesel engines only emit these elements [2]. Lead is also emitted together with particulate matter and can be transported with it surviving even for 10 days. Wet and dry deposition can affect surface waters and soils [7]. When environment is acidified it can turn into soluble form, which is available to plants. The level of a plant's tolerance to lead is different for various species. Cumulative properties are widely used for clearing an environment from this element as well as protecting barer against spread with wind to further distances [8]. Lead concentration in the atmosphere does not exceeded limited values according to European Union law, however it can be transported and cumulated in plants. Heavy metals occurred in soils and atmosphere can affect plant growth and development, and as a consequence decrease the yield [1]. Even small amount of heavy metal in the air can cause a plant's physiological disorder affecting biogeochemical balance and habitat stability [17]. There are however, some plant species, which can accumulate few percent of heavy metals in dry mass of above ground organs, and they are called hyperaccumulators [21]. Many investigations have been carried out to discover the mechanisms of these species high resistance to heavy metal concentrations. There are still many discussions concerning this problem [14]. The effect of heavy metal can cause negative response of photosynthetic activity, as well as disorders in water balance, respiration, mineral substances content [3]. The water balance is extremely important for proper photosynthesis process and plant growth. Water potential, transpiration intensity and relative water content are usually affected by exposure to heavy metal in ambient air. Chlorophyll content is one of the parameters, which is affected by imbalances in plant metabolism. Hence it can be also a proper marker of plant stress to some external factor, such as heavy metals in ambient air.

Italian ryegrass (*Lolium multiflorum* ssp. *italicum* L) cultivar 'Lema' was found as one of the most useful bioindicator for heavy metal concentration in ambient air, due to its wide range and fast growing [11]. The aim of this study was to evaluate the relation of lead accumulation in Italian ryegrass leaves with chlorophyll content. As well as determination of plant response in different locations and exposure series using canonical variate analysis.

## 2. MATERIALS AND METHODS

### 2.1. Sampling and samples preparation

The experiment was carried out in 2011 during growing season. Investigations schedule was provided according standardized method of German Engineering Association [20]. Similar amount of seeds were sown into 5L pots filled with the standard mixture of soil and peat. Plants were watered by deionized water (conductivity level between  $2\text{-}5\mu\text{S cm}^{-1}$ ; deionizer HLP 30, Hydrolab, Poland) to avoid the additional application of heavy metals. Additionally, plants were fertilized (according to their needs) during growing in the greenhouse, the last fertilizing was at least one day before transport to the exposure site. Every time when plants reached the height of 8-10 cm they were cut to 4cm, also one day before exposure. After six weeks of cultivation in the greenhouse conditions the pots with plants were transported to the exposure sites. Five sites were selected to presented investigations and were located in the city of Poznan and surrounding areas. The sites varied in the air quality characteristic - there were two city sites (site No 1 and 2), one site in suburb areas (No 4), one site representing agriculture area (No 5) and one site located in Agro-ecological Landscape Park (No 3) (Fig. 1).

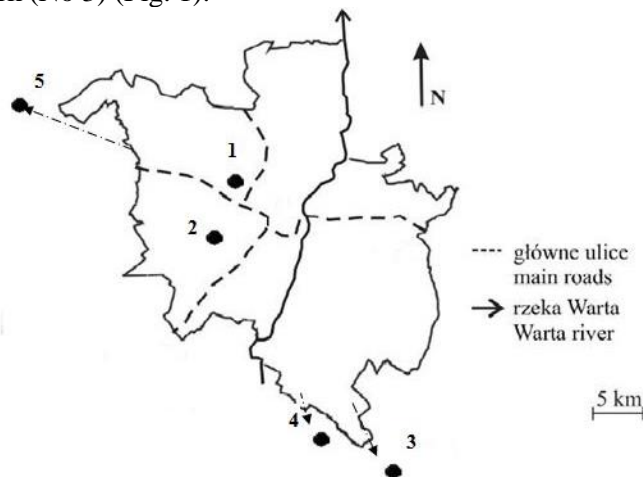


Fig. 1. Location of exposure sites in Poznan city and surrounding areas

Plants were exposed for  $28\pm 1$  days. Three exposure series were carried out in the 2011 year - 16.05-12.06; 13.06-10.07; 11.07-07.08. Five plants were exposed at every site. Similar set of plants were cultivated in greenhouse conditions as control. A continuous water supply was provided through glass fibre wicks placed in pots and dipped in specially constructed water reservoirs

with volume ca. 8L. Hence it was not necessary to check and water the plants every week. The construction made it possible to locate plants at 130cm height above ground at every site, so we received comparable results of plant response to air pollution by lead.

## 2.2. Chlorophyll and lead content measurements

Chlorophyll content  $a+b$ ,  $a$  and  $b$  in fresh and dry matter was investigated. Chlorophyll  $a+b$ ,  $a$  and  $b$  content in fresh matter was measured using the dimethyl sulfoxide extraction method [16, 9]. The level of chlorophyll was analysed by the spectroscopic method (spectrophotometer Hach DR 2000, Lange, USA) and calculated based on Arnon's pattern. Based on the percentage of dry matter content and chlorophyll in fresh weight, the chlorophyll content in dry weight was calculated.

Lead (Pb) concentrations in leaves were measured before and after every exposure series. For this purpose leaves were dried and 0.5 g of dry weight were deepened in 9mL ultrapure  $\text{HNO}_3$ . Samples were mineralized in microwave equipment MARS5. The whole process was divided into three phases: rich the certain parameters, maintains proper parameters (pressure 300 PSI, temperature  $175^\circ\text{C}$ ) for 15 minutes, cooling for the next 15 minutes. The digested samples were quantitatively transferred into 10mL volumetric flasks, and the final volume adjusted to the mark with deionized water. Pb was determined in each prepared sample using an inductively coupled plasma spectrometer equipped with a dynamic reaction cell (ICP-MS Elan DRCII, Perkin Elmer, Canada). An ICP-MS spectrometer equipped with a Meinhard concentric nebulizer, a cyclonic spray chamber, Pt cones and a quadruple mass analyzer were used for this study. Typical instrument operating conditions for the ICP-MS spectrometer were: RF power - 1150 W; plasma Ar flow rate - 15 L/min; nebulizer Ar flow rate - 0.98 L/min and auxiliary Ar flow rate - 1.2 L/min. Whilst tuning the ICP-MS, compromise conditions for a maximum signal intensity of the analyte ( $^{24}\text{Mg}^+$ ,  $^{115}\text{In}^+$ ,  $^{238}\text{U}^+$ ) and a minimum ratio of oxide ( $^{140}\text{Ce}^{16}\text{O}^+ / ^{140}\text{Ce} < 3\%$ ) and doubly charged ions ( $^{128}\text{Ba}^{2+} / ^{128}\text{Ba}^+ < 3\%$ ) were found. The proper conditions of ICP-MS working were checked by using a solution containing Mg, In, U at a concentration of  $1 \mu\text{g L}^{-1}$  and a Ba concentration of  $10 \mu\text{g L}^{-1}$  (Smart Tune Solution - Elan DRC II /plus, Atomic Spectroscopy Standard, Perkin Elmer Pure). Calibration curves were established using aqueous standards of Pb. The standards solution were prepared using  $10 \text{ mg L}^{-1}$  Multi-element ICP-MS Calibration Std 3 (Atomic Spectroscopy Standard, Perkin Elmer, Canada). The isotopes of Sc45 and Rh103 were prepared from appropriate solutions with a concentration of  $1000 \text{ mg L}^{-1}$  (Merck, Germany). All standards were prepared daily from after

subsequent appropriate dilution with high purity deionized water (Millipore, USA). The internal standards correction, scandium and rhodium at the concentration of  $1 \mu\text{g L}^{-1}$ , provided for dealing with matrix-induced various and instrumental drifts.

The correctness of the analytical results was assessed by the use of CRMs for Pb in water reference material NIST 1643e.

### 2.3. Statistical analysis

Let us adopt the structure of a model for the sample  $y_{ikj}$  coming from the content of lead ( $i=1$ ) or  $i$ -th chlorophyll forms ( $i = 2, \dots, I$ ; here  $I = 7$ ),  $k$ -th exposure site ( $k = 1, \dots, K$ ; here  $K = 6$ ), the  $j$ -th series ( $j = 1, \dots, J$ ; here  $J = 3$ ) and  $r$ -replications ( $r = 1, \dots, r_{k,j}$ ; here  $r_{k,j} = 5$  excluding  $r_{4,3} = 4$ ):

$$y_{ikjr} = \mu_i + \xi_{ik}^1 + \xi_{ij}^2 + \xi_{ikj}^{12} + e_{ikjr} \quad (2.1)$$

where for the  $i$ -th selected (one of six) chlorophyll content or content of lead,  $\mu_i$  is the general mean,  $\xi_{ik}^1$  is the  $k$ -th exposure site effect,  $\xi_{ij}^2$  is the  $j$ -th exposure series effect,  $\xi_{ikj}^{12}$  is the  $k, j$ -th effect of site  $\times$  exposure series interaction and  $e_{ikjr}$  is the random error. In addition, let  $N$  denote the number of all replications in the experiment, and equal  $N = \sum_k \sum_j r_{kj}$ .

All chlorophyll contents are related to each other, as well as connected to the Pb level in leaves. According to the above assumptions and relations, it is convenient to treat the results of experimental observations as multidimensional variates. The multivariate linear model can be written in the form:

$$\mathbf{Y} = \mathbf{1}_N \boldsymbol{\mu}' + \mathbf{X}_1 \boldsymbol{\Xi}_1 + \mathbf{X}_2 \boldsymbol{\Xi}_2 + \mathbf{X}_{12} \boldsymbol{\Xi}_{12} + \mathbf{U} \quad (2.2)$$

where  $\mathbf{Y}$  is the  $N \times I$  matrix of observations,  $\mathbf{1}_N$  is the  $N \times 1$  vector of every element equal 1,  $\boldsymbol{\mu}$  is the  $I \times 1$  vector of general means,  $\boldsymbol{\Xi}_1$  is the  $K \times I$  matrix of exposure sites effects,  $\boldsymbol{\Xi}_2$  is the  $J \times I$  matrix of exposure series effects,  $\boldsymbol{\Xi}_{12}$  is the  $KJ \times I$  matrix of sites  $\times$  series interaction parameters,  $\mathbf{X}_1$ ,  $\mathbf{X}_2$ ,  $\mathbf{X}_{12}$  are design matrices and  $\mathbf{U}$  is the  $N \times I$  matrix of errors.

Finally the considered model can be described in the form:

$$\mathbf{Y} = \mathbf{X} \boldsymbol{\Xi} + \mathbf{U} \quad (2.3)$$

where  $\mathbf{X} = [\mathbf{1}_N : \mathbf{X}_1 : \mathbf{X}_2 : \mathbf{X}_{12}]$  and  $\boldsymbol{\Xi}' = [\boldsymbol{\mu}' : \boldsymbol{\Xi}_1' : \boldsymbol{\Xi}_2' : \boldsymbol{\Xi}_{12}']$ .

Let us consider the hypotheses:  $H_{0;12} : \mathbf{C}_{12}\boldsymbol{\Xi}_{12} = \mathbf{0}$ , where  $\mathbf{C}_{12} = \mathbf{I}_{KJ} - \frac{1}{KJ}\mathbf{1}_{KJ}\mathbf{1}'_{KJ}$  ( $\mathbf{I}_{KJ}$  being the  $KJ \times KJ$  identity matrix). Then the best linear unbiased estimator for  $\boldsymbol{\Xi}_{12}$  is  $\hat{\boldsymbol{\Xi}}_{12} = (\mathbf{X}'_{12}\mathbf{X}_{12})^{-1}\mathbf{X}'_{12}\mathbf{Y}$  [17]. This hypothesis tests whether the lead concentration and chlorophyll contents of an experimental object (grass plants in individual site and certain series), reduced by the exposition site  $\times$  series interaction (means of Pb concentrations and chlorophyll contents in all exposition site and series over all exposition series), is zero. The hypothesis was tested using Lawley-Hotelling's statistics [12, 10].

The analyses conducted to determine the chlorophyll contents in Italian ryegrass made it possible to present the position of selected experimental objects in the space of the two first canonical variates. Canonical variate analysis is a method which enables a graphical presentation of the results of multidimensional experiments [12]. In the case of our investigation into the differences between sites  $\times$  series interaction (the lead concentration and chlorophyll contents), this method consists in transforming the matrix  $\mathbf{C}_{12}\hat{\boldsymbol{\Xi}}_{12}$  into a set of new variables which carry similar information, but are distributed in a multivariate Euclidean space [10, 12].

### 3. RESULTS AND DISCUSSION

Investigations revealed variability in Pb content in *Lolium multiflorum* leaves, as well as in all forms of chlorophyll content. The lowest lead concentrations were always noted at the control site in all series. While, chlorophyll concentrations were lower at all exposure sites in comparison to the control one. Differences between all chlorophyll forms in exposed plants and control were always significant at level  $\alpha=0.01$ . Decreased chlorophyll content in plant leaves in relation to selected trace elements was already noted in many horticultural and crop species [13]. While lead disturbed biosynthesis of chlorophylls in mustard [8]. The highest Pb level was observed in plants exposed at city sites (No 1 and 2), while lower and comparable levels were noted at agro-ecological park (No 3), sub-urban (No 4) and rural sites (No 5) (Tab. 1).

The highest chlorophyll (all forms) content was noted in control plants. Comparing exposure sites, excluding control, the highest average chlorophyll contents were observed at the sub-urban site (No 4) (Fig. 2.).

Table 1. Comparison of chlorophyll concentrations and Pb contents at individual exposure sites and the control. Average values of chlorophyll concentrations for control.

Parameters	Exposure sites (differences between individual exposure sites and control)					Control (average values)
	1	2	3	4	5	
$a+b$ (mg g <sup>-1</sup> FW)	-1.37**	-1.21**	-1.03**	-0.92**	-0.29**	4.31
$a$ (mg g <sup>-1</sup> FW)	-0.77**	-0.92**	-0.66**	-0.61**	-0.14**	2.71
$b$ (mg g <sup>-1</sup> FW)	-0.54**	-0.39**	-0.52**	-0.28**	-0.18**	1.34
$a+b$ (mg g <sup>-1</sup> DW)	-31.6**	-27.6**	-29.0**	-21.6**	-26.4**	46.9
$a$ (mg g <sup>-1</sup> DW)	-20.6**	-19.5**	-19.5**	-15.4**	-17.5**	30.5
$b$ (mg g <sup>-1</sup> DW)	-9.91**	-8.21**	-9.56**	-6.07**	-8.24**	14.2
Pb (μg g <sup>-1</sup> DW)	0.57**	0.59**	0.26**	0.30**	0.23**	0

\*\* $\alpha \leq 0.01$ - significance level

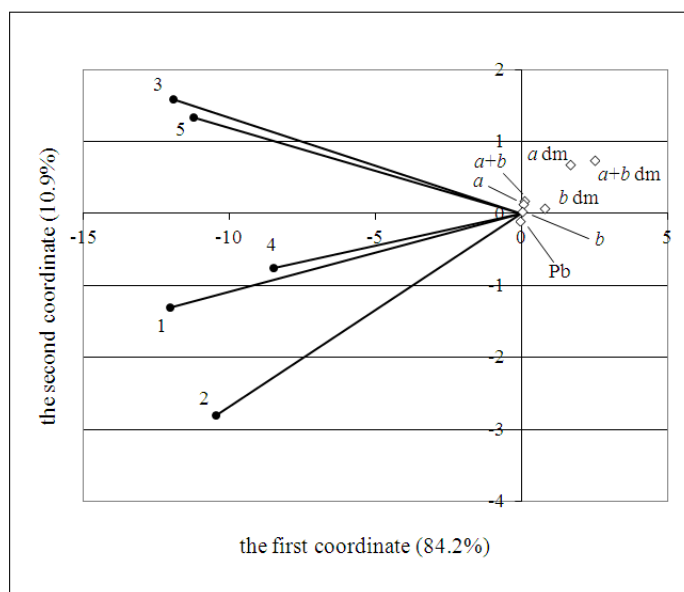


Fig. 2. Position of chlorophyll contents in plants at the exposure sites in relation to control in the first two canonical variates and spacing of the chlorophyll contents and Pb in dual space

This was affected by the highest chlorophyll contents in dry mass. While the highest levels of chlorophyll contents in fresh weight were recorded in plants located in the rural site (No 5). The lowest chlorophyll contents (excluding

chlorophyll a in fresh weight) were noted at one of the urban sites (No 1). Interesting relation was observed between Pb accumulation and chlorophyll content at sub-urban site. High chlorophyll contents in dry matter were noted, while Pb concentrations were comparable to these noted at site No 3 and 5. However, chlorophyll contents in fresh weight were at comparable level at these three sites (Tab. 1). Detailed comparison of individual experimental objects (values of observation factors in particular exposure series and site) confirmed the highest chlorophyll contents in control site in all exposure series (Fig. 3).

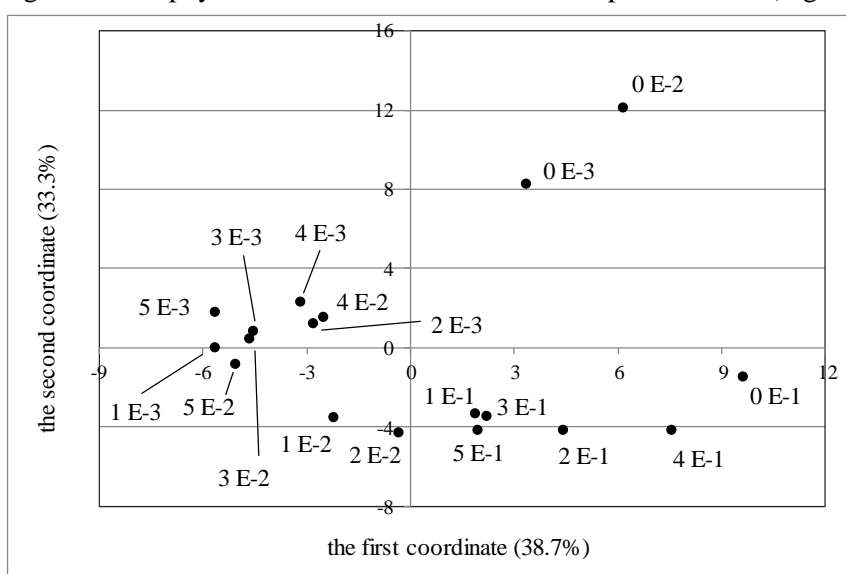


Fig. 3. Position of chlorophyll contents in plants at exposure sites and series in the first two canonical variates (1 e -1 denotes the first exposure site and the first exposure series)

This was valid for all chlorophyll forms, excluding chlorophyll in fresh weight after 3<sup>rd</sup> series (Tab. 2). Analysis of positioning of certain experimental objects in the canonical variates space revealed data dividing into two groups. At one site chlorophyll contents for all exposure sites of 1st series were located, while the 2<sup>nd</sup> and 3<sup>rd</sup> was at the other site of the plot. Lower differences in comparison to control were found for samples collected from plants after the 1st exposure series (Fig. 3). Chlorophyll contents in dry mass at both urban sites in the 2<sup>nd</sup> exposure series were the lowest for all experimental objects (Tab. 2), hence we could observe a greater distance to results at the rest of sites in the 2<sup>nd</sup> and the 3<sup>rd</sup> exposure series.



Table 2. Differences between individual exposure sites and series of all chlorophyll forms and Pb in comparison to means over all exposition sites and all exposition series

Site-series	Chlorophylls						
	<i>a+b</i>	<i>a</i>	<i>b</i>	<i>a+b</i> dm	<i>a</i> dm	<i>b</i> dm	Pb
0E-1	2.02	0.20	1.16	27.39	7.43	13.17	-0.32
1E-1	-0.75	-1.16	-0.02	-7.76	-8.91	-1.18	0.02
2E-1	0.88	-0.48	0.66	4.02	-4.07	3.67	-0.02
3E-1	-0.55	-1.03	0.04	-6.07	-7.96	-0.67	0.06
4E-1	0.84	-0.50	0.65	11.85	-1.00	6.66	0.07
5E-1	-0.46	-1.01	0.16	-7.48	-8.58	-0.66	0.16
0E-2	1.06	1.29	0.16	30.21	26.42	6.82	-0.32
1E-2	-0.96	-0.19	-0.45	-11.25	-4.91	-4.23	0.81
2E-2	-1.25	-0.61	-0.47	-11.56	-6.29	-4.05	0.93
3E-2	0.27	0.33	-0.34	-5.41	-2.48	-3.72	-0.08
4E-2	-0.83	0.00	-0.29	-5.62	0.18	-2.05	0.01
5E-2	1.75	1.30	0.42	0.36	1.17	-0.41	-0.16
0E-3	-0.66	0.04	-0.34	10.69	12.33	1.11	-0.32
1E-3	0.00	0.57	-0.20	-7.37	-1.87	-3.21	-0.09
2E-3	-0.84	-0.12	-0.39	-7.09	-1.87	-3.13	-0.11
3E-3	-0.38	0.27	-0.29	-7.26	-1.75	-3.17	-0.16
4E-3	-0.40	0.26	-0.30	-3.78	1.04	-2.41	-0.20
5E-3	0.26	0.82	-0.17	-3.87	1.10	-2.53	-0.28

E-exposure site

A smaller distance to the control site was observed for the sub-urban site (No 4) and the urban site (No 2) during the 1<sup>st</sup> exposure series (Fig. 3). This was caused by high values of chlorophyll *a+b* and *b* (Tab. 2). The lower level of chlorophyll content and photosynthesis activity at the exposure sites was related to lead accumulation. Disturbed photosynthesis process can cause smaller plant growth and development, as well as a lower level of elements related to photosynthesis activity, such as nitrogen, potassium, magnesium, and also can effect on cell membranes permeability. Chlorophyll inhibition was previously noted [5, 4] mainly due to high sensitivity of two key enzymes of the chlorophyll biosynthetic pathway: d-aminolaevulinic acid (ALA)-dehydratase (EC 4.2.1.24) and protochlorophyllide reductase [18].

#### 4. CONCLUSIONS

Canonical variate analysis revealed variability of Pb and contents of all chlorophyll forms in different exposure sites and series. Overall, the highest chlorophyll content was observed in the control plants, while the lowest were noted in plants exposed in one of the urban sites. The lowest level of Pb concentrations was noted at sub-urban site together with the highest level of contents of all chlorophyll forms. Canonical variate analysis could be a proper tool for graphical data presentation of the Pb level in plants exposed to various environmental conditions.

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ANALIZA ZMIENNYCH KANONICZNYCH ZAWAŃCZOŚCI CHLOROFILU  
W ROŚLINACH EKSPONOWANYCH NA RÓŻNE STĘŻENIA OŁOWIU  
W POWIETRZU ATMOSFERYCZNYM

Streszczenie

Praca prezentuje rezultaty biomonitoringu ołowiu w zróżnicowanych warunkach środowiskowych. Do oceny zawartości poziomu ołowiu wykorzystano rośliny życia wielokwiatowej. Dodatkowo w liściach oznaczono zawartość chlorofilu ( $a+b$ ,  $a$  oraz  $b$ ) w świeżej masie. Rośliny ekspozycyjne w okresie wegetacyjnym roku 2011 na pięciu stanowiskach badawczych różniących się parametrami środowiskowymi oraz w warunkach kontrolnych. Rośliny ekspozycyjne w 28-dniowych okresach badawczych. Wyniki zawartości Pb oraz poziomów chlorofilu w różnych miejscach ekspozycyjnych oraz seriach testowano z zastosowaniem wielowymiarowej analizy wariancji. Wykazano zmienność zawartości Pb oraz poziomów wszystkich form chlorofilu w różnych miejscach ekspozycyjnych i seriach. Najniższe zawartości ołowiu oraz najwyższe poziomy wszystkich form chlorofilu zaobserwowano na stanowisku podmiejskim. W pracy wykazano przydatność analizy zmiennych kanonicznych do graficznej prezentacji wyników biomonitoringu powietrza.

Słowa kluczowe: ołów, chlorofil, życia wielokwiatowa, analiza zmiennych kanonicznych

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