

**SOCIETY OF ECOLOGICAL CHEMISTRY AND ENGINEERING**

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**ECOLOGICAL CHEMISTRY  
AND ENGINEERING A**

**CHEMIA I INŻYNIERIA EKOLOGICZNA A**

**Vol. 20**

**No. 6**

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**OPOLE 2013**

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Ecological Chemistry and Engineering A / Chemia i Inżynieria Ekologiczna A  
is partly financed by Ministry of Science and Higher Education, Warszawa

ISSN 1898–6188

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Stanisław KALEMBASA<sup>1</sup> and Beata KUZIEMSKA<sup>1\*</sup>

## EFFECT OF VARIED CONTENT OF MOLYBDENUM AND BORON IN SOIL ON UREASE ACTIVITY

### WPLYW ZRÓŻNICOWANEJ ILOŚCI MOLIBDENU W GLEBIE NA AKTYWNOŚĆ UREAZY

**Abstract:** The aim of the study was to determine the effect of varied content of molybdenum and boron in soil on urease activity. Soil in a three-year pot experiment, to which different amounts of molybdenum (0, 0.05, 0.1 and 0.15 mgMo · kg<sup>-1</sup> of soil on a one-off basis as an aqueous solution of Na<sub>2</sub>MoO<sub>4</sub> · 2 H<sub>2</sub>O) and boron (5, 10, 15 mgB · kg<sup>-1</sup> of soil as an aqueous solution of borax Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> · 10 H<sub>2</sub>O) were used for analyses. Orchard grass and crimson clover were cultivated in the experiment. Four re-growths of the test plants were harvested during each vegetation period. Soil during each year of the experiment was analysed, after each re-growth of the test plants. Urease activity was determined by the Hoffman and Teicher method based on colorimetric determination of ammonia following enzymatic hydrolysis of urea.

The application of increased amounts of molybdenum significantly increased the enzyme activity in soil samples taken after the grass and clover were harvested. Urease activity in soil samples taken after clover harvest was higher each year than in soil taken after grass harvest. No effect of increasing boron content on urease activity was observed in soil taken after the cultivation of test plants.

**Keywords:** enzymatic activity, urease, molybdenum, boron, mineral fertilization

## Introduction

Acidic soils with limited molybdenum availability account for approx. 60 % of arable land in Poland [1–3]. Like other countries around the world where sandy and acidic soils dominate, they must be fertilised with this microelement [3, 4]. The average content of this element in soil ranges from 0.1 to 5 mg · kg<sup>-1</sup> of soil. Usually, the heavier the soil, the more molybdenum it contains. According to Starck [5], the content of available forms of this microelement in soil does not usually exceed 10 % of its overall content. Active molybdenum occurs in soil solutions at very small concentrations from 10<sup>-8</sup> to 8 · 10<sup>-8</sup> mole · dm<sup>-3</sup>, mainly as MoO<sub>4</sub><sup>2-</sup>. In soil, it forms

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various primary and secondary minerals, it is found in many organic compounds, it is bound on the surface of hydrated iron and aluminium oxides and silty minerals and it can be dissolved in the soil solution. Molybdenum undergoes various antagonisms in soil and its content decreases with decreasing pH and increasing temperature [6]. It is an essential element for correct growth and development of plants, animals and humans [7]. In plants, it is contained in enzymes (eg nitrate reductase and nitrogenase) which take part in processes of nitrogen fixing and nitrate reduction. It is also a component of enzymes – oxidases, which catalyse other processes in plants. In case of a shortage of this microelement in plants fed with nitrates,  $\text{NO}_3^-$  ions accumulate in soil and plants then contain small amounts of chlorophyll and ascorbic acid. The optimum concentration of molybdenum in plants does not exceed  $1 \text{ mg} \cdot \text{kg}^{-1}$  of d.m. Molybdenum is taken up from the soil passively, and its absorbability increases with increasing pH of soil, which can be explained by supplanting the adsorbed  $\text{MoO}_4^{2-}$  ions by  $\text{OH}^-$  ions in a less acidic environment and by the ion migration to the soil solution. In addition, an increase in the concentration of phosphate ions in soil increases the availability of molybdenum because those ions act like  $\text{OH}^-$  ions [5]. Physiological demand for the element is different for different plants. Higher demand is shown by legume and *Cruciferae* plants and those cultivated on soils (substrates) with high nitrate content. Insufficient levels of molybdenum lead to death of the apex, leaf necrosis and flower atrophy [8].

The content in soil of boron, which is the other element under study, ranges from 1 to  $210 \text{ mgB} \cdot \text{kg}^{-1}$  of soil. Such levels are insufficient in about 90–100 % of the soils in Poland. Although the type of soil is not so closely related to the content of the microelement in it, its highest concentrations are found in brown clayey, black and salty soils [7, 9, 10]. The process of the element uptake by plants is affected by: pH of soil (it is better absorbed from acidic and weakly soils and its absorption decreases with increasing pH), the presence of other ions in the soil solution ( $\text{Ca}^{2+}$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$  and  $\text{PO}_4^{3-}$  ions considerably reduce boron absorption) and water content (its absorption increases at higher moisture content values). Plants take up the element through their root system as the  $\text{H}_2\text{BO}_3^-$  anion or undissociated molecules of boric acid  $\text{H}_3\text{BO}_3$ . The mechanism of its uptake has not been fully elucidated. It has been suggested that there is more than one mechanism [11, 12].

Symptoms of boron shortage are observed mainly on freshly limed, light and sandy soils. They are aggravated by periods of drought, but also during spells of cold and rainy weather. A deficit of the element in plants results in inhibition of growth and atrophy of apices, both in shoots and roots, and in the plants losing their ability to form generative parts of flowers.

The literature contains little data on the effect of varied amounts of boron and molybdenum in soil on its enzymatic activity, although soil enzymes play a fundamental role as catalysts of reactions of organic matter decomposition [13]. In many scientific publications, they are increasingly often referred to as the coefficient of biochemical and microbiological activity of soils. Performing different agricultural procedures, such as: natural, organic and mineral fertilisation, the application of pesticides as well as the species and cultivar of the plants – all these are the factors which affect enzymatic activity, and consequent fertility of soils [13, 14]. Therefore, a study was taken up

whose aim was to determine the effect of a plant species (in terms of biological capability of nitrogen reduction) and varied content of molybdenum and boron in soil on urease activity. The grass *Dactylis glomerata* L. (which is unable to reduce nitrogen [N<sub>2</sub>] from the atmosphere) and crimson clover (*Trifolium pratense* L.) (which demonstrates a great capability for reducing nitrogen [N<sub>2</sub>]), were used as test plants. Therefore, it can be supposed that urease activity in the soil on which clover was grown should be higher than in orchard grass. Verification of this hypothesis was the aim of this study.

## Material and methods

Soil from a three-year pot experiment was used for the analyses. In the experiment, conducted in 2008–2010 at facilities of the University of Natural Science and Humanities in Siedlce, orchard grass and crimson clover were the test plants. This random experiment was conducted in triplicate, in 15 dm<sup>3</sup> pots, which were filled with 10 kg of soil each. The soil for the experiment was taken in spring 2008 from the humus horizon (Ap: 0–30 cm) of pseudogley soil [15], in Wysoczyzna Siedlecka (Siedlce Uplands). According to the soil characterisation, it was formed from boulder clay of the Wolstonian stage (Warthe Stage). When the samples were taken, the soil was used for agriculture and classified as complex four of agricultural soil usability (very good rye complex) and quality class IVa. Its basic properties are shown in Table 1.

Table 1

Some properties of soil used for pot experiment

Percentage granulometric fraction with a diameter [mm]			Group granulometric	pH 1 MKCl	Hh [cmol(+) · kg <sup>-1</sup> ]	C <sub>org</sub>	N <sub>tot</sub>	C:N	Mo <sub>tot</sub> [mg · kg <sup>-1</sup> soil]
fraction of sand 2–0.05	dust fraction 0.05–0.002	clay fraction < 0.002				[g · kg <sup>-1</sup> soil]			
71	22	7	sandy loam	4.8	2.83	8.2	0.75	10.9:1	0.088

Its granulation was typical of sandy clay [16], which corresponds to the average agronomical category of soil heaviness; its pH was acidic and the C:N ratio (10.9:1) was typical of humus horizon. The total molybdenum and boron content were typical of natural soils [7].

The following fertilisation options were examined:

1. control (no fertilisation – 0),
2. PK (phosphorus and potassium fertilisation),
3. NPK (nitrogen, phosphorus and potassium fertilisation),
4. NPK + Mo<sub>1</sub> (nitrogen, phosphorus and potassium fertilisation + 0.05 mgMo · kg<sup>-1</sup> of soil),
5. NPK + Mo<sub>2</sub> (nitrogen, phosphorus and potassium fertilisation + 0.1 mgMo · kg<sup>-1</sup> of soil),

6. NPK + Mo<sub>3</sub> (nitrogen, phosphorus and potassium fertilisation + 0.15 mgMo · kg<sup>-1</sup> of soil),

7. NPK + B<sub>1</sub> (nitrogen, phosphorus and potassium fertilisation + 5 mgB · kg<sup>-1</sup> of soil),

8. NPK + B<sub>2</sub> (nitrogen, phosphorus and potassium fertilisation + 10 mgB · kg<sup>-1</sup> of soil),

9. NPK + B<sub>3</sub> (nitrogen, phosphorus and potassium fertilisation + 15 mgB · kg<sup>-1</sup> of soil).

In the years 2008–2010 orchard grass (*Dactylis glomerata* L.) was grown which was sown and harvested four times in each year of the experiment; in the same years, crimson clover (*Triflorium pratense* L.), cultivar Jubilatka was grown, which was also sown in each year of the experiment. The clover was harvested during the phase between budding and the beginning of flowering, four times in each year of the experiment.

The soil was fertilised every year with nitrogen, phosphorus and potassium. Nitrogen was applied as 21 % ammonium sulphate at the following doses: under grass – 0.05 g · kg<sup>-1</sup> of soil, under clover – “0”. Phosphorus was applied as triple superphosphate containing 19 % P, both under grass and under clover, in the same amount of 0.08 gP<sub>2</sub>O<sub>5</sub> · kg<sup>-1</sup> of soil. Potassium was applied as potassium salt containing 40 % K (as with phosphorus) in the same amount under both the experimental plants: 0.12 gK<sub>2</sub>O · kg<sup>-1</sup> of soil. Molybdenum was applied as an aqueous solution of Na<sub>2</sub>MoO<sub>4</sub> · 2 H<sub>2</sub>O once in the year when the experiment was set up in increasing amounts: 0.05, 0.1 and 0.15 mgMo · kg<sup>-1</sup> of soil, and boron at the following doses 5, 10 and 15 mg of B · kg<sup>-1</sup> of soil as aqueous solution of borax Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> · 10 H<sub>2</sub>O.

The growth and development of the plants were observed during the entire vegetation period and the soil humidity was kept at 60 % of the field water capacity. If plant diseases or pests appeared, chemical plant protection agents were used.

Urease activity in soil samples taken after each re-growth of the test plants in each year of the experiment was determined by the Hoffman and Tiecher method, based on colorimetric determination of ammonia formed by enzymatic hydrolysis of urea [17].

The results were worked out statistically with the analysis of variance, using the Fisher-Snedecor distribution in accordance with program F.R.Anal.var.4.1, and the LSD<sub>(0.05)</sub> value was calculated according to Tukey's test.

## Results and discussion

Cultivation, fertilisation and contamination of soils with different types of toxic substances modify their biological properties, especially their enzymatic activity [14, 18–20]. An enzyme which is particularly often an object of research is urease – one of the hydrolases which catalyses decomposition of urea to ammonia and carbon dioxide. It is an absolutely specific enzyme because it catalyses only one chemical reaction.

The urease activity in soil taken after each grass re-growth during the three years of the experiment is shown in Tables 2–4. The activity of urease was similar throughout the experiment; the highest average value was determined in soil taken after the third year of the experiment: 3.49 (Table 4), and the lowest was in the soil taken after the first year – 3.25 mgN-NH<sub>4</sub> · kg<sup>-1</sup> · h<sup>-1</sup> (Table 2). Similar activities of the enzyme were measured earlier [19].

In this experiment, fertilisation significantly affected the enzyme activity in each year of the three-year cycle. Its significantly higher activity in soil as compared to control pots was determined even with the lowest doses of molybdenum of those used in the experiment. The highest urease activity was determined in the soil to which the highest amounts of this metal were introduced ( $0.15 \text{ mgMo} \cdot \text{kg}^{-1}$  of soil).

Table 2

Urease activity in soil ( $\text{mgN-NH}_4 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) after cultivation of grass (I year experiment)

Objects	Cuts				Means
	I	II	III	IV	
Without fertilization	3.06	2.96	3.09	2.90	3.00
PK	3.15	3.11	3.13	3.03	3.10
NPK	3.28	3.18	3.26	3.10	3.20
NPK + 0.05 mgMo	3.38	3.33	3.33	3.15	3.30
NPK + 0.1 mgMo	3.52	3.27	3.39	3.15	3.33
NPK + 0.15 mgMo	3.73	3.68	3.61	3.46	3.63
NPK + 5 mgB	3.30	3.20	3.26	3.10	3.21
NPK + 10 mgB	3.34	3.24	3.29	3.11	3.25
NPK + 15 mgB	3.33	3.23	3.29	3.10	3.24
Mean for molybdenum	3.54	3.43	3.44	3.25	3.42
Mean for boron	3.32	3.22	3.28	3.10	3.23
Mean in experiment	3.34	3.24	3.29	3.12	3.25

LSD<sub>0.05</sub> for fertilization 0.12;

LSD<sub>0.05</sub> for cuts 0.10.

Table 3

Urease activity in soil ( $\text{mgN-NH}_4 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) after cultivation of grass (II year experiment)

Objects	Cuts				Means
	I	II	III	IV	
Without fertilization	3.17	3.04	3.27	3.10	3.15
PK	3.22	3.18	3.36	3.18	3.23
NPK	3.38	3.27	3.42	3.23	3.32
NPK + 0.05 mgMo	3.62	3.49	3.66	3.54	3.58
NPK + 0.1 mgMo	3.74	3.61	3.79	3.63	3.69
NPK + 0.15 mgMo	3.73	3.61	3.80	3.75	3.72
NPK + 5 mgB	3.41	3.29	3.40	3.27	3.34
NPK + 10 mgB	3.45	3.25	3.44	3.26	3.35
NPK + 15 mgB	3.40	3.25	3.43	3.32	3.35
Mean for molybdenum	3.70	3.57	3.75	3.64	3.66
Mean for boron	3.42	3.26	3.42	3.28	3.35
Mean in experiment	3.46	3.33	3.51	3.36	3.42

LSD<sub>0.05</sub> for fertilization 0.21;

LSD<sub>0.05</sub> for cuts 0.09.

Table 4

Urease activity in soil ( $\text{mgN-NH}_4 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) after cultivation of grass (III year experiment)

Objects	Cuts				Means
	I	II	III	IV	
Without fertilization	3.23	3.16	3.28	3.08	3.19
PK	3.34	3.27	3.39	3.20	3.30
NPK	3.47	3.31	3.59	3.39	3.44
NPK + 0.05 mgMo	3.70	3.55	3.76	3.58	3.65
NPK + 0.1 mgMo	3.78	3.71	3.84	3.70	3.76
NPK + 0.15 mgMo	3.94	3.89	3.88	3.76	3.87
NPK + 5 mgB	3.46	3.31	3.54	3.33	3.41
NPK + 10 mgB	3.50	3.28	3.55	3.31	3.41
NPK + 15 mgB	3.51	3.31	3.59	3.42	3.43
Mean for molybdenum	3.81	3.72	3.83	3.68	3.76
Mean for boron	3.49	3.30	3.56	3.35	3.42
Mean in experiment	3.55	3.42	3.47	3.40	3.49

LSD<sub>0.05</sub> for fertilization 0.27;LSD<sub>0.05</sub> for cuts 0.27.

Increasing the content of molybdenum during the first year of the study increased the enzyme activity by 10 % to 12.1 %, in the second year by 11.4 % to 11.8 %, and in the last year of the experiment by 11.4 % to 12 % as compared to the control. No significant effect of increasing boron content in soil on urease activity was shown to exist in any year of study (Table 8).

Table 5

Urease activity in soil ( $\text{mgN-NH}_4 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) after cultivation of clover (I year experiment)

Objects	Cuts				Means
	I	II	III	IV	
Without fertilization	3.68	3.57	3.62	3.54	3.60
PK	3.75	3.65	3.79	3.69	3.72
NPK	3.94	3.75	3.89	3.87	3.86
NPK + 0.05 mgMo	4.16	4.07	4.21	4.01	4.11
NPK + 0.1 mgMo	4.28	4.17	4.32	4.08	4.21
NPK + 0.15 mgMo	4.42	4.33	4.38	4.10	4.31
NPK + 5 mgB	4.01	3.76	3.90	3.86	3.88
NPK + 10 mgB	4.07	3.81	3.94	3.89	3.93
NPK + 15 mgB	4.03	3.78	3.99	3.89	3.92
Mean for molybdenum	4.29	4.19	4.30	4.06	4.21
Mean for boron	4.04	3.78	3.94	3.88	3.91
Mean in experiment	4.04	3.78	3.94	3.88	3.91

LSD<sub>0.05</sub> for fertilization 0.21;LSD<sub>0.05</sub> for cuts 0.17.

Table 6

Urease activity in soil ( $\text{mgN-NH}_4 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) after cultivation of clover (II year experiment)

Objects	Cuts				Means
	I	II	III	IV	
Without fertilization	3.64	3.72	3.60	3.65	3.65
PK	3.72	3.67	3.84	3.77	3.75
NPK	3.80	3.80	3.95	3.88	3.86
NPK + 0.05 mgMo	4.14	4.12	4.19	4.13	4.14
NPK + 0.1 mgMo	4.30	4.19	4.41	4.22	4.28
NPK + 0.15 mgMo	4.40	4.30	4.61	4.48	4.45
NPK + 5 mgB	3.79	3.77	3.95	3.83	3.84
NPK + 10 mgB	3.85	3.83	3.88	3.78	3.84
NPK + 15 mgB	3.89	3.85	3.99	3.82	3.89
Mean for molybdenum	4.28	4.20	4.40	4.28	4.29
Mean for boron	3.84	3.82	3.94	3.81	3.85
Mean in experiment	3.95	3.92	4.05	3.95	3.97

LSD<sub>0.05</sub> for fertilization 0.16;LSD<sub>0.05</sub> for cuts 0.09.

Table 7

Urease activity in soil ( $\text{mgN-NH}_4 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) after cultivation of clover (III year experiment)

Objects	Cuts				Means
	I	II	III	IV	
Without fertilization	3.70	3.58	3.74	3.69	3.68
PK	3.75	3.58	3.79	3.85	3.74
NPK	3.86	3.81	3.88	3.83	3.84
NPK + 0.05 mgMo	4.20	4.10	4.09	4.00	4.10
NPK + 0.1 mgMo	4.33	4.23	4.32	4.06	4.23
NPK + 0.15 mgMo	4.44	4.42	4.55	4.37	4.45
NPK + 5 mgB	3.79	3.78	3.95	3.91	3.86
NPK + 10 mgB	3.86	3.75	3.98	3.90	3.87
NPK + 15 mgB	3.87	3.84	3.94	3.88	3.88
Mean for molybdenum	4.32	4.25	4.32	4.14	4.26
Mean for boron	3.84	3.79	3.96	3.90	3.87
Mean in experiment	3.98	3.90	4.03	3.95	3.96

LSD<sub>0.05</sub> for fertilization 0.16;LSD<sub>0.05</sub> for cuts 0.09.

The other factor evaluated in the study – the number of crop – had a varied effect on the urease activity in soil samples taken after grass harvesting. Its activity in the first and third years of the experiment was the highest in soil taken after the first grass re-growth, while in the second year it was the highest after the third re-growth. On the other hand, the lowest urease activity in the first and last years of the experiment was observed in soil taken after the fourth crop, while in the second year, it was the lowest in soil taken after the second crop of orchard grass. The activity of the enzyme in soil taken after each clover re-growth of crimson clover in the three-year experiment cycle is shown in Tables 5–7. The enzyme activity determined in the years of study was similar: it was equal to 3.91 in the first year, 3.97 in the second and 3.96  $\text{mgN-NH}_4 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  in the third.

The urease activity depended significantly on fertilisation during each year of the study. Both NPK fertilisation as well as introducing different amounts of molybdenum to soil significantly increased its activity. The increase was marked and statistically proven, even with the smallest dose of molybdenum –  $0.05 \text{ mgMo} \cdot \text{kg}^{-1}$  of soil – and it was equal to 11.4 % in the first and second years of the experiment and 11.1 % in the third year, as compared to the control. The increase in the amount of molybdenum to  $0.1 \text{ mgMo} \cdot \text{kg}^{-1}$  of soil in the first and second years of the experiment resulted in an increase in the activity of the enzyme by 11.7 %, and in the last year by 11.5 %, relative to the pots where the element was not added. The highest dose of molybdenum used in the experiment –  $0.15 \text{ mgMo} \cdot \text{kg}^{-1}$  of soil – increased the activity of the enzyme by 12.0 % in the first and third year and by 12.2 % in the second year, relative to the control. As in soil after grass cultivation, as mentioned above, no significant effect of the varied content of boron on the attribute under examination was shown, which is illustrated in Table 8.

Table 8

Increasing or decreasing the activity of urease in soil ( $\text{mgN-NH}_4 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) in three years experiment depending on application of boron or molybdenum and plant grass or clover

Plant	Year of experience			Mean
	I	II	III	
	molybdenum			
Grass	0.22	0.34	0.32	0.29
Clover	0.35	0.43	0.42	0.40
Mean	0.29	0.39	0.37	0.35
	boron			
Grass	0.03	0.03	-0.02	0.01
Clover	0.05	-0.01	0.03	0.03
Mean	0.04	0.01	0.01	0.02

LSD<sub>0.05</sub> for element 0.20.

An analysis of the time of taking samples for analysis in the years of study showed the highest activity of the enzyme in the first year of the experiment in the soil taken after the first re-growth, while in the second and third year it was after the third re-growth. The lowest activity of urease was observed after the second re-growth of crimson clover.

To summarise this study, it can be said that increasing amounts of molybdenum in soil significantly increased urease activity, both in samples taken after grass and clover harvest. No significant effect of boron – the other micro-element examined in the experiment – on urease activity was observed in soil taken after harvests of both test plants.

The highest average activity of the enzyme after the grass harvest was observed in the third year of the experiment, while in the soil after clover harvest it was in the third and second years of the experiment.

The time of taking samples for analysis had an ambiguous differentiating effect on the activity of the enzyme in soil after harvesting the test plants.

A comparison of the results obtained in both the analysed soils (after the grass and clover harvest) showed that the activity of urease in the soil taken after clover harvest in the first year of the experiment was higher by 21.8 % compared to the soil taken after grass harvest, and it was higher by 16.5 % in the second year and by 12.6 % in the third. This should be associated with the fact that clover has the ability to reduce nitrogen from the air, thereby increasing the amount of nitrogen in the soil. The correlation between nitrogen content in soil and urease activity has been shown in many publications [14, 19].

These results require further research because the effect of molybdenum and boron on the enzymatic activity of soil has not been sufficiently studied or determined.

## Conclusions

1. Using growing amounts of molybdenum significantly increased the activity of urease soil after the cultivation of grass and clover.
2. No effect of varied boron content on urease activity was found in soil samples taken after the cultivation of test plants.
3. The time of taking samples for analysis had an ambiguous differentiating effect on the activity of urease in soil.
4. Urease activity in soil samples taken after the clover harvest was higher each year than in soil taken after grass harvest.

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### WPLYW ZRÓŻNICOWANEJ ILOŚCI MOLIBDENU W GLEBIE NA AKTYWNOŚĆ UREAZY

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**Abstrakt:** Celem pracy było określenie wpływu zróżnicowanej ilości molibdenu i boru w glebie na aktywność ureazy. Do analiz wykorzystano glebę w ciągu trzyletniego doświadczenia wazonowego, w której stosowano zróżnicowane ilości molibdenu (0; 0,05; 0,1 i 0,15 mgMo · kg<sup>-1</sup> gleby jednorazowo w formie roztworu wodnego Na<sub>2</sub>MoO<sub>4</sub> · 2 H<sub>2</sub>O) i boru (w dawkach 5, 10, 15 mgB · kg<sup>-1</sup> gleby w formie roztworu wodnego boraksu Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> · 10 H<sub>2</sub>O). W doświadczeniu uprawiano trawę – kupkówkę pospolitą i koniczynę czerwoną. W każdym sezonie wegetacyjnym zbierano cztery odrosty roślin testowych. Analizie poddano glebę w każdym roku prowadzenia doświadczeń, po każdym odroście roślin testowych. Aktywność ureazy wyznaczono metodą Hoffmana i Teichera opartą na kolorymetrycznym oznaczeniu amoniaku po enzymatycznej hydrolizie mocznika.

Stosowanie zwiększonych ilości molibdenu wpłynęło istotnie na zwiększenie aktywności badanego enzymu w glebie pobranej po zbiorze trawy i koniczyny. Aktywność ureazy w glebie pobranej po zbiorze koniczyny, w każdym roku badań, była większa niż w glebie pobranej po zbiorze trawy. Nie stwierdzono wpływu wzrastających ilości boru na aktywność ureazy w glebie pobranej po uprawie roślin testowych.

**Słowa kluczowe:** aktywność enzymatyczna, ureaza, molibden, bor, nawożenie mineralne

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## PHYTOTOXICITY OF IONIC LIQUID CONTAINING PHOSPHORUS ATOM

### FITOTOKSYCZNOŚĆ CIECZY JONOWEJ ZAWIERAJĄCEJ ATOM FOSFORU

**Abstract:** Ionic liquids are low melting which have been accepted as new generation of polar solvents and catalysts. These compounds are non-volatile, non-flammable and their “green” character has usually been justified with their negligible vapor pressure. However, the marketing of any chemical substance, requires the determination of their impact on all the elements of nature. Determination of the potential ecotoxicity of new chemical compounds associated with the conduct of research on the effects of those compounds on the growth and development of selected organism.

In the present work, the influence of triphenyl-*n*-pentylphosphonium iodide, introduced to the soil on germination and early stages of growth and development of superior plants was investigated using the plant growth test based on the OECD/OECD 208/2006. In this test, the seeds of selected species of land superior plants – spring barley (*Hordeum vulgare*) and common radish (*Raphanus sativus* L. subvar. *radicula* Pers.) were planted in pots containing soil to which a test chemical compound had been added and in pots with control soil.

To evaluate the phytotoxicity of the triphenyl-*n*-pentylphosphonium iodide, the germination and (dry and fresh) weight of control plant seedlings were determined and compared with the germination and (dry and fresh) weight of the seedlings of plants grown in the soil with appropriate amounts of the test chemicals added. The visual assessment of any types of damage to the test species, such as growth inhibition, chlorosis and necrosis, based on the obtained results determined the size of the LOEC and the NOEC.

**Keywords:** ionic liquids, phytotoxicity, land superior plants, spring barley, common radish, yield, dry weight, chlorosis, necrosis

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## Introduction

The history of research on ionic liquids commenced at the end of the 19<sup>th</sup> century with the obtaining a substance called the “red oil” as a result of the Friedel-Crafts reaction. Then analytical methods did not enable the chemical constitution of this substance to be identified, therefore the structure of the “red oil” was understood relatively not long ago. In 1914, Walden obtained the first useful salt of this type – ethylammonium nitrate. The name *ionic liquid*, on the other hand, first appeared in specialist literature in 1974. A greater interest was aroused by ionic liquids only in the 90s with the obtaining of salts stable in the presence of air and moisture. From that time on, a dynamic growth of research activity related to the synthesis, properties and applications of ionic liquids in the chemical industry, electrochemistry, biotechnology or pharmacy has been observed [1–5].

Ionic liquids are chemical compounds that are built exclusively of ions. The cation is organic and, as a rule, is a large molecule of an asymmetric structure. The anion is most often an inorganic compound. Such a situation leads to obtaining a salt with a low crystalline energy which, as a consequence, reduces the compound’s melting point, which ranges between –20 °C and 100 °C. The ionic liquids that are liquid at room temperature are described by the acronym RTIL (*Room Temperature Ionic Liquids*), while the salts being solids under these conditions are referred to as *Ionic Liquids* (IL) [3, 6, 7].

Ionic liquids in the form of organic salts exhibit a number of desirable properties, such as very low vapour pressure, thermal and electrochemical stability, high ionic conductance, incombustibility, good catalytic properties; also, many of these compounds are recyclable. Ionic liquids are unmixable with many organic substances, while the majority of them exhibit excellent solubility in water; they are also very good solvents for many inorganic, organic and organometallic compounds. Yet the most important feature of ionic liquids is that they provide the possibility of obtaining substances of optimal properties for a given process through the modification of the cation structure and selection of the appropriate anion; therefore, the term *designer solvents* has been invented for these salts. The above-mentioned properties were decisive to ranking ionic liquids among very attractive solvents [3, 5, 8–12].

The all of the above characteristics and the relatively low production cost may soon lead to the situation, where huge amounts of these chemicals will be brought in to industry, agriculture and commercial trading. This might, at the same time, pose a potential hazard to the natural environment, to which ionic liquids would get in the form of production wastes, waste water discharges, dump effluents, etc. Hence the need arises for determining the degree of influence of these salts on the natural environment and the potential for their penetration and accumulation in particular living organisms or entire trophic chains. As for now, there is a huge number of report in the scientific literature, which concern with the research of the potential ecotoxicity of these compounds [13–19]. The cited literature demonstrates clearly that the potential toxicity of ionic liquids is determined by a number of factors, of which the primary role is played by the cation type and the length of the substituent in the cation; the anion type,

the concentration used and the habitat conditions of a specific organism are not without significance, either [3, 5, 13, 20, 21].

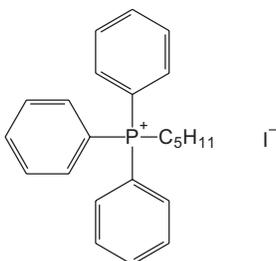
When planning ecotoxicological tests it should be borne in mind that the ultimate “store” of all substance, both nutritive and toxic ones, is the soil. Due to human activity, the soil becomes loaded with a number of chemical substances that may have an adverse effect, both on the edaphon and on the yield volume and the nutritive quality of cultivated plants [22]. As soon as large amounts of ionic liquids appear in the trade, their passage to the soil environment should be taken into account, where the phenomenon of soil sorption associated with the presence of humus and inorganic colloids may retain these salts in the near-root layer, which poses an immediate threat to the plants. Therefore, a lot of studies have already appeared in the literature, whose authors attempt to assess the degree of ionic liquid influence on all soil environment elements and on the growth and development of higher land plants [8, 18, 23–25].

The phytotoxicity determined in those studies is part of broadly understood ecotoxicity. When conducting ecotoxicological studies, one can use classic chemical analysis methods that enable the concentration of chemical compounds to be determined in the samples analyzed. However, in ecotoxicological tests, environmental bioanalysis and biomonitoring more and more often abandon classic chemical analysis due to high apparatus and operation expenditures. Chemical analysis may also be insufficient in providing information about a potential ecological risk, because it is not able to identify all possible toxic effects, which are often dependent on the bioavailability of the toxic substance, as well as on the synergic and antagonistic interactions within the whole population or individual organisms [26–28].

The purpose of the presented study was to evaluate the influence of triphenyl-*n*-pentylphosphonium iodide on the growth and development of higher land plants.

## Materials and methods

The presented study has determined the effect of triphenyl-*n*-pentylphosphonium iodide having the formula:



synthesized at the Department of Organic Chemistry of Jan Dlugosz University (AJD) in Czestochowa, on the emergence and growing of higher land plants. The compound under examination at room temperature is a solid body, quite sparingly soluble in water. The experiment to determine the phytotoxicity of the ionic liquid was carried out in the vegetation hall of the AJD’s Department of Biochemistry and Bioproduct Technology

based on the OECD/OCDE 208/2006 Guide [29] and standard PN-ISO 11269-2:2001 [30].

A monocotyledonous plant – the spring barley (*Hordeum vulgare*), and a dicotyledonous plant – the common radish (*Raphanus sativus* L. subvar. *radicula* Pers.) were used in the pot experiment. 90 mm-diameter flower pots were filled with the control soil and with a soil with the addition of triphenyl-*n*-pentylphosphonium iodide, respectively. The grain size analysis of the soil showed that it was light loamy sand (pgl) with a fine earth particle content of approx. 11 %, an organic carbon content of 0.9 %, and a pH(KCl) of 5.8. 20 identical seeds of the selected plants originating from the same source were sowed into each of so prepared pots.

The experiment to examine the potential phytotoxicity was composed of two testing cycles: preliminary tests and final tests. The preliminary tests were carried out to determine the range of the compound's concentrations influencing the soil quality; therefore, in accordance with standard PN-ISO 11269-2:2001 [30], triphenyl-*n*-pentylphosphonium iodide was applied in three concentrations: 0 mg, 1 mg, 10 mg, 100 mg and 1000 mg/kg dry soil mass. In the final tests, concentrations were selected in a geometric progression using a factor of 2. In the presented study, concentrations of 20 mg, 40 mg and 80 mg/kg dry soil mass, respectively, were used. The ionic liquid was introduced to the soil in the form of water solutions.

To assess the phytotoxicity of triphenyl-*n*-pentylphosphonium iodide, the emergence and mass (dry and green) of control plant sprouts with the emergence and mass (dry and green) of the sprouts of plants growing on the soil with the respective amount of the examined substance added were determined and compared. A visual examination of any damage to the plants, such as growth inhibition, chlorosis and necrosis, was also made, which is shown on the digital photographs. Based on the obtained results, the values of LOEC (*Lowest Observed Effect Concentration*) that is the lowest concentration causing statistically proved differences between the emergence and crop of plants growing on the soil with the ionic liquid addition and those of the control plants, and the values of NOEC (*No Observed Effect Concentration*) which is the highest concentration not causing any noticeable toxic effects, were also determined.

The significance of the obtained results was evaluated using variance analysis (the Fisher-Snedecor F test), while the values of  $LSD_{0.05}$  were calculated using the Tykey test.

## Results and discussion

The results related to the effect of triphenyl-*n*-pentylphosphonium iodide on the emergence and growth of spring barley and common radish at their early development stages are presented in Tables 1 and 2 and in Fig. 1.

The results obtained from the preliminary tests allow one to claim that triphenyl-*n*-pentylphosphonium iodide is a chemical substance exhibiting high toxicity towards higher land plants. Only the lowest of the substance concentrations applied in the experiment under discussion, namely 1 mg and 10 mg/kg dry soil mass, respectively, did not have any significant effect on the emergence and growth of the both experiment-

Table 1

Changes in basic parameters of the phytotoxicity test for spring barley following the introduction of triphenyl-*n*-pentylphosphonium iodide compound (in mg/kg of soil dry mass) to the soil

Sample	a	b	c	d	e	f	g	h	i	
Preliminary test										
0	20	19	100	2.699	100	0.139	100	0.1107	100	
1	20	20	105	2.862	106	0.143	102	0.1118	101	
10	20	20	105	2.572	95	0.131	94	0.1159	105	
100	20	20	105	1.242	46	0.063	45	0.1350	122	
1000	20	17	89	0.230	9	0.014	10	0.2452	222	
Final test										
20	20	20	105	1.848	68	0.094	67	0.1292	117	
40	20	20	105	1.818	67	0.091	65	0.1314	119	
80	20	19	100	1.430	53	0.074	53	0.1469	133	
	LSD <sub>0.05</sub> – 1			LSD <sub>0.05</sub> – 0.147		LSD <sub>0.05</sub> – 0.006		LSD <sub>0.05</sub> – 0.0073		

a – amount of seeds planted; b – number of plants; c – % germinations relative to the controls; d – crop fresh weight [g/pot]; e – % of crop relative to the control; f – mean weight of single plant [g]; g – % of single plant weight relative to the control; h – dry weight [mg/g f.m.]; i – % of dry weight relative to the control.

Table 2

Changes in basic parameters of the phytotoxicity test for common radish following the introduction of triphenyl-*n*-pentylphosphonium iodide compound (in mg/kg of soil dry mass) to the soil

Sample	a	b	c	d	e	f	g	h	i	
Preliminary test										
0	20	20	100	3.763	100	0.188	100	0.1269	100	
1	20	20	100	3.863	103	0.193	103	0.1240	98	
10	20	20	100	3.769	100	0.189	100	0.1252	99	
100	20	19	95	1.303	35	0.069	36	0.1555	123	
1000	20	17	85	—	—	—	—	—	—	
Final test										
20	20	19	95	2.953	78	0.159	94	0.1133	89	
40	20	18	90	2.451	65	0.139	74	0.1110	97	
80	20	20	100	1.780	47	0.091	48	0.1301	103	
	LSD <sub>0.05</sub> – 1			LSD <sub>0.05</sub> – 0.259		LSD <sub>0.05</sub> – 0.015		LSD <sub>0.05</sub> – 0.0074		

a – amount of seeds planted; b – number of plants; c – % germinations relative to the controls; d – crop fresh weight [g/pot]; e – % of crop relative to the control; f – mean weight of single plant [g]; g – % of single plant weight relative to the control; h – dry weight [mg/g f.m.]; i – % of dry weight relative to the control.

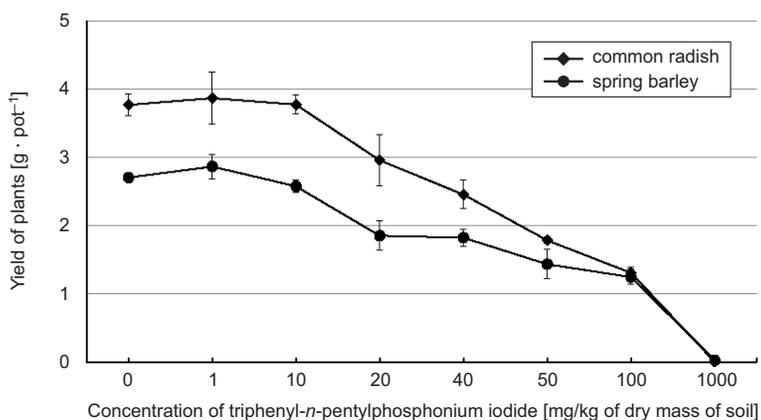


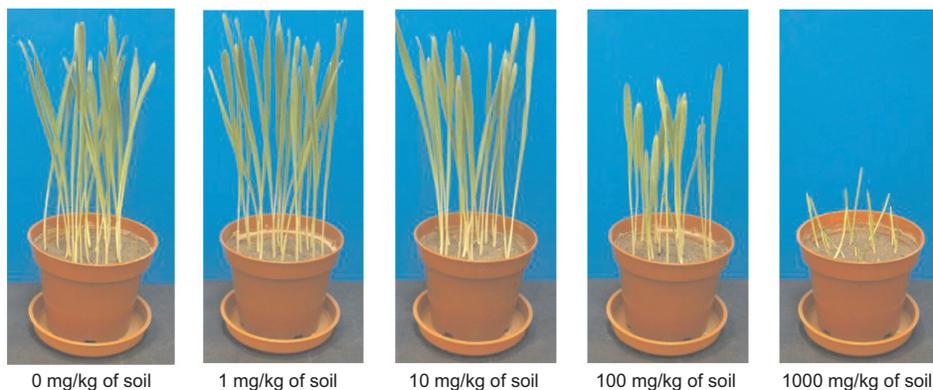
Fig. 1. Effect of triphenyl-*n*-pentylphosphonium iodide on the yield of fresh weight of plants

al plants. This conclusion is based on the fact that, as recognized by standard PN-EN 13432 [31], a substance does not exhibit toxicity if the germination rate of seeds and the overall fresh mass of plants growing on the ground with that substance do not differ by more than 10 % from those of the control sample. In the case of the visual examination of plants, too, no visible differences in appearance were observed between the test plants and the control plants; at the same time, no growth inhibition and alterations due to chlorosis and necrosis were found either (Figs. 2 and 3). Yet the toxicity of triphenyl-*n*-pentylphosphonium iodide became clearly apparent upon introducing 100 mg of this compound to the soil. The observed decrease of green sprout mass was in that case approx. 54 % for the barley and 65 % for the radish compared with the control. Alterations in the appearance of the plants were also observed; the barley seedlings were smaller than the control plants and showed distinct chlorotic changes; in the case of the radish, dwarfism was more conspicuous, and dark spots appeared on the leaves, indicating a progressing necrosis. After applying the highest concentration, *ie* 1000 mg/kg soil, the decrease in the fresh mass of barley seedlings exceeded 90 %, while the radish seeds were only able to germinate (Figs. 2 and 3).

The final tests showed that increasing the ionic liquid concentration in the soil up to 20 mg, 40 mg and 80 mg of the substance per one kg of dry mass led to a consistent decrease in the crop of fresh plant mass. For the above concentrations, the barley yield decrease was 32 %, 33 % and 47 %, respectively; and for the radish, a reduction in fresh plant mass by 22 %, 35 % and 53 %, respectively, was found compared with the control. Similar changes were observed for the fresh mass yield per one plant, and a steady increase in dry mass content was found for the spring barley (Tables 1 and 2). Visible chlorotic and necrotic changes on the barley seedlings and the radish plants were observed at a concentration of 80 mg/kg dry soil mass (Figs. 2 and 3).

Its adverse effect on the germination capacity of radish seeds and barley grains triphenyl-*n*-pentylphosphonium iodide only showed at the highest concentration (1000 mg/kg soil).

## PRELIMINARY TEST



## FINAL TEST

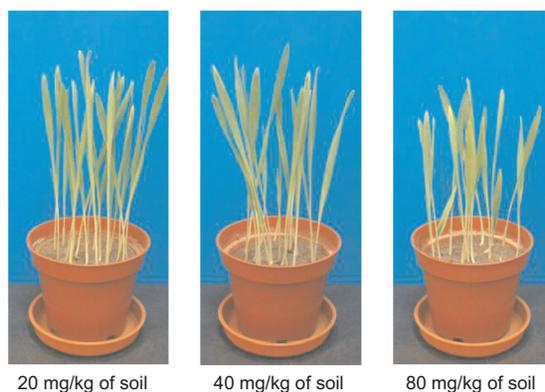


Fig. 2. Digital photographs of spring barley on the 14<sup>th</sup> day after introduction to the soil triphenyl-*n*-pentylphosphonium iodide (in mg/kg of soil dry weight)

The results obtained in the study under discussion are confirmed by available literature. A toxic effect of ionic liquids on the growth and development of higher land plants is reported by Balczewski et al [8], Biczak et al [18] and Matzke et al [23]. The phytotoxicity of ionic liquids observed in those studies was dependent chiefly on the applied concentration of the compound, but the factor determining the toxicity were the genetic features of the species and variety of plants used in the experiment. In addition, Matzke et al [19 and 24] and Studzinska and Buszewski [25] suggest that the toxicity of ionic liquids towards plants depends also on the structure of the compound and various environmental factors, including heavy metals and the organic and mineral colloid contents of the soil. Cybulski et al [5] have also found that the phytotoxicity of ionic liquids is dependent on the type of cation or anion, but also on the length of the alkyl chain. The above quoted authors have also demonstrated that in the case of chiral ionic liquids, their adverse influence on plants depends also on the rotation of the substance.

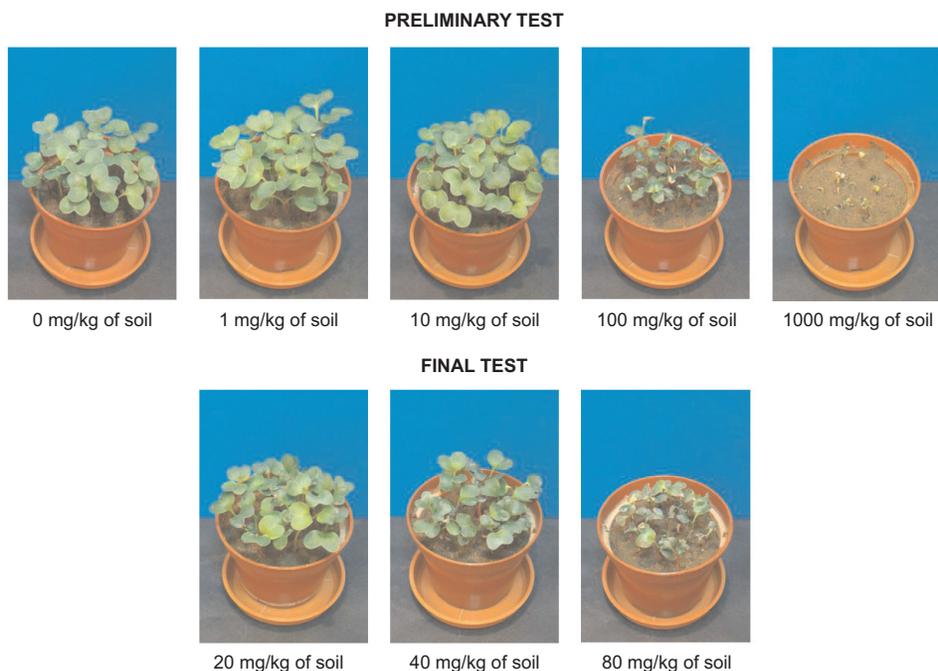


Fig. 3. Digital photographs of common radish on the 14<sup>th</sup> day after introduction to the soil triphenyl-*n*-pentylphosphonium iodide (in mg/kg of soil dry weight)

## Conclusions

The results obtained from the experiment discussed enable one to claim that triphenyl-*n*-pentylphosphonium iodide can be regarded as a chemical substance exhibiting quite high toxicity towards higher land plants. The observed toxic action of this ionic liquid was dependent chiefly on the concentration used. The highest concentration of the examined compound, which did not cause any distinct decrease in the emergence and growth of the plants (NOEC), was 10 mg/kg dry soil mass for spring barley seedlings and radish plants, while the lowest concentration causing a reduction of plant emergence/crops (LOEC) could be established at a level of 20 mg of the substance per 1 kg of dry soil mass for spring barley and common radish, respectively. Triphenyl-*n*-pentylphosphonium iodide only affected the germination capacity of the both plants' seeds to a small extent.

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## FITOTOKSYCZNOŚĆ CIECZY JONOWEJ ZAWIERAJĄCEJ ATOM FOSFORU

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**Abstrakt:** Ciecze jonowe mające niskie temperatury topnienia zostały zakwalifikowane do nowej generacji polarnych rozpuszczalników organicznych i katalizatorów. Związki te są nietlotne, niepalne, a ich „zielony” charakter jest związany z nieznacznym ciśnieniem par. Jednakże wprowadzenie do obrotu jakichkolwiek substancji chemicznych wymaga określenia ich wpływu na wszystkie elementy przyrody. Oznaczenie potencjalnej ekotoksyczności nowych związków chemicznych wiąże się z prowadzeniem badań dotyczących oddziaływania tych substancji na wzrost i rozwój wybranych organizmów.

W przedstawionej pracy wpływ jodku trifenylo-*n*-pentylfosfoniowego wprowadzonego do gleby w różnych stężeniach, na wschody i wczesne stadia wzrostu i rozwoju roślin wyższych określono w badaniach fitotoksyczności w oparciu o przewodnik OECD/OCDE 208/2006. W przeprowadzonym eksperymencie nasiona wybranych gatunków lądowych roślin wyższych – jęczmienia jarego (*Hordeum vulgare*) i rzodkiewki zwyczajnej (*Raphanus sativus* L. subvar. *radicula* Pers.) wysiano do wazonów zawierających glebę, do której dodano badany związek chemiczny i do wazonów zawierających glebę kontrolną.

Oceniając fitotoksyczność jodku trifenylo-*n*-pentylfosfoniowego, określono i porównano wschody i masę (suchą i zieloną) pędów roślin kontrolnych ze wschodami i masą (suchą i zieloną) pędów roślin rosnących na glebie, do której wprowadzono odpowiednie ilości związku. Dokonano ponadto oceny wizualnej wszystkich uszkodzeń badanych gatunków roślin, takich jak zahamowanie wzrostu, nekroza i chloroza oraz na podstawie otrzymanych wyników określono wielkości LOEC i NOEC.

**Słowa kluczowe:** ciecze jonowe, fitotoksyczność, lądowe rośliny wyższe, jęczmień, rzodkiewka, plon, sucha masa, chloroza, nekroza

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**TRACE ELEMENT CONTENT IN MEADOW SWARD  
AND SOIL ALONG ROAD NO. 957  
PASSING THROUGH ZAWOJA VILLAGE**

**ZAWARTOŚĆ PIERWIASTKÓW ŚLADOWYCH W RUNI ŁĄKOWEJ  
I GLEBIE WZDŁUŻ DROGI WOJEWÓDZKIEJ NR 957  
PRZEBIEGAJĄCEJ PRZEZ ZAWOJĘ**

**Abstract:** The aim of the research was to determine content of 5 heavy metals (Cu, Zn, Ni, Pb, Cd) in meadow sward and soil whose samples were collected from points along road No. 957 passing through Zawoja (Poland, Malopolska province). Samples were collected from 13 points, each one at a distance of 5 and 200 m from the road edge. In total, 26 samples of plant and soil material were collected. The content of metals was determined using ICP-AES method. The content of copper and zinc (trace elements that at the same time are bioelements indispensable for plants and animals) in the grass sward was at deficiency (Cu) or was within the range of optimal values (Zn). All the studied plant samples were characterized by higher than optimal lead content. Excessive cadmium content was found in four samples of the sward, which indicates necessity to exclude that biomass from fodder use. As a rule, the studied soils were characterized by a natural or increased content of heavy metals. Soil pollution with heavy metals occurred in particular spots. As a rule, no significant correlation between the content of studied elements in the sward and soil was proven (only the contents of nickel in the plants and soil were significantly positively correlated).

**Key words:** meadow sward, soil, trace elements, traffic pollution

Vehicle transport is an example of an anthropogenic source of pollution of environment. In 2010 road transport was a source of emission of 3.92 Mg Cu, 16.90 Mg Pb, 0.46 Mg Cd and 6.53 Mg Ni into the atmosphere (which constituted respectively 1.20 % of the total emission of Cu, 3.22 % of the total emission of Pb, 1.04 % of the total emission of Cd, and 3.97 % of the total emission of Ni) [1]. Discussed pollutants fall mainly on areas situated closest to roads, which may lead to a considerable

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accumulation of trace elements in soils and plants adjacent to traffic routes. Studying the content of trace elements in those plants and soil is important as it enables to direct management by introducing proper agricultural practices (*eg* liming) as well as by selecting suitable plants and purpose of cultivation.

The research aimed to assess the content of 5 heavy metals (Cu, Zn, Ni, Pb and Cd) in meadow sward and soil sampled from 13 points along traffic route 957 in the area of Zawoja (Poland, Malopolska province).

## Material and methods

The research material consisted of meadow sward and soil samples collected from 13 sampling points situated in the vicinity of the road No. 957 in Zawoja (Fig. 1). Zawoja is a village situated in the south of Poland (Malopolska province, Suski district). It is a popular tourist site located in a mountainous region – at the foot of the Babia Gora Mountain, in the area of two mountain ranges: Beskid Zywiecki and Beskid Makowski. Road No. 957 belongs to a category of regional roads. That road is the main traffic route in the vicinity of Zawoja. It runs through Skawica and Zawoja, and then it leads to the Babia Gora National Park (road No. 957 is the only road to that national park). Next, it crosses national road No. 7 (which is a part of European route E77) and regional road No. 958, and then it ends in Nowy Targ. In 2005, on average 4,827 vehicles per 24 hours passed the section of the road which leads to Zawoja, whereas in 2010 it was 5,845 vehicles [2, 3]. Between 2005 and 2010, the number of motorcycles passing

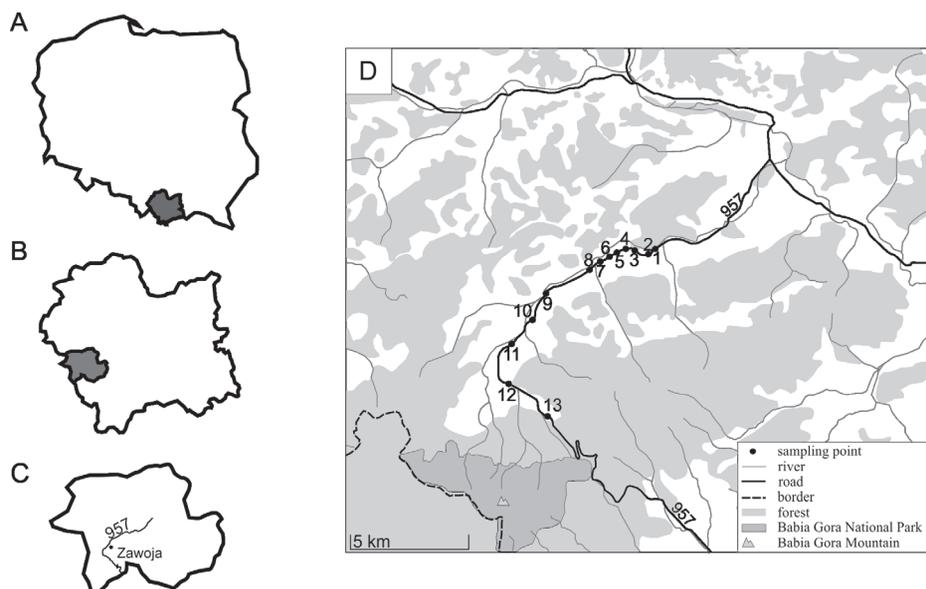


Fig. 1. Location of sampling points: A – Poland with marked Malopolska province; B – Malopolska province with marked Suski district; C – Suski district with marked Zawoja and road No. 957; D – sampling points along road No. 957

through increased by 261 %, the number of cars increased by 15 %, and the number of trucks increased by 91 %; only the number of buses and tractors decreased (by 39 % and 40 % respectively). In the following years, further growth of traffic volume on that section is forecasted – up to 6,842 vehicles a day in 2015, and 7,893 vehicles a day in 2020 (which will constitute 164 % of the number of vehicles passing through that road within 24 hrs in 2005) [4].

The samples were collected at the distance of 5 m and 200 m from edge of the road No. 957. The meadow sward samples were collected from permanent grasslands, and the soil samples were collected from those grass covered areas (0–10 cm layer).

The meadow sward was dried at 70 °C in a dryer with hot air flow. After that, to assess the content of heavy metals (Cu, Zn, Ni, Pb, Cd), the plant material was dry mineralized in a chamber furnace (450 °C, 5 hrs) and the remains were dissolved in nitric acid(V) solution 1:2 (HNO<sub>3</sub>:H<sub>2</sub>O, v/v) [5].

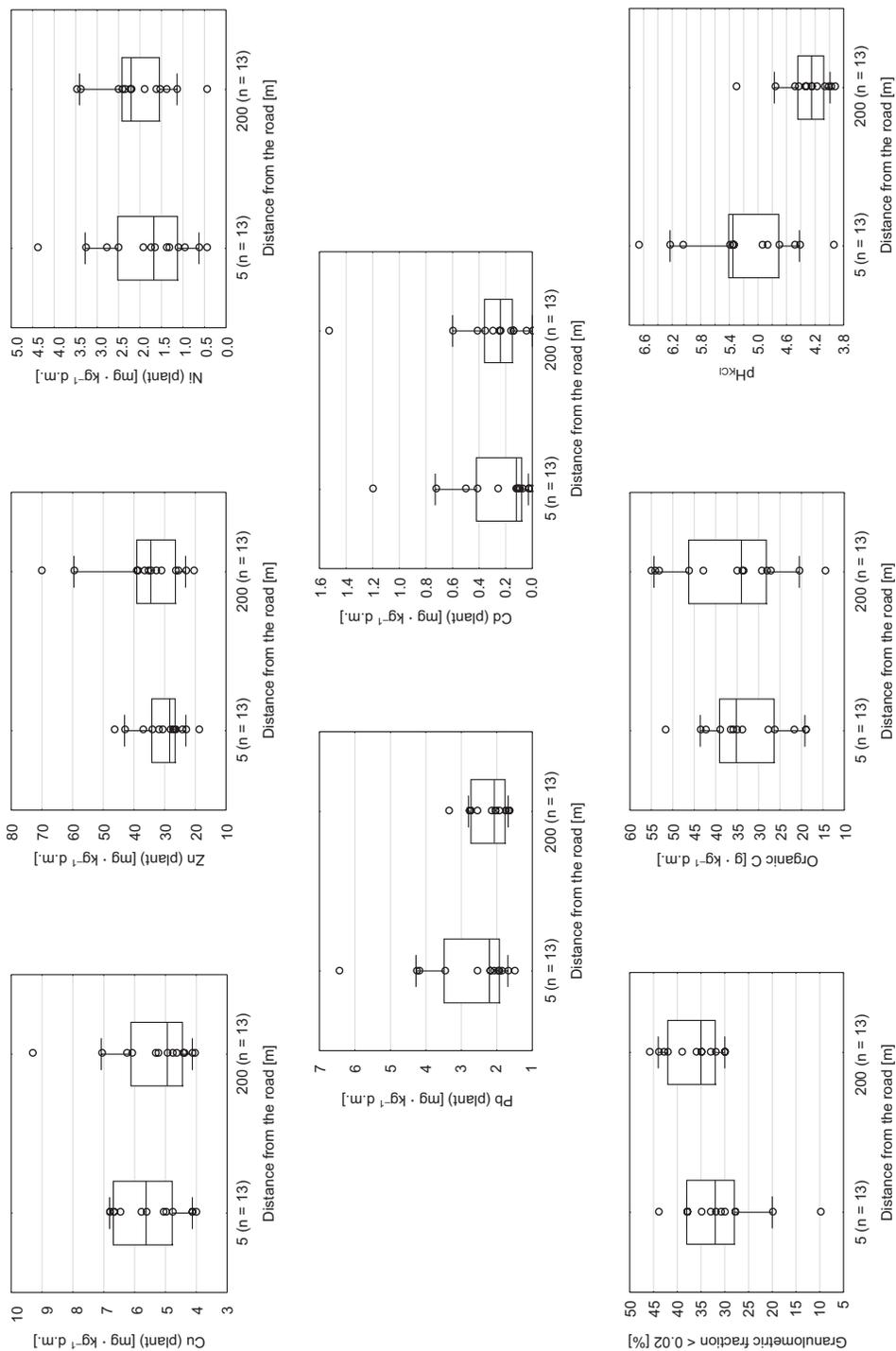
The air-dried soil samples were sifted through a sieve with 1 mm mesh. Granulometric composition was assessed using aerometric Bouyoucose-Casagrande method in Proszynski's modification, and organic carbon content was determined with Tiurin method. The soil pH was determined with potentiometer in 1 mol · dm<sup>-3</sup> KCl solution. Approximate to total contents of Cu, Zn, Ni, Pb and Cd were assessed in the soils after the samples dry mineralization (450 °C, 8 hrs), evaporating with a mixture of nitric(V) and chlorous(VII) acids and subsequent dissolving the remains in hydrochloric acid [5].

The content of 5 heavy metals in plant and soil samples was assessed using inductively coupled plasma atomic emission spectrometry (ICP-AES method) on JY 238 Ultrace apparatus. All assessments were conducted in two replications. The obtained results were elaborated statistically – minimum and maximum values were stated, and arithmetic mean and standard deviation were computed. Medium soil pH values were obtained after converting pH values of individual samples into hydrogen ions concentrations, determining mean values from obtained data and then their converting into pH values. All statistical analyzes were performed using the data analysis software system STATISTICA version 10 (StatSoft, Inc.).

## Results and discussion

Among the determined heavy metals there are elements recognized as micronutrients (Cu, Zn, Ni) which are indispensable for proper growth and development of plants and animals; level of those elements in a sward should meet nutrient requirements of those organisms. The remaining studied elements (Pb, Cd) are not necessary for proper growth and development of living organisms and, even in small amounts, have a toxic effect on them.

The mean copper content in the meadow sward (Table 1) was several times smaller than the maximum permissible content of that element in plants designated for fodder, provided by Kabata-Pendias et al [3]. According to Gorlach's assessment [7, based on different sources], such a low copper content in the studied sward was insufficient for animal feeding purposes. Only in nine sward samples the content of that element exceeded 6.0 mg Cu · kg<sup>-1</sup> d.m. (Fig. 2), which is the value considered as sufficient for



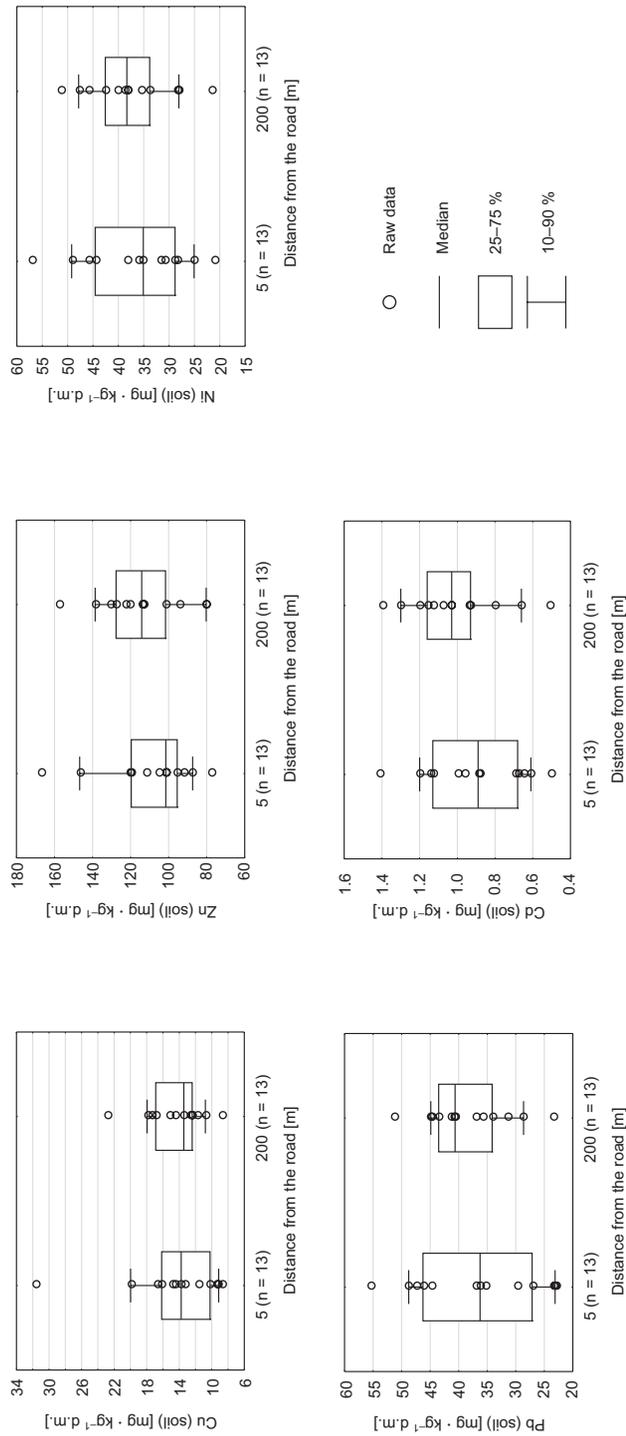


Fig. 2. Raw data obtained from conducted laboratory analyzes

satisfying nutrient requirements of animals. On the basis of the obtained results, no oriented relations between the distance from the road and the copper content in plants were found. However, Kolodziej et al [8] found a higher copper content in plants growing near traffic routes (particularly with high intensity of traffic).

Table 1

Selected statistical parameters of the contents of some trace elements in meadow sward from Zawoja region

Distance from the road [m]	Parameter	Cu	Zn	Ni	Pb	Cd
		[mg · kg <sup>-1</sup> d.m.]				
5 (n = 13)	Mean	5.54	30.72	1.87	2.80	0.29
	Minimum	4.02	18.75	0.47	1.52	0.01
	Maximum	6.81	46.33	4.40	6.47	1.20
	Standard deviation	1.09	7.88	1.12	1.43	0.35
200 (n = 13)	Mean	5.45	36.54	2.06	2.26	0.32
	Minimum	4.08	20.83	0.47	1.64	trace
	Maximum	9.34	70.08	3.49	3.38	1.53
	Standard deviation	1.47	14.02	0.85	0.54	0.40

As a rule, the zinc content in the sward stayed within the optimal range, and only in one sample it exceeded 60 mg Zn · kg<sup>-1</sup> d.m., which is the value given by Górlach [7, based on different sources] as the maximum optimal content in fodder. As the maximum permissible zinc content in fodder amounts to 100 mg · kg<sup>-1</sup> d.m. [6], plants from all sample points could be used for fodder. As a rule, a higher content of that element was found in the further (200 m) distance from the road. That view is not confirmed either by results of earlier researches conducted along the national road No. 4 in the Malopolska and Podkarpacie provinces [9, 10] or by results of researches by Kolodziej et al [8].

On the whole area of the research the nickel content in the plants was low, allowing for fodder use of the sward (according to assessments of Kabata-Pendias et al [6] and Górlach [7, based on different sources]). As a rule, the plant samples collected further from the road were characterized by a slightly higher nickel content. However, Kolodziej et al [8] did not find that relation between distance from traffic routes and nickel content in plants.

Assuming the lead content given by Górlach [7, based on different sources], amounting to 0.1–1.0 mg Pb · kg<sup>-1</sup> d.m., as safe in fodder, it was found that all samples of the analyzed sward contained more lead. According to guidelines established by Kabata-Pendias et al [6], fodder plants cannot contain more than 10 mg Pb · kg<sup>-1</sup> d.m. The studied plants contained no more than 6.5 mg Pb · kg<sup>-1</sup> d.m. In the case of that element, a tendency for a higher content in the samples collected closer to the road is noticeable, which is pointed out also by other authors [8, 10–12].

The cadmium content generally did not exceed 0.5 mg · kg<sup>-1</sup> d.m., which is the permissible cadmium content in fodder plants [6]. An excess of the given maximum

permissible value was found in four samples (two collected 5 m from the road edge from sampling points No. 9 and 12, and two collected 200 m from the road edge from sampling points No. 10 and 12), which excluded fodder use of that biomass. The observation of Jasiewicz and Buczek [11] as well as of Kolodziej et al [8] about a higher content of that element in samples collected closer to road was not confirmed in own research. The sward collected 200 m from the road was characterized by a higher content of that element. Higher cadmium content in further distances from road were found also by Filipek-Mazur et al [10].

To summarize, from among the analyzed heavy metals only lead was the element whose higher content was found in the plants growing closer to the road. Cadmium was the element whose content in the sward excluded fodder use of plants – an excessive content of that element was found in four samples (two collected 5 m from the road edge and two collected 200 m from the road edge).

Medium (53.8 %) and heavy (38.5 %) deposits were dominant among the studied soils (Table 2). The soils located in the distance of 200 m from the road were characterized by a slightly higher mean organic carbon content than soils located in the distance of 5 m. Based on  $\text{pH}_{\text{KCl}}$  values, most of the studied soils were classified as very acid (53.8 %) and acid (34.6 %). The soils located in the distance of 200 m from the road were characterized by lower pH values, which is in accordance with other authors' observations [12, 13].

Table 2

Selected statistical parameters of some properties of soils from Zawoja region

Distance from the road [m]	Parameter	Granulometric fraction $\phi < 0,02 \text{ mm}$ [%]	Organic C [g · kg <sup>-1</sup> d.m.]	$\text{pH}_{\text{KCl}}$
5 (n = 13)	Mean	31.15	33.3	4,73
	Minimum	10.00	19.0	3.94
	Maximum	44.00	51.9	6.67
	Standard deviation	8.74	10.0	—
200 (n = 13)	Mean	36.54	36.5	4.25
	Minimum	30.00	14.5	3.92
	Maximum	46.00	55.1	5.31
	Standard deviation	5.70	13.1	—

The studied soils were mainly characterized by a natural copper content (Table 3), an increased content of that element was found only in three samples (evaluation of the degree of soil pollution was conducted based on criteria established by Kabata-Pendias et al [14]). Similarly to Modlingerova et al [15], no distinct diversity of the copper content in soil depending on the soil distance from the road was found in the authors' own research. However, Własniewski [13], Chen et al [16], Klimowicz and Melke [17] as well as Pagotto et al [18] found a higher copper content in samples collected closer to traffic routes.

Table 3

Selected statistical parameters of the contents of some trace elements in soils from Zawoja region

Distance from the road [m]	Parameter	Cu	Zn	Ni	Pb	Cd
		[mg · kg <sup>-1</sup> d.m.]				
5 (n = 13)	Mean	14.61	109.8	36.28	36.72	0.90
	Minimum	8.66	77.4	20.93	23.00	0.50
	Maximum	31.63	166.7	56.95	55.39	1.41
	Standard deviation	6.11	24.4	10.37	11.00	0.27
200 (n = 13)	Mean	14.38	115.0	37.74	38.28	1.01
	Minimum	8.71	80.2	21.49	23.44	0.51
	Maximum	22.88	157.5	51.33	51.39	1.40
	Standard deviation	3.71	22.2	8.41	7.58	0.25

The studied soil samples, with an exception of two weakly polluted samples (the first one collected 5 m from the road edge from sampling point No. 3, and the second one collected 200 m from the road edge from sampling point No. 11), were characterized by an elevated zinc content (according to guidelines established by Kabata-Pendias et al [14]). A slightly lower cadmium content was found in the samples collected closer to the road. However, Modlingerova et al [15], Chen et al [16], Klimowicz and Melke [17], Pagotto et al [18] as well as Viard et al [19] found a higher zinc content in soils located closer to traffic routes.

According to criteria given by Kabata-Pendias et al [14], three soil samples were characterized by a natural nickel content, three were weakly polluted, the remaining ones were characterized by an increased nickel content. Two samples, from among the weakly polluted samples, came from the 5 m distance from the road edge (from sampling points No. 3 and 13), and one sample from the 200 m distance (from sampling point No. 8). No distinct relation between the metal content in the soil and the soil distance from the road was found in the authors' own research. Wlasniewski [13] and Modlingerova et al [15] found a lower nickel content in soils located further from road. However, Chen et al [16] did not show that relationship.

According to assessment of Kabata-Pendias et al [14], the studied soils were characterized by a natural (14 samples) or increased (12 samples) lead content. The lead content in the samples was not clearly diversified depending on the distance of soils from the road. However, bibliographical data confirm a higher lead content in samples collected closer to road [11, 13, 15–19].

One soil sample with natural cadmium content was found; the other samples were characterized by an increased content of that element (according to guidelines given by Kabata-Pendias et al [14]). Lower cadmium content was found in the samples collected closer to the road. Wlasniewski [13] did not show statistically significant differences in a cadmium content between soils located in different distances from road. Klimowicz and Melke [17] also did not show a relation between distance from road and a cadmium content. Jasiewicz and Buczek [11], Chen et al [16], Pagotto et al [18] as well as Viard

et al [19] found higher accumulation of cadmium in soils located closer to road, whereas Modlingerova et al [15] – in soil samples collected further from road.

To summarize, in the carried out research no distinct relation between the distance from the road and the content of heavy metals was found. It indicates an influence of other factors, including for example geological properties of soil, on the content of trace metals in soil. According to criteria given by Kabata-Pendias et al [14], in total two samples of soil collected 5 m from the road and two samples collected 200 m from the road were characterized by weak pollution with trace metals. Most of the studied soils were characterized by a natural or increased content of trace metals, therefore there is no reason to exclude them from agricultural production (the only limitation is about not cultivating plants for production of food with particularly low content of harmful elements on soils with an increased content of trace metals) [14]. A low content of heavy metals in the soils as well as properties of those soils were the cause of the low content of metals in the studied meadow sward. All the analyzed soil samples were characterized by lower contents of trace metals than the permissible metal contents in soils of agricultural lands which are given in the Regulation of Minister of Environment on the soils and earth quality standards [20]. Determined contents of individual elements in the soils were generally higher than mean contents in soils of the Malopolska province amounting to: 13.4 mg Cu, 79.2 mg Zn, 15.3 mg Ni, 29.1 mg Pb and 0.57 mg Cd · kg<sup>-1</sup> [21]. Comparison of the obtained results with given by Tokarz and Turzanski [22] mean contents for soils of the Malopolska province (amounting to 15.60 mg Cu, 75.22 mg Zn, 18.86 mg Ni, 41.05 mg Pb and 0.83 mg Cd · kg<sup>-1</sup>) and for soils of Zawoja (31.5 mg Cu, 150.3 mg Zn, 54.0 mg Ni, 48.6 mg Pb and 1.61 mg Cd · kg<sup>-1</sup>) is considerably less rigorous.

As a rule, no statistically significant correlation between the content of determined elements in the sward and their total content in the soil was found (only in the case of nickel a significantly positive correlation was found – Table 4).

Table 4

Coefficients of correlation between contents of trace elements and properties of soils from Zawoja region

Parameter	Cu (soil)	Zn (soil)	Ni (soil)	Pb (soil)	Cd (soil)
H <sup>+</sup>	-0.31	0.39*	0.02	0.40*	0.42*
Organic C	-0.17	0.59 **	-0.18	0.51**	0.57 **
Granulometric fraction $\phi < 0.02$ mm	0.04	0.01	0.49 *	0.30	0.29
Cu (plant)	0.10	-0.33	0.37	0.06	-0.05
Zn (plant)	-0.31	0.16	-0.15	0.13	0.10
Ni (plant)	0.04	-0.05	0.41*	-0.12	-0.12
Pb (plant)	-0.23	0.04	-0.34	-0.21	-0.05
Cd (plant)	-0.15	-0.06	0.12	-0.09	-0.13

\* Significant at  $p < 0.05$ ; \*\* significant at  $p < 0.01$ .

A statistically significant positive correlation was shown between the organic carbon content in the soils and the content of zinc, lead and cadmium in the soils as well as

between the concentration of hydrogen ions and the content of those three elements. The nickel content in the soils was statistically significantly positively correlated with the content of fraction with diameter lesser than 0.02 mm.

## Conclusions

1. The content of heavy metals which at the same time are bioelements indispensable for plants and animals was insufficient (Cu) or was within the range of optimal values (Zn). All the studied plant samples were characterized by higher than optimal lead content. Moreover, four of the examined sward samples were characterized by an excessive cadmium content, which indices the necessity to exclude that biomass from fodder use.

2. As a rule, the studied soils were characterized by a natural or increased content of trace elements. Enrichment of some of the samples in trace elements took place in particular spots.

3. From among the analyzed heavy metals only lead was the element whose higher content was found in the plants growing closer to the road. No distinct relation between the distance from the road and the content of heavy metals in the soils was found.

4. As a rule, no statistically significant correlation between the content of the determined elements in the sward and their total content in the soil was found (the exception was nickel, whose contents in the sward and soil were significantly positively correlated).

## Acknowledgements

The research results carried out within the subject No. 3101 were financed from the subsidy for science granted by the Polish Ministry of Science and Higher Education.

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## ZAWARTOŚĆ PIERWIASTKÓW ŚLADOWYCH W RUNI ŁĄKOWEJ I GLEBIE WZDŁUŻ DROGI KRAJOWEJ NR 957 PRZEBIEGAJĄCEJ PRZES ZAWOJĘ

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**Abstrakt:** Celem badań było określenie zawartości 5 metali ciężkich (Cu, Zn, Ni, Pb, Cd) w runi łąkowej i glebie pobranych punktowo wzdłuż drogi nr 957 przebiegającej przez Zawoję (województwo małopolskie). Próbkę pobrano w 13 punktach, w każdym w odległości 5 i 200 m od skraju jezdni. Łącznie pobrano po 26 próbek materiału roślinnego i glebowego. Zawartość metali oznaczono metodą ICP-AES. W runi trawiastej zawartość metali ciężkich będących równocześnie biopierwiastkami niezbędnymi dla roślin i zwierząt była niedoborowa (Cu) lub mieściła się w zakresie wartości optymalnych (Zn). Wszystkie badane próbki roślinne cechowały się większą od optymalnej zawartością ołowiu. W czterech próbkach runi stwierdzono nadmierną zawartość kadmu, co wskazuje na konieczność wyłączenia tej biomasy z użytkowania paszowego. Badane gleby z reguły cechowały się naturalną lub podwyższoną zawartością metali ciężkich. Zanieczyszczenie gleb metalami ciężkimi miało charakter punktowy. Z reguły nie udowodniono istotnej zależności między zawartością badanych pierwiastków w runi i w glebie (jedynie zawartość niklu w roślinach i glebie była istotnie dodatnio skorelowana).

**Słowa kluczowe:** ruń łąkowa, gleba, pierwiastki śladowe, zanieczyszczenia komunikacyjne



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## SPENT BLEACHING EARTH – OBTAINING AND DIRECTIONS OF UTILIZATION

### ZAOLEJONA ZIEMIA BIELĄCA – POWSTAWANIE I KIERUNKI ZAGOSPODAROWANIA

**Abstract:** Fat factories both in Poland and in the world produce a great of waste – spent bleaching earth (SBE). This waste is a source of pollution of natural environment. The utilization of SBE is difficult because of complex chemical compounds which change in time. The physicochemical methods used in utilization are non effective because of high energy and costs. The autochthonous lipolytic microorganisms used both in natural or modified environment may be the future alternative methods of fat biodegradation (biostimulation, bioaugmentation).

**Keywords:** bleaching earth (BE), spent bleaching earth (SBE), utilization, autochthonous microorganisms

Due to diverse composition, the utilization of industrial waste depends on its physical and chemical properties and therefore presents many difficulties. Over the years, attempts were made to dispose of this waste using various costly physical and chemical methods, but biological methods seem to be the most promising in the long run. They are economically viable and do not introduce additional chemical contaminants to the ecosystem.

The development of effective methods of waste management is contingent on the knowledge of interactions between microorganisms of a given ecosystem and the structure of chemical compounds, which are the main components of the waste, as well as possible decomposition products. The biotechnological processes of waste management use the adaptability of microorganisms, especially autochthonous microorganisms, adapted to large concentrations of major chemical contaminants. These compounds are a source of nourishment and energy for such microorganisms, and thus are used in catabolic processes, reducing at the same time the amount of contaminants.

Waste fat introduced into the environment in the form of spent bleaching earth (SBE) is one of the largest sources of energy and nutrients for microorganisms. In Poland, the oil and fat industry plants generate annually approximately 40,000 Mg of SBE,

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similarly as in Great Britain [1]. In Japan, the amount of this waste generated each year ranges from 50,000 to 80,000 Mg [2–4]. SBE is formed as the result of the oil refining (bleaching) process, where various types of bleaching earth (BE) are used as an adsorbent.

## Bleaching earth

The bleaching earth (BE), also known as the Fuller's earth, is formed from bentonites, which are in 80 % composed of hydrated aluminum silicate (montmorillonite or kaolinite), a natural clay mineral containing aluminum, silicon, oxygen and hydroxyl groups [5, 6].

The bleaching earth purifies and stabilizes vegetable oils, mineral oils and animal fats against oxidation. It also accelerates coagulation and then adsorbs on its surface the contaminants dissolved in fat [3, 7, 8].

Factors that account for the bleaching and clarification properties of BE include the following: the layer spatial structure, a large specific surface, adsorbate particle sizes of 20–50  $\mu\text{m}$ , a significant affinity to contaminants by forming chemical bonds with external chemical groups, such as OH and SH [2, 3, 9].

Kaolinite has the simplest structure. It is composed of one tetrahedral layer and one octahedral layer. Due to this structure, hydrogen bonds are formed between oxygen atoms present in the tetrahedral layer and OH groups present in the octahedral layer, thus making it possible for other groups and cations to enter between the layers. Thanks to the exchange of  $\text{H}^+$  ions from hydroxyl groups present on the surface of the mineral, kaolinite has low sorption and ion-exchange properties.

Montmorillonite is composed of three layers in a 2:1 ratio. The three-layer spatial structure manifests itself in overlapping of tetrahedral  $[\text{SiO}_4]$  and octahedral  $[\text{AlO}_6]$  forms – two tetrahedral layers of silica are separated by an octahedral layer of aluminum oxide. As the result, no hydrogen bond between the layers is formed and oxygen charges are not saturated, which increases the adsorption properties of the carrier. Water molecules and cations (K, Na, Ca, Fe, *etc.*) can penetrate the layers without being involved in an ion exchange reaction [10].

A large specific surface of BE, which is conducive to the adsorption of contaminants, is the property that accounts for its suitability for the fat refining process. This process may be physical, where the Van der Waals forces appear between the adsorbate and adsorbent (at the temperature below 60 °C), or chemical (chemisorption, activated adsorption) with the involvement of forces of a chemical bond formed at the place of contact between the adsorbate and adsorbent phases (at approx. 100 °C).

Two types of bleaching earth are used in the oil and fat industry [11]:

- natural earth (neutral, Fuller's earth), which is used in the process of refining oils containing trace amounts of chlorophyll and a small amount of phospholipids, soaps and metals,

- acid-activated earth (the earth activated by means of sulfuric or hydrochloric acid), which is used in the process of refining rapeseed oil containing a significant amount of chlorophyll.

The activation of BE involves grinding of the material, formation of an aqueous suspension and adding to this suspension concentrated hydrochloric acid in the amount of approximately 30 % of the weight of the material [8, 9] or sulfuric acid [2, 4]. The obtained mixture is then filtered on presses, washed with water, dried by exhaust gases and ground. As the result of this treatment, the structure and physical and chemical properties of bentonite change, leading to the increased adsorption capacity (the increased specific surface, micropore volume, fineness of the grains and oil absorbency properties). After chemical activation, the bleaching earth has the composition and properties depending on the type of mineral [2].

The highest activity of the bleaching earth is achieved by complete leaching of  $\text{Al}_2\text{O}_3$  from the octahedral layer by  $\text{Mg}(\text{II})$  and  $\text{Fe}(\text{II})$  ions with insignificant amounts of  $\text{Zn}(\text{II})$ ,  $\text{Ni}(\text{II})$ ,  $\text{Li}(\text{I})$  or  $\text{Cr}(\text{III})$ . As far as the tetrahedral layer is concerned, up to 15 % of the silicon is replaced by aluminum ions [9, 10].

In addition to the montmorillonite-based bleaching earth, the oil and fat industry uses other types of adsorbents (active at 90–110 °C, 10–90 min), such as Engelhard F-105, Jeltar-100 and Tompsin Optimum 214 FF [12].

In the fat bleaching process, it is usually sufficient to add 0.5–1 % of the bleaching earth. Larger amounts cause greater losses of oil, which is also adsorbed. Oil is usually mixed with the adsorbent at 60–100 °C, using vacuum and protecting it against oxidation. At higher temperatures (above 120 °C), the content of free fatty acids increases and the structure of polyunsaturated fatty acids changes, leading to the formation of conjugated polyenes that are more prone to oxidation and polymerization [11].

The efficiency of the bleaching process can be improved by using two different adsorbents:

- Trisyl synthetic silica, used before the adsorbate contacts BE, adsorbs phospholipids and metals, increasing the absorption of dyes by BE,
- activated carbon, used before using BE, adsorbs polycyclic aromatic hydrocarbons, trace amounts of soaps, phospholipids and proteins.

The objective of the bleaching earth used in the processing of oil is to remove the following: [3, 7, 8] gossypol, anthocyan, chlorophyll (which causes rancidity of oil), mucus, ingredients having peroxidative properties, sulfur and phosphorus compounds, undesirable components formed during deacidification, substances responsible for bad taste, residues of soaps, phospholipids, macromolecular alcohols (sterols, tocopherols), polycyclic aromatic hydrocarbons (anthracene, benzopyrene, phenanthrene), pesticides and heavy metals (Fe, Cu, Cd, Hg, Pb), and to keep the carotenoids (natural antioxidants).

The disadvantage of this type of adsorbent is the surface acidity, which contributes to acidification of oils, increasing the amount of free fatty acids formed as the result of hydrolysis of triacylglycerols (TAG), adsorption of sodium ions from soaps and isomerization of alkyl chains of TAG. Attempts were made to eliminate this disadvantage by developing a mixture of Filtrrom Grade 105 activated bleaching earth and the Y zeolite substituted with magnesium ions, adsorbing free fatty acids, and by conducting the oil bleaching process at temperatures not exceeding 105 °C [11].

## Spent bleaching earth

The *spent bleaching earth* (SBE) is non-standard waste that contains, apart from an inorganic substance – the adsorbent (the bleaching earth), also significant amounts of organic compounds. Depending on the refining technology and the type of processed oil, SBE contains 25–40 % of oil and water-insoluble substances, vegetable dyes, macro- and micronutrients and traces of toxic metals [2, 8, 13–17]. In Poland, the fat substance content in SBE ranges from 8–28 %, in Canada 28–34 %, in Iraq 41 % and in Japan 40 % [4, 18].

SBE is waste with a natural acidic pH of 3.8–4.2 and a high content of oleic acid, which determines the microbiological composition and the direction of fat transformations. Due to diverse chemical composition, lack of permeability to water and air and strong adsorption properties, it is difficult to manage. The system of interactions: the diatomaceous earth – adsorbed fat – metals – microorganisms capable of growing in the presence of fat or using fat for their vital functions as the only source of carbon, constitutes another problem.

## Utilization of the spent bleaching earth

The spent bleaching earth management includes its direct utilization or oil recovery, adsorbent recovery and recycling.

So far, no cost-effective method, making it possible to reuse SBE in the fat refining process, has been developed. After being used once, its activity strongly decreases and the process of removing oil and contaminants requires the use of large amounts of solvents.

Also treating SBE as a source of oil in combination with fresh oleaginous seeds did not bring satisfactory results, since it resulted in an increase in the content of mineral substances in the extracted meal and bad oil aftertaste. Therefore, the attempts to recover the fat-containing adsorbent to be reused in the oil refining process were abandoned. This has led to intensified research into alternative ways of SBE utilization, especially since storing SBE outdoors leads to caking and hardening, poses a self-ignition risk [19] and involves penetration of contaminants into groundwater and even water-bearing layers [20].

Most SBE utilization methods consisted in using physical and chemical processes (displacing of oils by water or saponification of fats and soap separation and the recovery of the carrier itself).

Fat was recovered as the result of extraction by means of organic solvents or CO<sub>2</sub> at the pressure of 300–700 atm at 20–80 °C [7, 8, 21, 22], displacement of oil by water, heat treatment, saponification of fat, soap separation and annealing in a rotary kiln in a nitrogen atmosphere [8, 23]. The result of these processes was the oxidation of unsaturated fatty acids present in SBE and their isomerization, leading to the formation of technical fats. The recovered oil was used for the production of surfactants, biodegradable polyesters, lubricants, antirust products or biofuels.

The adsorbent was recovered by heating at 300–400 °C and activation by HCl [5, 6, 8, 24]. The recovered adsorbent was used to remove organic compounds, particularly petroleum products, from water and wastewater and to remove dyes and heavy metals from effluents [24–29] as a binder for soil stabilization in the road building industry [30] and for soil conditioning and fertilization [31].

The above-mentioned methods required large amounts of solvents and high costs, which made them unprofitable.

Also direct utilization of SBE as a small additive to compound feedingstuffs [18, 19, 32], coal briquettes (boiler fuel) [5, 6, 33] and in the production of ceramic building materials (cement clinker) [34] failed to solve the problem because of high costs and low level of utilization of this waste.

In recent years, several reports have been published regarding the biological recycling of SBE involving microorganisms. However, the processes of decomposition of contaminants with the participation of autochthonous microorganisms are very slow or are completely inhibited due to high saturation of SBE with water-insoluble substances. To make the biodegradation process effective, it is necessary to improve physical properties of the waste, usually by the addition of sawdust, straw, peat, manure, brown coal [13, 31], and to increase pH by using CaCO<sub>3</sub> and by adding bio-activators [31] or microbial vaccines [16, 35] or by using earthworms [36]. The obtained mixture is loose, is not prone to caking and is more susceptible to biodegradation.

The optimum use of this waste in the biodegradation process is dependent on its chemical composition, which varies over time [35].

Biological decomposition of organic contaminants by microorganisms is one of the most important and most effective ways to remove them from the environment. Biodegradation of these compounds in the natural environment (*in situ*) is usually a multi-step process that occurs with the participation of synergistically acting autochthonous microorganisms. Determinants of this process include the following: the composition and activity of microorganisms, the presence of antagonistic microorganisms, the properties and the “age” of contaminants, the presence of other compounds, temperature, oxygen supply, the content of nutrients and physical and chemical properties of the environment.

Biological treatment occurs in the presence of aerobic heterotrophic microorganisms, which use the contaminants as a source of energy and cell synthesis leading to the increase in the biomass and reduction of fat content in the waste.

Fat adsorbed on SBE is converted in the process of bioconversion by lipolytic microorganisms, including the following: *Candida utilis*, *Candida rugosa*, *Candida curvata*, *Geotrichum candidum*, *Aspergillus niger*, *Penicillium roqueforti*, *Penicillium citrinum*, *Penicillium sp.*, *Galactomyces geotrichum*, *Mucor sp.*, *Apiotrichum curvatum*, *Bacillus subtilis* and *Streptomyces sp.* [16, 35, 37, 38]. They have the ability to biosynthesize many exoenzymes affecting the water-insoluble substrates [39–42].

The bioconversion of fat from SBE occurs with the participation of lipases produced by microorganisms and activated on the fat-water interphase surface [43, 44]. Lipases can occur in two main groups: carboxylesterases (EC3.1.1.1), which hydrolyze

short-chain substrates that are partially soluble in water, and true lipases (EC3.1.1.3), which show the activity in relation to long-chain, poorly water-soluble substrates, resulting in the formation of emulsions or colloids. Microorganisms can produce various groups of enzymes, *eg* in the case of bacteria, 47 types of lipases have been identified [45, 46]. Lipases have a wide range of properties, depending on a source of nourishment, taking into account the positional specificity, fatty acid specificity, the optimum pH value and thermostability [43].

The bioconversion of fatty substances can be carried out for the following:

a) processing of SBE into the form of fertilizer assimilable by plants [19, 30] and the production of biocompost [13, 31], which, however, due to the presence of hydrocarbons, should not be widely used [47] and should be used only as a top cover of landfill sites for planting;

b) production of biogas in the fermentation processes;

c) composting in combination with vegetable waste or straw and sewage sludge [48];

d) production of specific compounds, such as:

– fatty acid methyl esters (FAMES) with the participation of *Candida cylindracea* [2, 3, 49, 50], used for the synthesis of hydroxy acids, surfactants, biodegradable polyesters, lubricants, antirust products and biofuels as alternative energy sources [3, 17, 22, 51, 52];

– riboflavin (by fermentation) with the participation of the *Ashybia gossypii* strain [2, 3] and *Bacillus subtilis* [53], which accounts for approximately 80 % of the annual production and results in complete recovery of the adsorbent with the possibility of being reused in the vegetable oil refining process. A high content of riboflavin was achieved, while the content of palm oil in SBE was reduced five times, accompanied by the bleaching effect [54]. Similar experiments were performed using waste from the production of rapeseed oil and equally good results were obtained [4, 54, 55];

– oxalic acid in the acid environment by a *Aspergillus niger* mutant [56–58];

– single-cell proteins (SCP) [37, 38, 59–61];

– secondary metabolites, mainly in the citrate fermentation process [62].

The enzymatic fat hydrolysis methods used in these processes, as compared to their chemical hydrolysis, allow for the optimum utilization of fat, make it possible to obtain products with a specific configuration, do not cause the formation of by-products (ecologically clean processes), are applied in energy-saving conditions (hydrolysis temperature: 30–40 °C) and reduce capital expenditure [63].

The majority of research on bio-utilization of SBE was conducted in the neutral or slightly alkaline environment in the presence of vaccines of lipolytic microorganisms whose activity in this environment was the highest. The hydrolysis of fat of various compositions is possible thanks to low substrate specificity lipases [64], active within a broad range of pH values and temperatures (ranging from 4–6 °C to 75 °C) [65–67]. They demonstrate the highest activity with the pH value of 8–9 and at 30–40 °C [68]. For most bacteria, the optimum pH value of the medium is neutral or alkaline and ranges from 7.0 to 9.0. Since SBE is characterized by a natural acidic reaction, during research this waste was neutralized by chemical substances, which increased the cost of the process and resulted in additional contamination of the environment. Currently,

more and more attention is paid to microbial lipases, whose activity is the highest in the acidic environment [67, 69–71], therefore, the bioconversion of fat in these conditions should take place without any obstacles. For the majority of *Pseudomonas* bacteria, the pH of the medium plays a minor role, since they produce lipase in a wide range of pH values. For example, *Pseudomonas fluorescens* produces extracellular lipase in the pH range from 4.0 to 10.0 [73], in contrast to lipase of *Pseudomonas nitroreducans* active in strongly alkaline environments [74]. Lipases of *Kurtzmanomyces* I-11 and *Kluyveromyces lactis* are active in the pH range from 1.9 to 7.2 [67, 72]. Given the unique ability of microorganisms to adapt to conditions in the environmental and to use fat as a source of energy and nourishment, the extent of bioconversion will depend on biological, physical and chemical properties of the waste in which the process occurs. An attempt to transform waste fat in SBE in its natural acidic environment using autochthonous lipolytic strains and stimulators that speed up decomposition of fat opens new prospects for SBE utilization [16].

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**ZAOLEJONA ZIEMIA BIELĄCA  
– POWSTAWANIE I KIERUNKI ZAGOSPODAROWANIA**

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**Abstrakt:** Zakłady tłuszczowe zarówno w Polsce, jak i na świecie produkują znaczne ilości odpadów pod nazwą zaolejonej ziemi bielącej (ZZB). Odpad ten jest źródłem skażenia środowiska naturalnego. Jest on trudny do utylizacji ze względu na złożony skład chemiczny zmieniający się w czasie. Stosowane fizykochemiczne metody zagospodarowania nie przynoszą spodziewanych efektów i są energo- i kosztochłonne. Perspektywną alternatywą są metody biologiczne z wykorzystaniem w warunkach naturalnych (*in situ*) autochtonicznych mikroorganizmów o aktywności lipolitycznej w procesie samorzutnej biodegradacji i w procesach sterowanych (biostymulacja, bioaugmentacja).

**Słowa kluczowe:** ziemia bieląca (ZB), zaolejona ziemia bieląca (ZZB), utylizacja, mikroorganizmy autochtoniczne



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## LIPOLYTIC ACTIVITY OF *Bacillus* sp. ISOLATED FROM THE NATURAL ENVIRONMENT

### AKTYWNOŚĆ LIPOLITYCZNA *Bacillus* sp. WYIZOLOWANYCH Z ŚRODOWISKA NATURALNEGO

**Abstract:** In the study 10 bacterial strains of *Bacillus* kind were screened (*B. cereus*, *B. firmus*, *B. mycoides*, *B. pumilus* i *B. subtilis*) for their ability to synthesize lipases on the media containing the following fatty substrates as carbon sources: tributyrin and Tween 40, 60, 80. The growth media were incubated for 15 days and the results were recorded after 2, 4, 8 and 15 days. The results were presented as the amount of liberated  $\mu\text{moles}$  of fatty acids [ $\mu\text{mol}$ ] and the units of lipolytic activity  $\text{U}/\text{cm}^3$ . Tested bacterial strains displayed different levels of activity of extracellular lipases depending on the type of the fatty substrate source and the time of culturing. For most of the tested strains the maximum production of lipases took place on the 15<sup>th</sup> day of culturing. The most effective growth medium in the process of enzymes biosynthesis was the medium with tributyrin, and both *B. cereus* A96 and G10 strains distinguished from the others by the highest activity liberating on average 97.5 and 69.375  $\mu\text{moles}$  of fatty acids. The value of lipolytic activity was increasing during the experiment from the value of 0.958 to 1.375  $\text{U}/\text{cm}^3$  for A96 strain and from 0.625 to 3.125  $\text{U}/\text{cm}^3$  for G10 strain. Taking into account all tested growth media, the lowest lipolytic activity was displayed by *B. pumilus* Tw3 strain.

**Keywords:** *Bacillus* sp., lipases, tributyrin, Tween

Lipases, defined as hydrolases of glycerol esters EC 3.1.1.3, are the enzymes of high catalytical potential. They catalyse the hydrolysis and trans-esterification of triacylglycerols, enantioselective synthesis, and hydrolysis of a variety of esters. They are produced by plants, animals and microorganisms, of which the last group remains in the centre of attention. Many bacteria have the ability to produce them but the most important in the process are the species: *Pseudomonas*, *Staphylococcus* and *Bacillus* [1–3].

Bacterial lipases are the extracellular water-soluble enzymes, produced in the late phase of logarithmic growth. All known bacterial lipases belong to the group of  $\alpha/\beta$  – hydrolases of 3-dimentional structure. Lipases are characterized by their unique ability to catalyse reactions at the interface of a lipid phase and the aqueous phase [1, 4].

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A wide interest in bacterial lipases is linked to their role as biocatalysts in many biochemical processes. They are used, among others to produce detergents, food, paper, pharmaceuticals and in the environmental protection. However, according to data from literature [1, 2] the microorganisms are varied in terms of their enzymatic activity, which depends on the species of the microbes and the culturing conditions (eg pH of the growth medium, temperature, source of nitrogen and presence of lipids in the medium). Therefore, bearing in mind immense application abilities of microbiological lipases, there has been research done in order to find new strains, able to synthesize significant amounts of highly active enzymes.

The aim of presented research was the evaluation of the ability to synthesize extracellular lipases by selected *Bacillus* sp. strains isolated from the natural environment, depending on the source of fatty substrate and incubation time.

## Materials and methods

The objects of the study were the following 10 bacteria strains:

- 5 *Bacillus pumilus* strains marked as: *M1*, *Tw3*, *A115*, *G4*, *G8*,
- *Bacillus subtilis* *G2*,
- 2 *Bacillus cereus* strains marked as: *A96* and *G10*,
- *Bacillus mycoides* *G3*,
- *Bacillus firmus* *Tw4*.

The source of fatty substrates in the growth media were 10 % solutions of the following: tributyrin, Tween 40, Tween 60 and Tween 80. The cultures were maintained in Erlenmeyer flasks of 250 cm<sup>3</sup> capacity containing 50 cm<sup>3</sup> of the respective growth medium and placed on a rotary shaker for 15 days at 30 °C. The cultures were introduced with an inoculum of density equal to E = 2 (standardized with the use of a spectrophotometer) obtained from the 48-hour culture on a nutrient broth.

Samples for the analysis were collected after 2, 4, 8 and 15 days of culturing and centrifuged for 20 minutes at 4000 rpm. In the obtained supernatant the extracellular lipolytic activity was marked by means of titration towards the same substrates previously added to the growth medium (the proper treatment). In the control treatment the supernatant was replaced with water. The amount of liberated fatty acids was estimated by titration with 0.05 M NaOH solution against 2 % phenolphthalein as an indicator, and calculated as a subtraction between the proper treatment and the control treatment results. The results were presented as the amount of liberated μmoles of fatty acids [μmol] and in the units of lipolytic activity. The unit was expressed as the amount of μmoles of 0,05 M NaOH required to neutralize fatty acids liberated by the lipases contained in 1 cm<sup>3</sup> of post-culture liquid within 1 minute. The lipolytic activity was expressed in the unit U/cm<sup>3</sup>.

## Results

In the presented paper 10 bacterial strains of *Bacillus* kind were screened, within 15 days, for their ability to synthesize lipolytic enzymes on the growth media containing

different sources of fatty substrates. The obtained results proved the variety among tested *Bacillus* strains in terms of extracellular lipases production according to the source of fatty substrate in the growth medium and incubation time.

In the presented research the most effective source of fatty substrate in the process of extracellular lipases biosynthesis was tributyrin (Fig. 1). After 15 days of culturing the highest amount of fatty acids was liberated by *B. cereus* G10 strain, on average 97.5  $\mu\text{mol}$ . The value of lipolytic activity for this strain was increasing from 0.625  $\text{U}/\text{cm}^3$  on the second day to the value of 3.125  $\text{U}/\text{cm}^3$  on the 15<sup>th</sup> day of culturing. Similar activity was displayed by the other strain under study *B. cereus* A96, however its lipolytic activity was considerably lower and fluctuated during the test from 0.958 to 1.375  $\text{U}/\text{cm}^3$  (Fig. 2). But for the strain *B. mycoides* G3, which belongs to the same group, the correlation was different. The lipolytic activity was decreasing from 1.0 to 0.167  $\text{U}/\text{cm}^3$ . The lowest amount of fatty acids was liberated by *B. pumilus* Tw3, on average 13.125  $\mu\text{mol}$  (Fig. 1). Tributyrin was not a favourable environment for the extracellular lipases production, as with time the lipolytic activity was decreasing from the value of 0.333 to 0.208  $\text{U}/\text{cm}^3$  (Fig. 2). The remaining strains of *B. pumilus* were

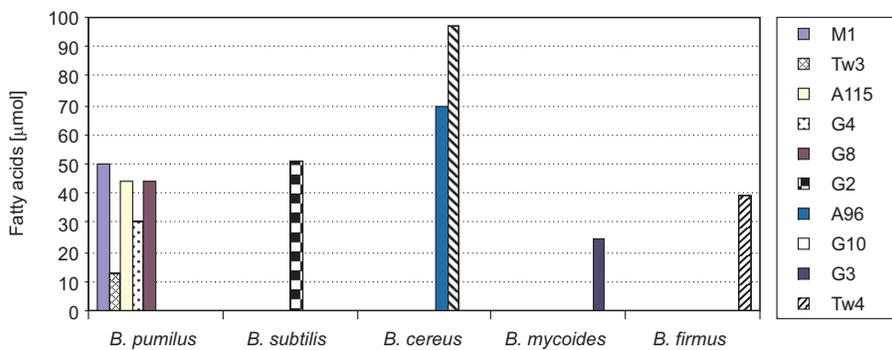


Fig. 1. The influence of tributyrin on extracellular lipases production by selected *Bacillus* strain

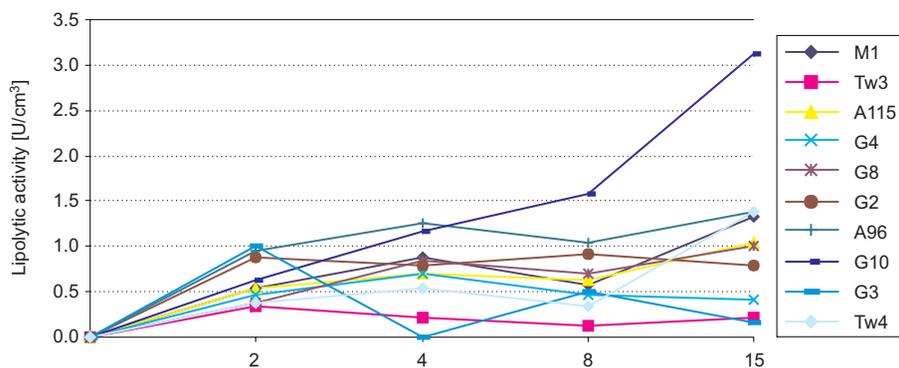


Fig. 2. The influence of the culturing time on extracellular lipases production in the presence of tributyrin on selected *Bacillus* strains

liberating much more of fatty acids, reaching the following values: on average 30.625  $\mu\text{moles}$  for the *G4* strain, 43.75  $\mu\text{moles}$  for the *A115* and *G8* strains and 50  $\mu\text{moles}$  for the *M1* strain (Fig. 1). The lipolytic activity was increasing of 0.5–0.7 unit for most of the mentioned strains during the experiment. Only in case of *G4* strain the activity was decreasing slightly from 0.458 to 0.416  $\text{U}/\text{cm}^3$  (Fig. 2).

*B. subtilis* strain *G2* belonging to the same group as *B. pumilus*, liberated on average 50.625  $\mu\text{moles}$  of fatty acids and its lipolytic activity was decreasing from 0.875 to 0.792  $\text{U}/\text{cm}^3$  (Fig. 1, 2).

The last strain under study, *B. firmus* *Tw4*, produced on average 39.375  $\mu\text{moles}$  of fatty acids and its activity increased of 1 unit within 15 days, from the initial value of 0.375 to 1.375  $\text{U}/\text{cm}^3$  (Fig. 1, 2).

Generally, the highest values of extracellular lipases activity in the presence of tributyrin were recorded on the 15<sup>th</sup> day of the experiment. The only exception were the strains: *Tw3* and *G3*, for which the maximum values were obtained on the 2<sup>nd</sup> day, and the strains *G4* and *G2* with the highest values on the 4<sup>th</sup> and 8<sup>th</sup> day respectively (Fig. 2).

In the presented study the lipolytic activity was also assessed towards long-chain synthetic lipids: Tween 40, 60, 80. The most favourable growth medium for the extracellular lipases production was the medium with the addition of Tween 40. The highest amount of fatty acids on this medium was produced by *B. subtilis* *G2* – on average 34.375  $\mu\text{moles}$  (Fig. 3). Also high mean values of  $\mu\text{moles}$  of fatty acids were obtained with the strains of *B. pumilus*: *G4* and *G8*, and recorded values were 30 and 31.25  $\mu\text{moles}$  respectively. The lowest amount of fatty acids was liberated by the strains *B. firmus* *Tw4* (on average 16.25  $\mu\text{moles}$ ) and *B. pumilus* *Tw3*, on average 14.37  $\mu\text{moles}$  (Fig. 3).

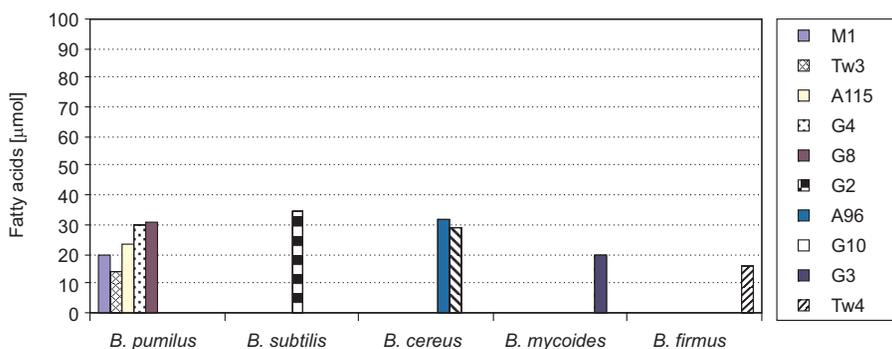


Fig. 3. The influence of Tween 40 on extracellular lipases production by selected *Bacillus* strains

It is difficult however to find the correlation between the time of culturing and lipolytic activity of tested strains on Tween 40 (Fig. 4). In case of the following strains: *B. cereus* *A96*, *B. subtilis* *G2* and *B. mycooides* *G3* the maximum values were obtained on the 2<sup>nd</sup> day and was decreasing on the following days. The highest lipolytic activity was noted for *B. cereus* *A96* strain and amounted 0.833  $\text{U}/\text{cm}^3$  which corresponded with the highest amount of  $\mu\text{moles}$  of fatty acids obtained on the medium with the addition of Tween 40.

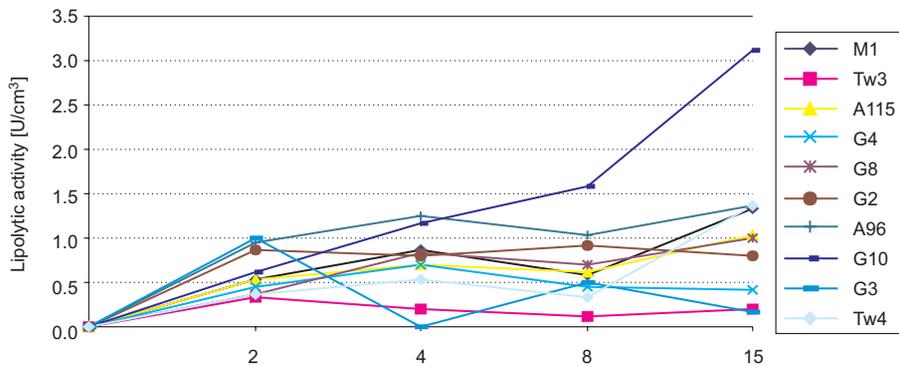


Fig. 4. The influence of the culturing time on extracellular lipases production in the presence of Tween 40 on selected *Bacillus* strains

While the maximum amounts of lipolytic activity for *B. cereus* G10 were obtained on the 4<sup>th</sup> day, for the strains *B. pumilus* Tw3, A115 and G8 they were recorded on the 8<sup>th</sup> day, and for the strains *B. pumilus* M1, *B. firmus* Tw4 and *B. pumilus* G4 on the last day of the experiment (Fig. 4).

The application of Tween 60 as a fatty substrate in the growth medium did not promote the extracellular lipases biosynthesis. The values of  $\mu$ moles of liberated fatty acids were the lowest for this medium in case of all the bacterial strains when compared with other fatty substrates. In the presence of Tween 60, the amount of liberated fatty acids did not exceed 30  $\mu$ moles (Fig. 5). The lowest mean values of fatty acids were noted for *B. pumilus* Tw3 and amounted 5  $\mu$ moles, and the highest 29.375  $\mu$ moles were noted for *B. pumilus* G4.

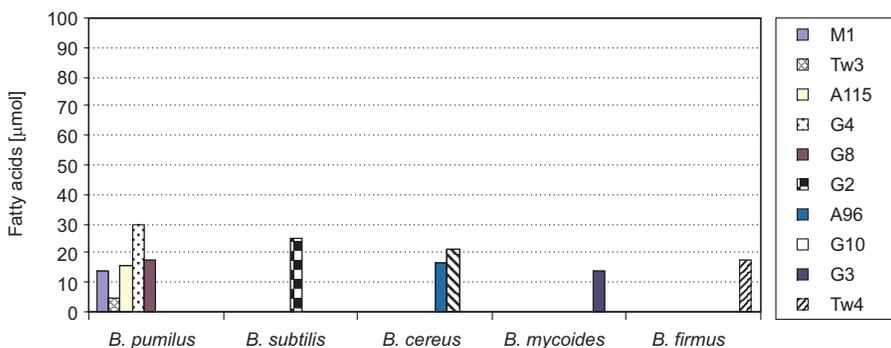


Fig. 5. The influence of Tween 60 on the production of extracellular lipases by selected *Bacillus* strains

In Tween 60 environment, the lipolytic activity was increasing with the time of the research (Fig. 6). Most of tested strains obtained the maximum activity on the 15<sup>th</sup> day of culturing. The highest activity of 0.75 U/cm<sup>3</sup> was measured for *B. pumilus* G4 strain, slightly lower value of 0.625 U/cm<sup>3</sup> was recorded for *B. cereus* G10 strain and 0.5 U/cm<sup>3</sup> value characterized the following strains: *B. pumilus* G8, *B. subtilis* G2 and

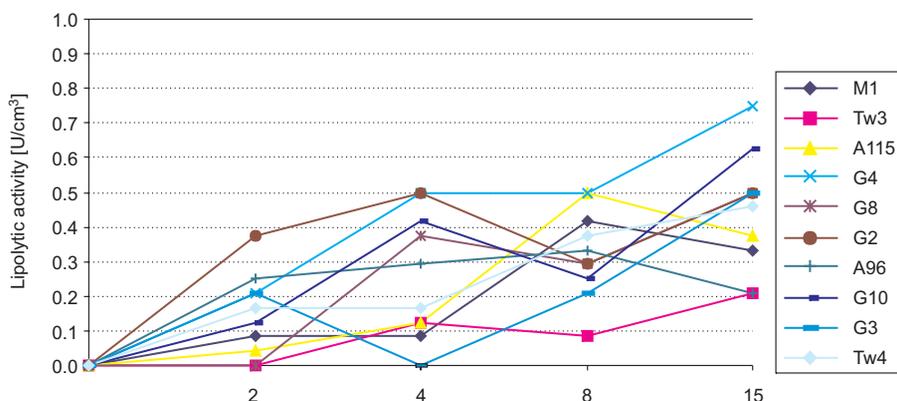


Fig. 6. The influence of the culturing time on extracellular lipases production in the presence of Tween 60 on selected *Bacillus* strains

*B. mycooides* G3. Only 2 strains obtained the maximum value of lipolytic activity on the 8<sup>th</sup> day of the experiment and they were *B. pumilus* M1 and A115.

It's worth noticing that 2 of the strains under study, namely *B. pumilus* Tw3 and G8 liberated extracellular lipases only on the 4<sup>th</sup> day of the research. It confirms the thesis that a fatty substrate in the growth medium induces the biosynthesis of lipolytic enzymes.

Tween 80 – the last tested synthetic source of fatty substrate was a favourable source for extracellular lipases production. During the experiment, the most vigorous strain was *B. subtilis* G2 liberating on average 33.125  $\mu$ moles of fatty acids similarly to the amounts liberated on Tween 40. For the rest of the bacterial strains the amount of liberated fatty acids fluctuated around 20  $\mu$ moles. The lowest results were recorded for the strains *B. pumilus* A115 and *B. mycooides* G3, obtaining 15.0 and 15.625  $\mu$ moles respectively (Fig. 7).

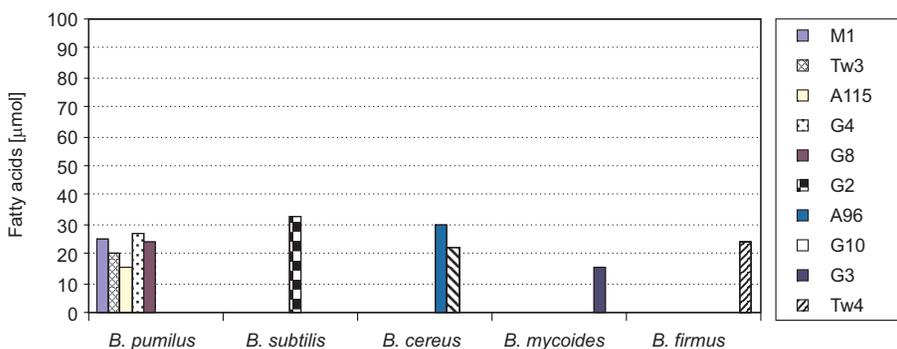


Fig. 7. The influence of Tween 80 on the production of extracellular lipases by selected *Bacillus* strains

Tween 80, analogous with Tween 40, revealed no clear correlation between incubation time, lipolytic activity, the species or the bacterial strains (Fig. 8). The lipolytic activity was decreasing during the experiment in case of the strains *B. cereus*

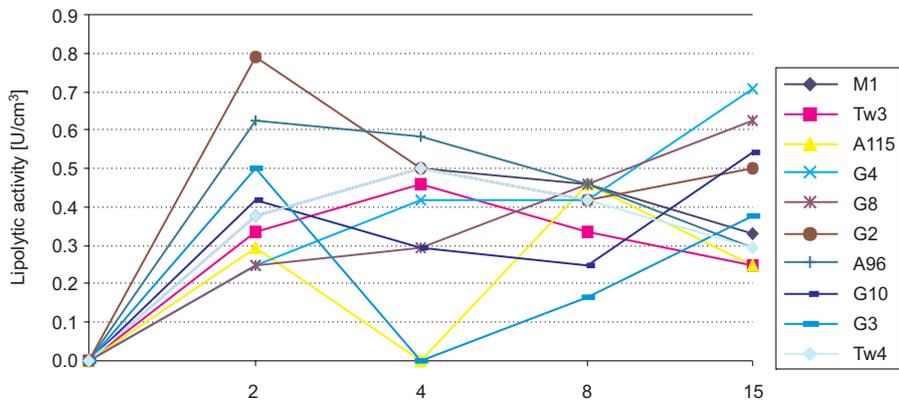


Fig. 8. The influence of the culturing time on extracellular lipases production in the presence of Tween 80 on selected *Bacillus* strains

*A96*, *B. subtilis* *G2* and *B. mycoides* *G3* (Fig. 8), similarly to the growth medium with the addition of Tween 40. For the rest of the strains the activity was increasing, to obtain its peak on the 4<sup>th</sup> day of culturing for the strains *B. pumilus* *M1* and *Tw3*, on the 8<sup>th</sup> day of culturing for the strains *B. pumilus* *A115* and *B. firmus* *Tw4* and finally on the 15<sup>th</sup> day of culturing for the strains *B. pumilus* *G4* and *G8*, *B. cereus* *G10*. The values obtained on the 15<sup>th</sup> day were among the highest recorded values and amounted 0.708 U/cm<sup>3</sup> for the *B. pumilus* *G4* strain, slightly lower 0.625 and 0.542 U/cm<sup>3</sup> for the strains *B. pumilus* *G8* and *B. cereus* *G10* respectively (Fig. 8).

Generally, the highest values of lipolytic activity on the analyzed growth media were obtained on the 8<sup>th</sup> and 15<sup>th</sup> day of incubation, which may suggest that the substrate was not used up completely. But it is still difficult to explain the activity drop on the following days of culturing. One possible reason [5–9] for the activity decrease is said to be the substrates exhaustion and growing pH of the environment. The activity drop could also be caused by the inhibitory interaction of forming products. Therefore, the study is to be continued towards setting the optimal culturing conditions, which may play an important role in the process of fatty wastes managing and the environmental protection as well.

## Summary and conclusion

Conducted research proved significant diversity between particular *Bacillus* strains in terms of their lipolytic activity, when different sources of fatty acids were considered and enabled to state that:

1. Biosynthesis of lipases catalysed by *Bacillus* strains was the most intense on the medium with tributyrin, and the least intense on the medium with the addition of Tween 60.
2. The extracellular lipases of tested bacterial strains were highly specific towards tributyrin and low towards Tween 60, which proves the process of their biosynthesis to be induced by the lipids.

3. Bacteria of *Bacillus* kind display higher preference of extracellular lipases to hydrolyze ester bonds formed by the long-chain butyric acids (contained by tributyrin) than long-chain fatty acids in Tween 40, 60 and 80.

4. All of tested *Bacillus* strains had the ability to produce the extracellular lipases. In terms of the sources of fatty substrates applied in the experiment the most vigorous strains were *Bacillus cereus* G10 and A96, and the least vigorous was *Bacillus pumilus* Tw3.

5. There was no clear correlation found between the time of incubation and the lipolytic activity, which indicates that it is specific for particular bacterial strains and depends on both the type of a fatty substrate in the growth medium and the environment of the strain isolation.

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## AKTYWNOŚĆ LIPOLITYCZNA *Bacillus* sp. WYIZOŁOWANYCH Z ŚRODOWISKA NATURALNEGO

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**Abstrakt:** W pracy przebadano 10 szczepów bakterii z rodzaju *Bacillus* (*B. cereus*, *B. firmus*, *B. mycoides*, *B. pumilus* i *B. subtilis*) pod względem możliwości syntezy lipaz w podłożach zawierających jako źródło węgla substraty tłuszczowe: tributyrinę oraz Tween 40, 60 i 80. Inkubację podłoży prowadzono przez 15 dni, a odczyty wykonano po 2, 4, 8 i 15 dniach. Wyniki podano jako ilość uwolnionych  $\mu\text{moli}$  kwasów tłuszczowych [ $\mu\text{mol}$ ] oraz w jednostkach aktywności lipolitycznej  $\text{U}/\text{cm}^3$ . Wśród badanych szczepów wykazano zróżnicowanie w poziomie aktywności zewnątrzkomórkowych lipaz w zależności od źródła substratu tłuszczowego i czasu hodowli. Dla większości badanych szczepów maksymalna produkcja lipaz występowała w 15 dniu hodowli. Najefektywniejsze w biosyntezie enzymów okazało się podłoże zawierające tributyrinę, a szczepy *B. cereus* A96 i G10 wyróżniały się najwyższą aktywnością, uwalniając średnio

97,5 oraz 69,375  $\mu\text{moli}$  kwasów tłuszczowych. Wartość aktywności lipolitycznej wzrastała w trakcie doświadczenia z 0,958 do 1,375  $\text{U}/\text{cm}^3$  dla szczepu *A96* oraz z 0,625 do 3,125  $\text{U}/\text{cm}^3$  dla szczepu *G10*. Natomiast, uwzględniając wszystkie testowane podłoża, najmniejszą aktywnością lipolityczną charakteryzował się szczep *B. pumilus Tw3*.

**Słowa kluczowe:** *Bacillus* sp., lipazy, tributyna, Tween



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## SPATIAL DIFFERENTIATION OF TOTAL NITROGEN CONTENT AND THE ACTIVITY OF N-TRANSFORMING ENZYMES IN A SOIL

### ZRÓŻNICOWANIE PRZESTRZENNE ZAWARTOŚCI AZOTU OGÓLEM ORAZ AKTYWNOŚCI ENZYMÓW PRZEMIAN AZOTU W GLEBIE

**Abstract:** The objective of this study was to evaluate and compare the spatial differentiation of total N ( $N_{TOT}$ ) content and urease (UR), nitrate reductase (NR) and arginine deaminase (ADA) activities in the surface horizon of Luvisol and Phaeozem of the Pomerania and Cuiavia region. 50 soil samples from both study areas were collected in April 2007 in a square sampling grid ( $90 \times 40$  m). The results were evaluated with the use of geostatistical methods. Spatial variability of the investigated parameters was evaluated by using empirical semivariograms with adjusted theoretical mathematical model of variograms. Raster maps of the studied properties were drawn. The concentration of chemical properties (TN, TOC,  $pH_{KCl}$ ) and the activity of UR and ADA was significantly higher in Phaeozem compared to Luvisol. Only the nitrate reductase activity was similar in samples of both types of soils. To characterise the spatial variability of the properties studied, spherical or mixed (spherical/linear) models with or without the nugget effect (only NR activity in Luvisol), were fitted to the calculated semivariograms. Total N content, NR activity in Phaeozem and ADA activity in Luvisol were in the strong variability class (the nugget effect < 25 %), while UR activity in both soil types and ADA activity in Phaeozem were situated in the moderate variability class (the nugget effect between 25 and 75 %). The ranges of influence calculated for properties studied ranged from 9.0 to 17 m. The raster maps showed that the distribution of each variable had a different pattern on the area studied. A specific variable was distributed in both topsoils in a different way.

**Keywords:** Luvisol, Phaeozem, spatial variability, total-N, urease, nitrate reductase, arginine deaminase activity

The amount of N contained in soils in organic forms by far exceeds that which is present in soluble inorganic forms ( $NO_3^-$  and  $NH_4^+$ ) [1]. Organically bound nitrogen in

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soil undergoes complex biochemical transformations as the result of which plant available mineral nitrogen forms are produced [2]. On every stage of these processes specific soil enzymes take part. They are produced by microorganisms and act exclusively intracellularly or are secreted to the soil environment and act extracellularly [3, 4]. Some of the enzymes are produced by plants in response to the presence of a specific substrate. Decomposition of urea to  $\text{NH}_3$  (and  $\text{CO}_2$ ) is brought about through the action of urease, an enzyme found practically in all soils [1]. Plants revealed the urease activity in the presence of urea, while they did not show it when the ammonium nitrate was the source of nitrogen [5]. Similarly, dissimilatory nitrate reductase is an adaptive enzyme produced only in the presence of nitrates as the substrate [6]. Nitrate reductase is the rate limiting enzyme in the initial process of denitrification (reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ ) [7]. At present one of the most often used indicators of soil biological activity is the ability of soil to ammonification of arginine. The process occurs only intracellularly and is associated with microbial biomass activity [8]. The level of the activity of specific enzymes involved in the N-cycle determines the intensity of soil nitrogen compounds transformation and could be the indicator of the nitrogen availability [9, 10].

Although a lot of attention has been devoted in the literature to the transformation of soil nitrogen and enzymes taking part in these processes in the agricultural space [11–13], most of research is carried out as laboratory experiments or in microplots systems under precisely controlled conditions. Very few studies however have concentrated on the spatial variability of those properties in the field scale, what is of a great theoretical and practical significance [14, 15]. Soil enzymatic activity, similarly to other biological properties, shows a high spatial variability, determination of which allows to estimate the real changes of the soil environment. The research of soil properties on the given area (eg arable field) is often done in one or a few points and on the basis of some results the decisions regarding soil utilization are undertaken.

The aim of the study was to determine the spatial variability of total nitrogen content and the activities of enzymes involved in the N transformation in the surface horizon of Luvisol and Phaeozem using geostatistical tools.

## Material and methods

The research was carried out in an 80-ha agricultural field located at the village of Orlinek near Mroczka in the Pomerania and Cuiavia region, northwest Poland. The two areas of  $90 \times 40$  m were selected for the research within the arable field. One area was covered with typical Luvisol and the second one with Phaeozem [16]. Chosen soils were situated as near as possible to avoid the influence of other factors on properties studied. In spite of the same material parent both soils were characterized by different morphological structure and other physicochemical, microbiological and biochemical properties. Winter wheat (*Triticum aestivum* L.) was cultivated after winter rape (*Brassica napus* L.) as the forecrop. Fifty soil samples were collected on each area at the stage of the winter wheat spreading on 12 April 2007 at regular intervals (10 m) from the 0–20 cm top layer.

Each sample consisted of 10 individual sub-samples taken randomly from a circle area with a radius of 2 m from the node point. Field-moist samples were sieved

(< 2 mm) and stored at 4 °C for not less than 2 days in order to stabilize microbial activity and then were analyzed for enzymatic activity within two weeks. Soil samples were analyzed for physical and chemical properties after air-drying at room temperature and sieving (< 2 mm).

Arginine deaminase activity (ADA) was measured using the Kandeler [17] method. After the addition of an aqueous L-arginine solution (11.5 M), soil samples were incubated for 3 h at 37 °C. The same procedure was followed for the control as for the enzyme assay but the control samples were stored immediately at -20 °C. After the incubation ammonium released by ADA was extracted with 2M KCl. For photometric analysis at 630 nm the filtrate was mixed with sodium phenolate solution (0.12 M), sodium nitropruside solution (0.17 M) and sodium hypochlorite (0.005 M NaOCl in 0.125 M NaOH) and allow to stand for 30 minutes at room temperature for colour development [18].

Soil urease activity was assayed as described by Kandeler and Gerber [19]. Briefly, 1 g of moist soil was incubated with 4 cm<sup>3</sup> of borate buffer (pH 10.0) and 0.5 cm<sup>3</sup> of urea solution for 2h at 37 °C. After the incubation ammonium released by UR was extracted with 2 M KCl. After filtering the resulting suspension, the concentration of ammonium ions were determined. To assess the ammonium content the filtrate was mixed with the Na salicylate/NaOH solution and the sodium dichloroisocyanide and allow to stand at room temperature for 30 minutes prior the measuring the optical density at 690 nm.

Assimilatory nitrate reductase activity (NR) was determined according to Kandeler [17]. Field moist soil samples were incubated for 24 hours at 25 °C under waterlogged condition with 0.9 mM 2,4-DNP (dinitrophenol) solution, substrate (25 mM KNO<sub>3</sub>) and destilated water. Controls were prepared the same way but they were incubated at -20 °C. After incubation 10 cm<sup>3</sup> of 4 M KCl solution was added to both samples and controls, the contents of test tubes were mixed briefly and filtered immediately. For spectrophotometric analysis 5 cm<sup>3</sup> of filtrates, 3 cm<sup>3</sup> of ammonium chloride buffer (0.19 M, pH 8.5) and 2 cm<sup>3</sup> of colour reagent (sulfanilamide and N-(1-naphthyl)-ethylenediamine hydrochloride) was added to the test tubes, mixed, and allowed to stand for 15 minutes at room temperature. Extinction of samples and controls was measured at 520 nm against the reagent blank.

All enzymatic assays were performed in triplicate. The data were corrected for oven-dry (105 °C) moisture content. One unit of arginine deaminase and urease activities were defined as the number of mg of product released by 1 kg of dried soil at 37 °C per 1 hour ( $\text{mg N-NH}_4^+ \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) while values of NR activity was expressed as  $\text{mg N-NO}_2^- \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ .

Physicochemical properties were determined according to standard methods and each sample was analyzed in triplicate. A particle-size was carried out using the Cassagrande'a method as modified by Proszynski; sand fraction content was determined using the sieving method; the pH in 1 M KCl was measured using the potentiometric method in 1 : 2.5 soil: solution suspensions; total organic carbon (TOC) and total nitrogen (TN) contents were determined using a dry combustion CN analyzer (Vario Max CN).

The spatial structure of properties studied has been characterized by using some geostatistical tools. A semivariogram was determined for each studied parameter in order to characterize the degree of spatial variability between neighboring samples, and the appropriate model function was fit to the semivariogram. Three basic parameters of semivariogram: sill, nugget effect and range were calculated [20]. The spatial variability of the properties studied was categorized into classes based on the percentage of total variance present as random variance:  $[\text{Co}/(\text{Co} + \text{C})] \cdot 100$ , as proposed by Cambardella et al [21].

In order to choose the best models adjusted the empirical variograms, a cross-validation procedure was used. The criterion to select the best fitting models was the mean squared deviation ratio (MSDR) calculated from the squared errors and kriging variances [22, 23]. Finally, the best-fit semivariograms were used to model the spatial distribution of variables by punctual kriging [24] and the maps illustrating the spatial variance of the parameters were drawn. The geostatistical calculations were done using Isatis software (Geovariance Co.).

## Results and discussion

Analysis of variance showed that the concentration of chemical properties (TN, TOC,  $\text{pH}_{\text{KCl}}$ ) and the activity of UR and ADA was significantly higher in Phaeozem compared with Luvisol ( $P < 0.05$ ). The  $\text{pH}_{\text{KCl}}$  values ranged from 4.11 to 5.76 in Luvisol and from 6.45 to 7.13 in Phaeozem, respectively. Organic carbon content in Luvisol ranged 5.51–9.0  $\text{g} \cdot \text{kg}^{-1}$  with mean value of 7.27  $\text{g} \cdot \text{kg}^{-1}$ , while in Phaeozem TOC concentration amounted for 13.1–25.1  $\text{g} \cdot \text{kg}^{-1}$  with mean 18.7  $\text{g} \cdot \text{kg}^{-1}$ . The basic statistical parameters of the TN content and enzymatic activity (mean, minimum, maximum) are presented in Fig. 1. Average ADA activity in Phaeozem was more than three times higher

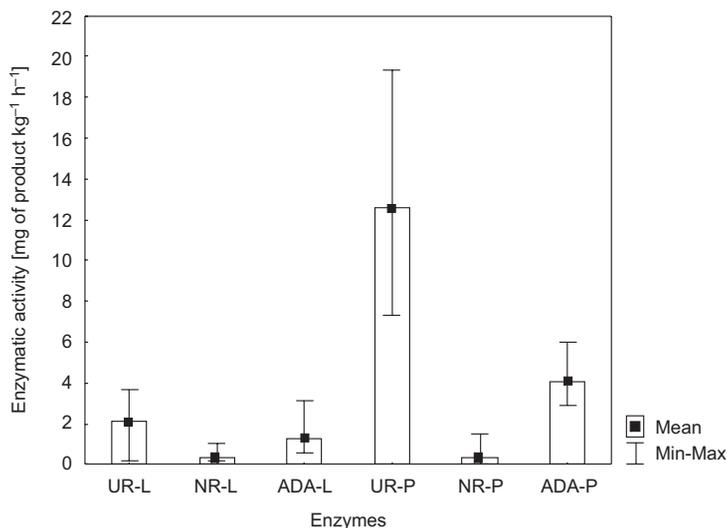


Fig. 1. Basic statistical parameters of variables studied ( $n = 50$ ): L – Luvisol, P – Phaeozem, UR – urease, NR – nitrate reductase, ADA – arginine deaminase activity

than that in Luvisol, while the UR activity overpassed the other one six times. Only the nitrate reductase activity data were not significantly different in both soil types.

The spatial variability of the soil properties studied differed significantly in the pattern of variation, which was shown in particular by the semivariogram parameters and kriged maps drawn (Table 1, Figs 2–3). To characterize the spatial variability of the properties, linear (UR activity), spherical (NR activity in Luvisol) or mixed (TN, ADA in Phaeozem) models with or without the nugget effect were fitted to calculated semivariograms.

Table 1

Parameters of variogram models

Property	Soil type	Model <sup>a</sup>	Nugget (Co)	Sill (Co + C)	Co/(Co+C) [%]	Range [m]	MSDR <sup>b</sup>	SD <sup>c</sup>
TN	Luvisol	SF, L, NE	0.0003	0.0016	20.4	15	1.05	S
	Phaeozem	SF, L, NE	0.0085	0.043	19.8	12	0.89	S
UR	Luvisol	L, NE	0.120	0.173	69.4	—	0.92	M
	Phaeozem	L, NE	0.635	1.835	34.6	—	1.00	M
NR	Luvisol	SF	—	0.0195	—	17	1.01	—
	Phaeozem	L, NE	0.005	0.020	0.25	—	1.16	S
ADA	Luvisol	L, NE	0.041	0.375	10.9	—	1.17	S
	Phaeozem	SF, L, NE	0.094	0.130	72.3	9	1.00	M

<sup>a</sup> SF – spherical, L – linear, NE – nugget effect, <sup>b</sup> MSDR – mean squared deviation ratio, <sup>c</sup> SD – spatial dependence, S – strong, M – moderate, TN – total nitrogen content [ $\text{g} \cdot \text{kg}^{-1}$ ]; UR – urease activity [ $\text{mgN-NH}_4^+ \text{kg}^{-1} \cdot \text{h}^{-1}$ ]; NR – nitrate reductase activity [ $\text{mgN-NO}_2 \text{kg}^{-1} \cdot \text{h}^{-1}$ ], ADA – arginine deaminase activity [ $\text{mgN-NH}_4^+ \text{kg}^{-1} \cdot \text{h}^{-1}$ ].

The spatial variability of the variables studied was categorized into two classes based on the percentage of total variance (*sill*) presents as a random variance [ $\text{Co}/\text{Co} + \text{C}$ , %] (Table 1). The above ratio is an important index for investigating spatial structures and enables comparison of the relative size of the nugget effect among soil properties. When

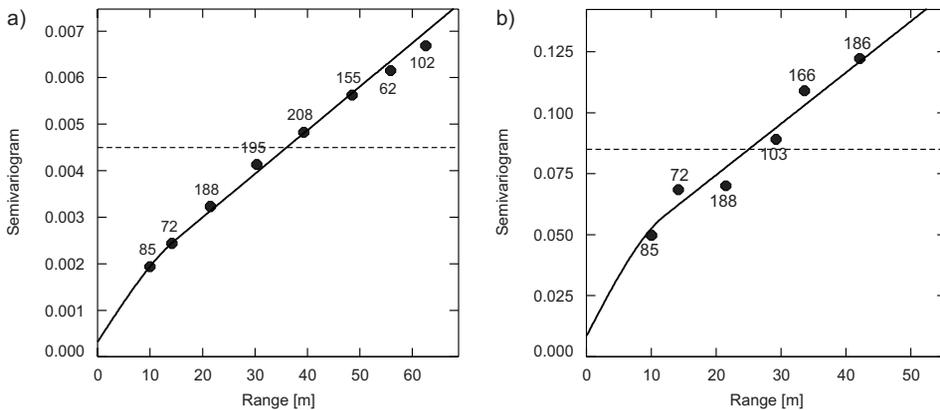


Fig. 2. Experimental semivariograms of (a) TN content in Luvisol, (b) TN content in Phaeozem

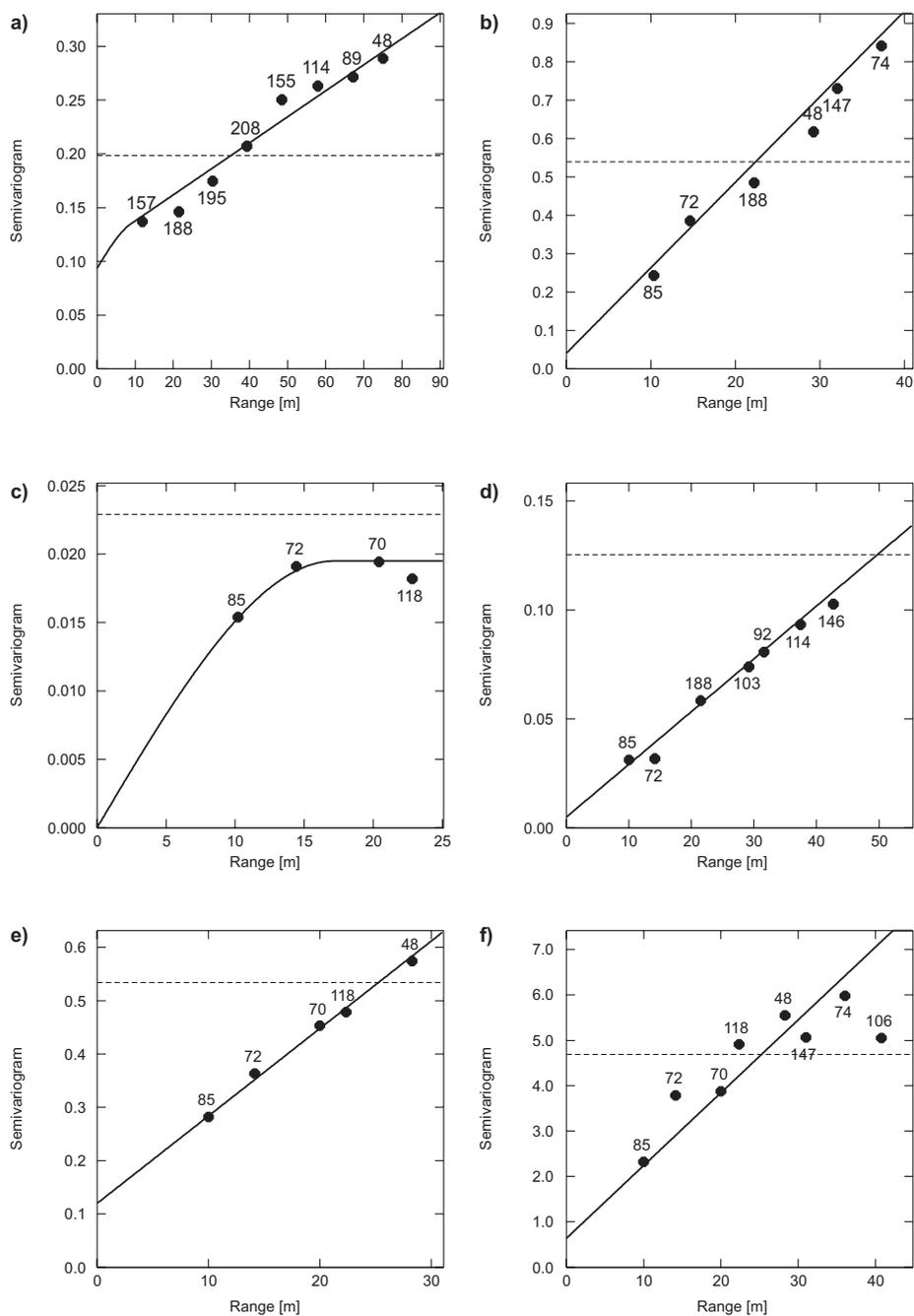


Fig. 3. Experimental semivariograms of (a) ADA activity in Luvisol, (b) ADA activity in Phaeozem, (c) NR activity in Luvisol, (d) NR activity in Phaeozem, (e) UR activity in Luvisol, (f) UR activity in Phaeozem

the ratio was less than 25 %, the variable had a strong spatial dependence; if the ratio was between 25–75 %, the variable had a moderate dependence; otherwise, the variable was considered randomly correlated (pure nugget effect) [21]. Nitrate reductase activity in Phaeozem and ADA activity in Luvisol showed a nugget/sill ratio of 0.25 and 10.9 % respectively, indicating a strong spatial variability, which can be influenced by variability in natural factors, such as soil texture and mineralogy [21]. Nugget semivariance for UR activity measured in both soil types was large compared with total variance (69.4 and 34.6 % of *sill*), suggesting a moderate spatial structure. Similarly, moderate contribution of nugget effect to total variance in UR activity (31.3. %) was noted by Aşkin and Kizilkaya [14]. Urease activity in the same Luvisol determined in August 2007 showed a lower [Co/Co + C], %] ratio of 19.8 suggesting that spatial structure of a given property has been changing in time [15]. The results indicated that only 27.7 % of the ADA activity in Phaeozem was due to structural variance and the random variability accounted for more than 70 % (Table 1). A strong class of spatial variability was noted in both soils for TN content. Some other studies have shown similar contribution of nugget variance to total variance (*sill*) in TN variability [25–27]. Total N content determined in the same Luvisol in August 2007 showed however moderate spatial variability [15].

The ranges of influence calculated for the variables studied ranged between 9 and 17 m (Table 1, Figs 2–3). Since the range is the maximum distance over which results are correlated [28] the sampling scheme for the properties studied (10 m) was suitable. If the sampling distance is bigger than the range, the data will no longer be spatially correlated, and as a result the geostatistics cannot be used [29]. The same property can vary significantly within the range due to the sampling distance; usually the longer sampling distance the higher range of influence. For example the range of influence for urease activity was 19 km when the soil was sampled at 4.5 km intervals [30], it reached 125 m when samples were collected in the distance of 15 m [14]. Different range values were noted for total nitrogen contents: 19.2–25 m [27], 42.5 m [31], 50 m [25] and between 208 and 650 m [26].

Spatial distribution of soil properties in both soil types are shown in Fig. 4a–f. Higher values have been noted for each property in Phaeozem as compared to Luvisol. In each figure, a light shading represents the lowest values, while a darker one is associated with the highest values. A band of relatively higher TN content in Phaeozem ran vertically from the north to the south of the field at 40–60 m of the field length, whereas TN concentration in Luvisol was spatially more uniform (Fig. 4a and b). The higher part of the Luvisol area was covered by TN values ranging from 0.8 to 1.1 g · kg<sup>-1</sup>. Lower values tended to be located at 0–45 m of the field length and 20–40 m of area width. The lowest values of ADA in Luvisol were noted in the centre of the field on the whole field width, while higher values were obtained in west and east part of the area studied (Fig. 4c). The highest ADA activity in Phaeozem was shown at 20–50 m of length and the whole width, while the lowest values were obtained at 0–10 and 50–70 m of the field length (Fig. 4d). Urease activity in Luvisol was similar all over the area except for lower values in the transect extended along the western part of the field (Fig. 4e).

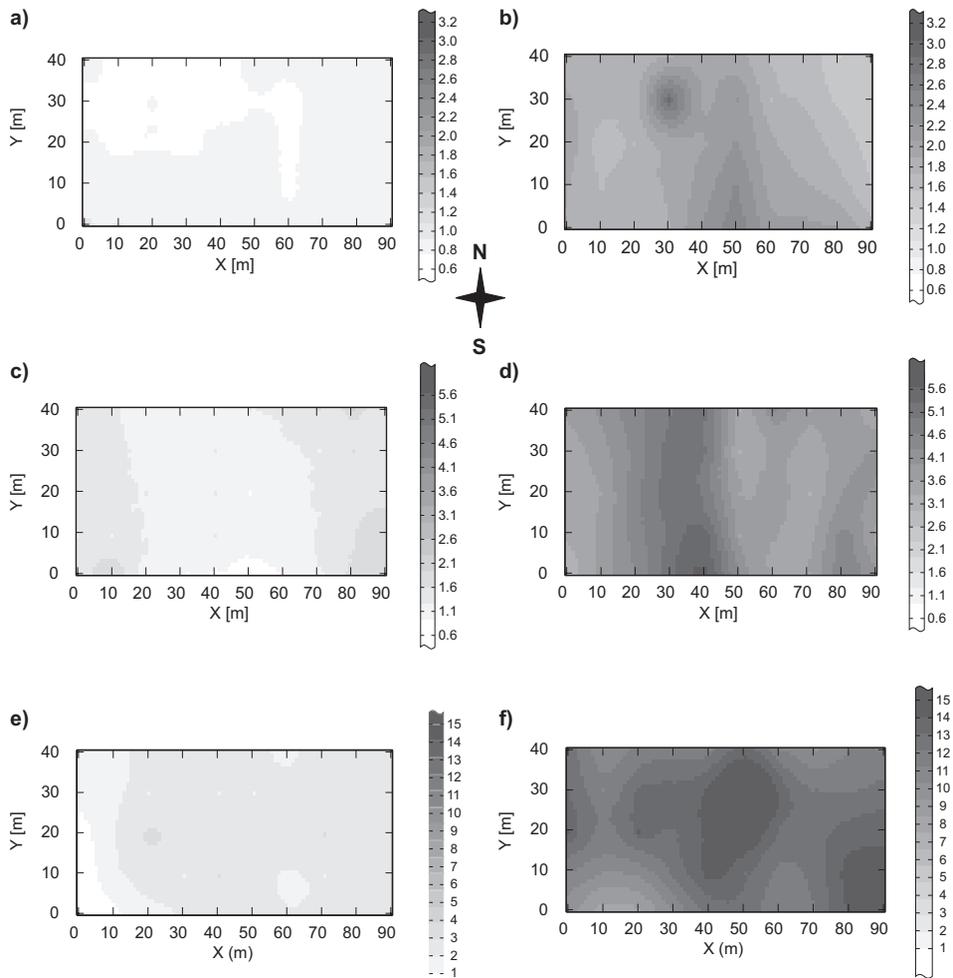


Fig. 4. Spatial distribution of TN content in Luvisol (a) and in Phaeozem (b), the rate of ADA in Luvisol (c) and in Phaeozem (d), UR activity in Luvisol (e) and in Phaeozem (f)

Urease activity in Phaeozem was clearly higher in the centre and in the south-east corner of the field (Fig. 4f).

Many studies have been devoted to examining the relationship between enzymatic activity and soil physico-chemical properties and results have varied with positive, negative, or no correlations being reported [32, 33, and many others]. In this study no significant correlation coefficients were found between total N content and N-cycle enzymes, except for ADA activity in Luvisol (Table 2).

This fact was earlier explained by McGill and Cole [34] who stated that the enzymes involved in N mineralization are less responsive to changes in N demand than the P-mineralizing enzymes are to P demand. According to those authors nitrogen in

Table 2

Correlation matrix (n = 50)

a) Luvisol

	pH <sub>KCl</sub>	TOC	ADA	NR	UR	TN
TN	— <sup>a</sup>	0.778	0.496	—	—	x
UR	0.385	0.569	—	-0.548	x	—
NR	—	—	—	x	**	—
ADA	0.374	—	x	—	—	**
TOC	—	x	—	—	**	***
pH <sub>KCl</sub>	x	—	*	—	*	—

b) Phaeozem

	pH <sub>KCl</sub>	TOC	ADA	NR	UR	TN
TN	—	0.913	—	—	—	x
UR	—	0.343	—	—	x	—
NR	—	—	—	x	—	—
ADA	0.409	—	x	—	—	—
TOC	—	x	—	—	*	***
pH <sub>KCl</sub>	x	—	*	—	—	—

TN – total nitrogen content ( $\text{g} \cdot \text{kg}^{-1}$ ), UR – urease activity ( $\text{mgN-NH}_4^+ \text{kg}^{-1} \cdot \text{h}^{-1}$ ); NR – nitrate reductase activity ( $\text{mgN-NO}_2 \text{kg}^{-1} \cdot \text{h}^{-1}$ ), ADA – arginine deaminase activity ( $\text{mgN-NH}_4^+ \text{kg}^{-1} \cdot \text{h}^{-1}$ ), TOC – organic carbon content ( $\text{g} \cdot \text{kg}^{-1}$ ), <sup>a</sup> – not significant, \*\*\* Correlation is significant at the 0.001 level, \*\* Correlation is significant at the 0.01 level \* Correlation is significant at the 0.05 level.

organic matter is bound between carbon atoms in a varied configuration, and inorganic N can only be released through multi-step pathways involving a set of enzymes that selectively eliminate particular types of C-N bonds. Moreover, the early products of the decomposition of organic nitrogen compounds often have fates other than those of a complete mineralization. From among studied enzymes only UR activity was significantly related to TOC, which has often been found in other studies [14, 28]. The significant relationship between enzymatic activity and organic C is likely due to higher C levels supporting greater microbial biomass and activity [33]. Additionally, increasing organic matter content provides a better environment for stabilizing and protecting enzymatic proteins in soil [35].

## Conclusions

Despite the areas selected for the research showed a high surface homogeneity, confirmed by a preliminary morphological and chemical study, the research showed a high spatial variability of soil properties studied within the same area. Higher ranges and spatial variability of the data were shown by soil properties of Phaeozem compared with Luvisol. Investigation of spatial variability of both chemical and biological parameters in the field scale are both of theoretical and practical significance. They

allow to estimate real changes of soil properties as the results of different agrotechnical procedures, what is efficacious in a better management of soil resources. Because of a high spatial distribution of the data decisions regarding soil utilization based on the research of soil properties on the given area done in one or a few points and on the basis of some results seems not to be adequate and can lead to under- or over-estimation of the real values.

## Acknowledgements

This research was financially supported by the Polish Ministry of Science and Higher Education (project no. N 310 030 32/1588, between 2007 and 2010).

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## ZRÓŻNICOWANIE PRZESTRZENNE ZAWARTOŚCI AZOTU OGÓŁEM ORAZ AKTYWNOŚCI ENZYMÓW PRZEMIAN AZOTU W GLEBIE

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**Abstrakt:** Celem badań było określenie zmienności przestrzennej zawartości N-ogółem (TN) oraz aktywności ureazy (UR), nitroreduktazy (NR) i poziomu deaminacji argininy (ADA) w poziomie powierzchniowym gleby pólnej oraz czarnej ziemi regionu Pomorza i Kujaw. W kwietniu 2007 r. z obu obszarów pobrano po 50 próbek glebowych z punktów zlokalizowanych w sztywnej siatce kwadratów (90 × 40 m). Wyniki zmienności

przestrzennej badanych parametrów określono za pomocą empirycznych variogramów oraz map rastrowych. Zawartość parametrów chemicznych (TN, TOC,  $\text{pH}_{\text{KCl}}$ ) oraz aktywność UR i ADA były większe w czarnej ziemi w porównaniu do gleby płowej. Jedynie aktywność nitroreduktazy była zbliżona w obu typach badanych gleb. Zmienność przestrzenną badanych parametrów przedstawiono za pomocą sferycznych lub mieszanych (sferyczno-liniowych) modeli semivariogramów. Zawartość N ogółem, aktywność NR w czarnej ziemi oraz ADA w glebie płowej znajdowały się w niskiej klasie zmienności (wariancja samorodka < 25 %) natomiast aktywność UR w obu typach gleb oraz ADA w czarnej ziemi zaliczono do średniej klasy zmienności. Zakresy autokorelacji badanych zmiennych wynosiły od 9 do 17 m. Mapy przestrzennego rozmieszczenia wyników badanych zmiennych wykazały, że rozmieszczenie wartości każdej z nich wykazywało inny kierunek. Ponadto wartości danej cechy były odmiennie rozmieszczone w obu typach gleb.

**Słowa kluczowe:** gleba płowa, czarna ziemia, zmienność przestrzenna, N-ogółem, ureaza, nitroreduktaza, poziom deaminacji argininy

Elżbieta STUDZIŃSKA-SROKA<sup>1\*</sup> and Daria ZARABSKA-BOŻEJEWICZ<sup>2</sup>

## THERAPEUTIC PROPERTIES OF EPIGAEIC LICHENS FROM SOWIA GORA

### WŁAŚCIWOŚCI LECZNICZE POROSTÓW NAZIEMNYCH SOWIEJ GÓRY

**Abstract:** One of the aim of nature protection should be improving the knowledge of society about properties of observed organisms. It can be helpful in creating the properly ecological conspicuous. Some species of lichens, *eg* genus *Usnea*, *Ramalina*, *Pseudevernia*, *Cetraria*, were commonly used in medicine from antiquity time. Their therapeutic properties are connected with the presence of secondary metabolites, *eg* cetraric acid, fumarprotocetraric acid, usnic acid. Nowadays few of the lichens have still application in producing some pills, tablets, syrups, toothpastes.

During field trips in Sowia Gora (Puszcza Notecka) some epigaeic lichens known from therapeutic properties were recorded. Suggestion of necessity of putting some information connected with therapeutic properties of species in the didactic table along the road of nature complexes characterized by high diversity of lichens is given. It permits to increase the level of interest in this group of species and *ipso facto* contribute to protection of lichens.

**Keywords:** *Cetraria*, *Cladonia*, *Peltigera*, secondary metabolites, nature protection

## Introduction

Among premises accompanying the process of taking action connected with nature protection, health motives are also distinguished. Maintenance of suitable quality of environment can be important in relation with sanity and physical health of human. Biodiversity of fungi, plants and animals matter *eg* in acquisition of natural compounds with potential biological activity. Carrying out of research on these organisms do not have to lead to destroying their stands of occurrence. Progress in organic syntheses and achievements in biotechnology caused that natural compounds, previously obtained

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only with use of substrates extractions, are nowadays both available in bigger amount and without influencing the environmental balance.

## Methodology

During field trips to Sowia Gora (central Poland) some lichens which are known from their therapeutic properties were recorded. Specimens which determination was not carried in the field, were collected and identified using methodological procedures. Morphological features of species were checked and analyzes of chemical reaction were made. *Thin Layer Chromatography* (TLC) of some specimens was carried out according to Culberson and Amann [1]. Nomenclature followed Faltynowicz [2]. Analyzes of lichens compound in the aspect of their therapeutic properties were based on the available literature.

## List of special interest species recorded in Sowia Gora

Sowia Gora is located in the south part of Miedzyrzecze Warty and Noteci [3], ca 12 km far away to north from Międzychód town. Pine forests are the most common communities growing here. In previous forest of Puszcza Notecka were often destroyed during some insects invasion and fires. Until now there are a few information about occurrence if epigaeic lichens observed in Puszcza Notecka [4–8]. Analyzes of species composition of plants and fungi in the community of Sowia Gora confirmed the presence of characteristic lichens both for *Leucobryo-Pinetum* and *Cladonio-Pinetum* forest [8].

During field trip in autumn 2008 species from three genus whose are known from their therapeutic properties were recorded:

I. *Cladonia* (*Cl. arbuscula* ssp. *squarrosa*, *Cl. arbuscula* ssp. *mitis*, *Cl. ciliata* ssp. *tenuis*, *Cl. coccifera*, *Cl. coniocraea*, *Cl. chlorophaea*, *Cl. cornuta*, *Cl. deformis*, *Cl. furcata*, *Cl. gracilis*, *Cl. grayi*, *Cl. merochlorophaea*, *Cl. pleurota*, *Cl. portentosa*, *Cl. pyxidata*, *Cl. rangiferina*, *Cl. subulata*, *Cl. squamosa*, *Cl. uncialis*);

II. *Cetraria* (*C. aculeata*, *C. islandica*);

III. *Peltigera* (*P. rufescens*).

## Therapeutic properties of recorded lichens

Curing properties of plants are known since ancient time. In the Ebers' papyrus (1550 BC) list of ca 900 prescription of different drug, 877 suggestions of treatment of injury and a few hundreds of name of medicinal plants were given. Hippocrates suggested some therapies with using of natural substances, and unusual rich in plants drug medicine of East (Chinese, Indian) believed in their unlimited curing potential [9, 10]. According to some ancient opinion, shape of plants and fungi could influence the efficiency of the treatment of ill organs [10].

Related to lichen substances first studies come from the middle of 19<sup>th</sup> century. A big contribution in the developing of this part of photochemistry had German botanist and chemist Wilhelm Zopf [11]. In his work "Die Flechtenstoffe" 150 lichens substances were described [12]. Another available contribution to the recognition of lichens chemistry has Japanese researchers Yasuhiko Asahina and Shōji Shibata. They defined structures of many lichens secondary compounds, and also developed the technique that allowed identification of lichens substances by using of microcrystallography [11]. Attention was also given to the biological activity of secondary compounds. The antibacterial properties were observed as one of the first ones. Further studies brought also other precious advantageous to the light, *eg* antiviral, antitumour, antioxidative, photoprotected and properties of enzymes inhibitions [11, 13].

Found near Sowie Góra some epigaeic species represent genus *Cladonia*, *Cetraria* and *Peltigera* whose therapeutic protection are known for a long time. Discovery concerns *Cetraria islandica* known under many names (*eg* Iceland moss) [14]. Species is still used to the receiving of extraction which help in treatment of illness of the respiratory system. Studies proved that discussed drug contained so-called acidic lichens (compounds included in the different chemical group characteristic for lichens). According to the literature data cetraric acid, fumarprotocetraric acid (aromatic compounds belonged to  $\beta$ -orcinol depsidones), protolichesterinic acid (an aliphatic  $\gamma$ -lactone), usnic acid (reported in the small amount or not detected, a dibenzofuran derivate) [15] can be distinguished. Moreover, polysaccharides, principally lichenan (lichenin), isolichenan (isolichenin), and galactomannans were also mentioned in the article. Above that some other constituents were discovered: naphthoquinone (naphthazarin) [13, 16], minerals (iron, magnesium, calcium, lead, arsenic, cadmium, mercury), carotenoids, [14], fatty acids (linoleic, oleic and linolenic acids), sterols (ergosterol, ergosterol peroxide), triterpenes (lupeol,  $\alpha$ -amyrin), the sesquiterpene lactone bakkenolide and monoterpenes (carvone, camphor, borneol) [15]. Thanks to that research it was proved that compounds belonging to polysaccharide group, isolated from higher plant, fungi, and lichens [17] have immunomodulatory, antiviral, radioprotective, anti-ulceric and anti-atherosclerotic properties [17, 19]. The experiments carried out on rats proved that polysaccharides decrease number of infections after operation. The results obtained difference in biological activity depending on place of this activity (pro-inflammatory or anti-inflammatory) [18]. Researches on polysaccharides of *C. islandica* conducted on human dendritic cells confirmed immunomodulating lichenin effect. Tests based on similar method of isolichenin, and also two other secondary metabolites (protolichesterinic acid, fumarprotocetraric acid) didn't revealed the similar activity [19].

In Poland, a drug (thallus of *C. islandica*) is an imported product and it is used to prepare the mixtures and extracts, element of complex drug (Pectosol – drops used in the inflammation of respiratory tract, Isla-moos and Isla-mint – pills for suction used in cough and hoarseness, Activ-angidin, Junior-angin, Fiorda, Herbitussin – for sore throat and inflammatory of oral cavity, Padma 28 – pills used to reinforce the immune system). Also toothpaste from BlanX series combines the extraction from Iceland moss is available which gives additional antibacterial effect on the tooth. Because of bitter

taste of compounds of the material, *C. islandica*, is also used to improve the digestion, and to increase appetite. Antibacterial activity comes out from presence protolichensterinic and protocetraric acid in the material [20]. Interesting may appear the fact that *C. islandica* as pulmonary tuberculosis (tuberculosis) drug was given also to F. Chopin [19]. Moreover, researches proved immunomodulating, anti-inflammatory, antiviral, antioxidant [19, 21] properties of extract. The gastroprotective activity of *C. islandica* is related to presence of protolichensterinic acid in its thallus. The research demonstrated that this element is active against *Helicobacter pylori*, bacterium responsible for development of gastric and duodenum ulcer [13].

*Cetraria aculeata* was the next species found in Sowie Góra. According to the literature species reveals antibacterial [22], antigenotoxic in bacterial systems and cytotoxic properties of extraction [23]. The research did not confirm antifungal properties [22].

*Peltigera rufescens* is representative of lichens from *Peltigera* genus in the investigated region. The extract prepared from thallus of mentioned above lichens revealed antioxidative properties in *in vitro* research. Although there is a lack of information concerning its use in traditional medicine, some sources shows that other species of this genus were used for the certain disorders. *Exempli gratia Peltigera canina* can be given which powdered thallus was use in order to prepare antirabies drug [18, 24]. In India and Ireland, *P. canina* was used to cure liver disease, this is also know for its laxative effect. Other species from discussed genus – *Peltigera aphthosa* was traditionally used as vermifuge [24].

In the vicinity of Sowie Góra was also recorded an important presence of species from genus *Cladonia*. Traditionally, some of them (eg *Cladonia rangiferina*, *Cl. pyxidata*) were used to remove fevers, colds, coughs, whooping cough, and other problems such as: arthritis, constipation, convulsions or tuberculosis [24]. One of the often occurring secondary metabolites in *Cladonia* genus is usnic acid [25]. Universality of usnic acid favors of development researches on its biological properties.

The compound was used in many countries as antibacterial medication in skin diseases. First mention about antibacterial properties of usnic acid come from fifty years of 20<sup>th</sup> century [26], also antiprotozoal, antiviral, anti-inflammatory, analgesic, antipyretic, antimytotic (inhibition of cell division), and antitumour activity of usnic acid was proved [27, 28]. Research conducted on rats showed also protection activity for mucous membrane of stomach [29]. The extract containing of usnic acid can protect against harmful radiation UVB. Moreover, they may reduce inflammatory reaction of skin connected with UV radiation, inflammatory reaction of skin [30]. Nowadays other formula of an usnic acid – “Usno” is known, medicament used in clinical bacterial infection. Usnic acid is also use as compound of toothpastes and toothrinses [31]. Above those curative properties, it is important to emphasize that the occurrence of allergic reaction to usnic acid is very unlikely, and furthermore this process is normally weak [28]. Some other results of research also show that hepatotoxicity properties can be observed while taking bog doses (it may damage or influence functioning of liver) [32].

## Conclusion

Epigeaic species recorded in Sowie Góra represent a few genus of lichens known from their therapeutic properties. One of the observed species – *Cetraria islandica* is well-known in traditional medicine, and also matters in modern phototherapy. One of the important lichens substances possessing of biological activity confirmed by research is presented in the text usnic acid (extracted *eg* from some *Cladonia* species).

In order to develop people awareness about therapeutic properties of lichens, some information should be given on the notice board, putting them especially where many species of lichens can be found, may increase interest in those organisms, till now commonly used mainly as bioindicators [33–35].

## Acknowledgements

The authors are deeply indebted to Prof. UM dr hab. Wiesława Byłka and Prof. dr hab. Karol Latowski (UAM) for helpful suggestion. They also sincerely thank mgr Ewa Grabowska for revision of English.

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## WŁAŚCIWOŚCI LECZNICZE POROSTÓW NAZIEMNYCH SOWIEJ GÓRY

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**Abstrakt:** Jednym z celów ochrony przyrody powinno być poszerzenie wiedzy społeczeństwa na temat właściwości obserwowanych organizmów. Może być to pomocne w kreowaniu właściwej świadomości prośrodowiskowej. Niektóre gatunki porostów, np. z rodzaju *Usnea*, *Ramalina*, *Pseudevernia*, *Cetraria*, były powszechnie wykorzystywane w medycynie od czasów starożytnych. Ich lecznicze właściwości powiązane są z obecnością metabolitów wtórnych, takich jak kwas cetrarowy, kwas furmaprotocetrarowy, kwas usninowy. Obecnie istnieje grupa porostów, która jest wykorzystywana przy produkcji tabletek, syropów, past do zębów.

Podczas badań terenowych w okolicach Sowiej Góry (Puszcza Notecka) udało się zaobserwować interesujące porosty naziemne wykazujące właściwości lecznicze. W artykule zasygnalizowano konieczność umieszczania informacji na temat właściwości leczniczych porostów na tablicach o charakterze dydaktycznym w kompleksach przyrodniczych cechujących się dużym bogactwem gatunkowym porostów. Pozwoli to zwiększyć zainteresowanie tą omówioną grupą organizmów i tym samym przyczynić się do ochrony porostów.

**Słowa kluczowe:** *Cetraria*, *Cladonia*, *Peltigera*, metabolity wtórne, ochrona przyrody

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## YIELDING, TOTAL PROTEIN AND CRUDE FIBER CONTENT IN SELECTED FAMILIES AND CULTIVARS OF MEADOW TIMOTHY

### PLONOWANIE, ZAWARTOŚĆ BIAŁKA OGÓLNEGO I WŁÓKNA SUROWEGO W WYBRANYCH RODACH I ODMIANACH TYMOTKI ŁĄKOWEJ

**Abstract:** The paper presents an assessment of selected cultivars and families of meadow timothy in terms of dry mass yields, total protein and crude fiber content.

The experiment was conducted at the Plant Breeding Station in Skrzyszowice which belongs to Malopolska Plant Growing Company – HBP LLC. The research was done on degraded chernozem developed from loess. The standard cultivar – Karta, two accompanying cultivars; Obra and Skala and seven families, like SzD-T-133, SzD-T-134, SzD-T-135, POB-T-78, POB-T-80, POB-T-81 and POB-T-82 were investigated in this research. In the analyzed families and cultivars of meadow timothy total nitrogen was assessed by Kjeldahl's method and crude fiber by gravimetric method by Goering and Van Soest.

Obtained results of family value assessment point to their high breeding potential. Analyzed families and cultivars were characterized by high yields of dry mass where differences reached even 46 %. Individual researched objects differed also by total protein content ranging between 5.2 to 17.25 depending on the cut and years of harvest. On the other hand, the values for crude fiber fluctuated from 8.4 to 32.6 %.

The highest concentration of total protein as well as the lowest concentration of crude fiber was found in POB-T-78 and SzD-T-135. Concentration of these components was significantly different in POB-T-78 and SzD-T-135 than in standard and accompanying cultivars. The highest concentration of total protein was found in POB-T-81, although the plants of POB-T-80 had the highest crude fiber content.

**Keywords:** cultivar of timothy, family, total protein, crude fiber, yield

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Meadow timothy (*Phleum pratense* L.) belongs among species commonly cultivated in cool and wet regions of the world. It adapts very well to various kinds of soil, tolerates pH in the range between 4.5 and 7.8, but does not tolerate salinity and alkalinity of soil. The main hindrance in timothy cultivation is its shallow root system and resulting intolerance to drought. Timothy reveals good winter hardiness and high fodder value, however harvest dates must be strictly observed because after inflorescence withering great protein losses are noted in timothy. The nutritional value of meadow timothy depends of many factors like: suitable cultivation, harvest date, cuts, cultivars, fertilization or meteorological conditions. Improving the nutritional value can be achieved also through the breeding works.

Improvement of nutritional value of fodder grasses is an objective of many breeding works. The basic criterion for breeding selection of meadow timothy is increasing its yield, however reaching considerable improvement by means of breeding is quite difficult [1]. Numerous investigations have confirmed an opposite relationship between dry mass content and nutritional value [2–4]. Therefore breeding works aiming at increasing dry mass content may lead to a decrease in nutritional value.

The improvement of dry matter yield and nutritive value require identification of genotypes associated with a weaker negative correlation between dry matter yield and nutritive value [5–7]. A very important feature of fodder grasses is their ability to produce stable and high yield of dry matter. Level of productivity and stability, mostly depends on genetic potential of the grasses [8, 9]. In the available literature can be found different reports about dry matter yield in grasses. It is difficult to compare these results as the studies were carried out in different periods, in which there were different climatic conditions. Moreover the yield depends not only on the cultivar but also on plant's family.

Therefore, presented investigations aimed at an assessment of diversification of meadow timothy families and cultivars in terms of dry mass yields, contents of total protein and crude fiber.

## Materials and methods

The experiment was conducted in 2005–2007 at the Plant Breeding Station in Skrzyszowice near Krakow (220 m a.s.l), on degraded chernozem developed from loess.

Skrzyszowice is situated in the northern part of Malopolska province, 25 km to the north-east of Krakow, in the Proszowice Plateau (Malopolska Upland). The soil of this area contains a deep layer of loess mixed with humus, the base consists of calcareous . In some places there are shallow rendzinas.

The chemical compounds of analysed soil, were as follow:  $\text{pH}_{\text{KCl}} - 7.2$ , available P – 54.0; K – 127.2 and Mg – 48.1  $\text{g} \cdot \text{kg}^{-1}$ .

This region is characterized by good climatic conditions for agriculture The climate is temperate. The field works begin in the third decade of March and finish in the first decade of November.

Annual rainfall total for the experimental period (2005–2007) fluctuated from 569.5 mm to 722.0 mm (Table 1).

Table 1

Total rainfall in Plant Breeding Station in Skrzyszowice [mm]

Month Year	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Amount
2005	29.0	25.0	9.0	53.0	82.0	85.0	91.0	116.5	15.0	16.0	34.0	46.0	601.5
2006	41.0	36.5	69.0	41.5	65.0	64.0	31.0	101.5	22.0	14.0	57.0	27.0	569.5
2007	54.0	38.0	57.0	27.0	67.0	84.0	60.0	64.0	163.0	54.0	34.0	20.0	722.0

Table 2

Mean monthly air temperatures in Plant Breeding Station in Skrzyszowice [°C]

Month Year	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Average
2005	-2.8	-6.9	-2.1	8.1	12.4	15.3	17.6	16.0	12.0	8.2	1.0	0.6	6.6
2006	-9.8	-4.3	-2.0	7.9	12.5	15.4	19.0	15.9	13.0	6.4	5.1	1.1	6.7
2007	1.5	-0.3	3.7	5.4	11.5	16.6	16.6	15.5	9.4	4.7	-0.5	-1.9	6.9

Obtained data showed the lower rainfall total in 2006 (second year of the research) and the highest in 2007. Whereas average rainfall totals during the vegetation period (April–September) ranged between 325.0 and 465.0 mm.

Table 2 presents the mean monthly air temperatures of Skrzyszowice, measured during the research. Mean annual temperature in the years of investigations fluctuated between 6.6 and 6.9 °C and in the April–September period from 12.5 to 14.0 °C.

The results showed that 2007 was generally the warmest period of the research, however the highest air temperature during vegetation period was found in 2006.

In the experiment, set up in randomised block arrangement in three replications (plots of  $1 \cdot 9.8 \text{ m}^2$ ) comprised standard Karta c.v., two accompanying cultivars: Obra and Skala and seven families: SzD-T-133, SzD-T-134, SzD-T-135, POB-T-78, POB-T-80, POB-T-81 and POB-T-82. The experiment was set up on 23 May 2005. The standard sowing rate was  $10 \text{ kg} \cdot \text{ha}^{-1}$ .

In autumn phosphorus fertilizers were sown in the amount of  $70 \text{ kgP}_2\text{O}_5 \cdot \text{ha}^{-1}$  as triple superphosphate and potassium fertilizers  $100 \text{ kgK}_2\text{O} \cdot \text{ha}^{-1}$  as potassium salt. Nitrogen fertilization, dosed  $20 \text{ kgN} \cdot \text{ha}^{-1}$  as ammonium nitrate was applied pre-sowing. The second dose of nitrogen fertilization,  $50 \text{ kgN} \cdot \text{ha}^{-1}$  was used after seeds sowing.

In the years of full utilization the following fertilizers were used:  $80 \text{ kgN} \cdot \text{ha}^{-1}$  under the first cut and  $60 \text{ kgN} \cdot \text{ha}^{-1}$  as ammonium nitrate after the second and third cuts. Phosphorus,  $120 \text{ kgP}_2\text{O}_5$  as triple superphosphate and potassium dosed  $60 \text{ kgK}_2\text{O} \cdot \text{ha}^{-1}$  as 57 % potassium salt, were applied once, in autumn preceding the harvest.

After plant drying and grounding, total nitrogen was assessed by Kjeldahl's method and crude fiber by gravimetric method in weighted average plant samples.

The obtained results were verified by the analysis of variance. Differences between means were assessed using Tukey multiple range test and then the least significant difference was calculated.

## Results and discussion

Average content of dry mass in the analyzed families and cultivars of meadow timothy fluctuated from 24.5 (2006) to 26.2 % (2007) and was significantly diversified (Table 3). This component was affected most by the air temperature and rainfall. Considering the compared families and cultivars, markedly higher content of dry mass – 26.7 % characterized SzD-T-133 family. The least of dry mass (22.6 %) was registered in plants from the first cut, whereas significantly more in plants from the third cut (26.1 %) and the second cut (28.2 %).

The highest concentration of total protein (12.0 %) was found in meadow timothy in 2006. Diversification in the content of discussed component was due to the weather course. In the years with higher air temperature meadow timothy contained higher amounts of protein. Minson [10], Cwintal and Wilczek [11] registered a similar relationship in red fescue. The analyzed meadow timothy families revealed significantly higher total protein concentrations in comparison with its cultivars. Considering the harvest date, the highest changeability of total protein was noted in subsequent cuts.

Table 3

Content of dry mass, total protein and crude fiber in meadow timothy [g · kg<sup>-1</sup>d.m.]  
– years of full utilization

Research factors	Object	Dry matter	Crude protein	Crude fibre
A. Years	2006	245.0	120.0	245.0
	2007	262.0	106.0	184.0
LSD <sub>0.05</sub>		18.7	15.4	61.6
B. Strain, cultivars	SzD-T-133	266.7	113.2	206.0
	SzD-T-134	262.5	108.6	215.0
	SzD-T-135	264.6	123.2	199.6
	POB-T-78	239.0	128.0	192.4
	POB-T-80	256.8	108.6	218.0
	POB-T-81	252.3	98.8	217.4
	POB-T-82	250.4	119.8	207.2
	OBRA	246.3	110.6	203.8
	SKALA	255.9	103.6	217.2
	KARTA*	259.0	101.2	211.0
LSD <sub>0.05</sub>		15.6	12.1	15.5
C. Cut	1	225.7	79.3	297.6
	2	282.4	145.0	168.1
	3	260.8	109.2	112.6
LSD <sub>0.05</sub>		22.2	28.8	68.3
Interaction	A × C	24.5	29.8	86.5

\* Standard cultivar.

The lowest concentrations of this component were noted in the first cut and the highest in the second. Obtained results were partly corroborated by literature data [12, 13]. With reference to crude fiber, a marked diversification was noted in the discussed experiment, depending on the analyzed factors. As well known rule concerning the opposite content of protein and fiber in grass plants has not been always confirmed [14, 15]. Dry mass yields fluctuated from 92.9 to 155.2 dt · ha<sup>-1</sup> (Table 4). Higher yields may be connected with higher rainfall total in 2007 (465 mm during the vegetation period). Among the analyzed families and cultivars, higher amount of dry mass was harvested from meadow timothy cultivars. Significantly higher yields of dry mass were registered for the first cut. No such differences were noted between the second and third cut. Similar results referring to dry mass were reported by Mason and Lachance [13].

Total protein yields per 1 ha were significantly diversified by the weather in the individual years, cultivars, families and cuts. A positive cooperation between years and objects was found, which shaped the discussed yields. Significantly higher total protein mass was obtained in the following objects: POB-T-82; POB-T-78 and SzD-T-135. Total protein yield depended mainly on dry mass yields. This dependence was confirmed by research conducted by other authors [15–17].

Table 4

Yield of dry mass and total protein [ $\text{dt} \cdot \text{ha}^{-1}$ ] – years of full utilization

Research factors	Object	Dry matter	Crude protein
A. Years	2006	92.9	9.6
	2007	155.2	13.5
LSD <sub>0.05</sub>		31.5	2.1
B. Strain. cultivars	SzD-T-133	116.3	10.3
	SzD-T-134	119.3	11.0
	SzD-T-135	117.9	12.0
	POB-T-78	121.3	13.3
	POB-T-80	123.0	10.8
	POB-T-81	133.8	11.5
	POB-T-82	131.9	14.0
	OBRA	126.0	11.6
	SKALA	129.2	10.7
	KARTA*	122.3	10.0
LSD <sub>0.05</sub>		7.7	1.5
C. Cut	1	88.6	6.9
	2	22.3	3.2
	3	26.5	2.9
LSD <sub>0.05</sub>		20.4	1.7
Interaction	A × C	25.5	2.0

\* Standard cultivar.

Analyzed populations of cultivars and families of meadow timothy are diversified with regard to many features. Accumulated genotypes widen changeability within the timothy genus, which may be used in breeding and research works [18–20].

## Conclusions

1. Both cultivars and families differed considerably among themselves regarding dry mass yields, total protein and crude fiber contents.
2. The factor diversifying cultivars and families were meteorological conditions.
3. On the basis of many-year observations it was stated that detailed observations should be conducted further to indicate valuable families.
4. The content of analyzed organic compounds in dry mass of researched families and cultivars of meadow timothy testifies their high fodder value.

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## PLONOWANIE, ZAWARTOŚĆ BIAŁKA OGÓLNEGO I WŁÓKNA SUROWEGO W WYBRANYCH RODACH I ODMIANACH TYMOTKI ŁĄKOWEJ

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**Abstrakt:** Praca prezentuje ocenę wybranych odmian i rodów tymotki łąkowej pod względem plonów suchej masy, zawartości białka ogólnego oraz włókna surowego.

Badania przeprowadzono na czarnoziemie zdegradowanym wytworzonym z lessu, na terenie Stacji Hodowli Roślin w Skrzyszowicach należącej do Małopolskiej Hodowli Roślin – HBP Kraków. W doświadczeniu uwzględniono odmianę wzorcową Karta, dwie odmiany towarzyszące Obra i Skala oraz siedem

rodów: SzD-T-133, SzD-T-134, SzD-T-135, POB-T-78, POB-T-80, POB-T-81 i POB-T-82. W przygotowanym materiale roślinnym oznaczono azot ogólny metodą Kjeldahla, a włókno surowe metodą wagową Goeringa i Van Soesta.

Uzyskane wyniki badania wartości rodów wskazują, że mają one wysoki potencjał hodowlany. Badane rody i odmiany charakteryzowały się dużymi plonami suchej masy, gdzie różnice sięgały nawet 46 %. Poszczególne badane obiekty różniły się także zawartością białka ogólnego, które wynosiły od 5,2 do 17,2 % w zależności od pokosu i lat zbioru. Natomiast dla włókna surowego wartości te kształtowały się od 8,4 do 32,6 %. Najwięcej białka ogólnego oraz mniej włókna surowego zawierały POB-T-78 i SzD-T-135, gdzie stwierdzono istotne różnice w stosunku do odmiany wzorcowej, jak i odmian towarzyszących. Spośród badanych rodów POB-T-81 wypadł najmniej korzystnie pod względem zawartości białka ogólnego, z kolei najwięcej włókna surowego odnotowano w roślinach POB-T-80.

**Słowa kluczowe:** odmiana tymotki, ród, białko ogólne, włókno surowe, plon

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## ASSESSMENT OF THE CHEMICAL COMPOSITION OF JERUSALEM ARTICHOKE (*Helianthus tuberosus* L.) AS ENERGY FEEDSTOCK

### OCENA SKŁADU CHEMICZNEGO SŁONECZNIKA BULWIASTEGO (*Helianthus tuberosus* L.) JAKO SUROWCA ENERGETYCZNEGO

**Abstract:** This work concerns the analysis of variation of chemical composition of ash of aboveground part of Jerusalem artichoke as a renewable energy source. Factors of the experiment were a cultivars of *Helianthus tuberosus*: Albik and Rubik, grown on sandy soil, good rye complex under constant fertilization and full dose of manure. In the ash were determined: the content of macro- and microelements and some heavy metals. Phenotypic variation of Jerusalem artichoke, in terms of each trait was the combined effect of genetic variation and environmental. Decisive share of cultivars, the total variance, had the contents of potassium, magnesium, nitrogen, phosphorus and manganese. Dominant role in the overall volatility of chlorine, zinc, cadmium, cobalt and copper play environmental variability, in the case of sodium, sulphur, molybdenum, iron, aluminium, chromium and lead interaction of varieties and years. Biomass of aboveground parts of Jerusalem artichoke contain smaller amounts of cadmium, lead and zinc than the limit values set out in the German DIN 51731st.

**Keywords:** Jerusalem artichoke, cultivar, security of biomass, macroelements, microelements, heavy metals

## Introduction

Jerusalem artichoke (*Helianthus tuberosus* L.) stands out as a species with a high capability of solar energy fixation and conversion to organic matter. This allows to achieve high and stable tuber and overground mass yield on light soils, which can be used in energy generation. Kays and Nottingham [1] report that the yield of dry matter of overground parts of Jerusalem artichoke, cultivated in conditions close to optimal, ranges from 10 to 15 Mg · ha<sup>-1</sup>. Stems of Jerusalem artichoke contain large amounts of

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dry matter, which can be used directly in burning, even immediately after harvesting. This applies mainly to biomass (stem and leaves), which are usually harvested in late autumn and winter [2–6]. Combustion is the simplest method of production of thermal and electrical energy. However, burning produces a lot of ash and these substances determine the potential for use of plant biomass for combustion. According to Izsaki and Kadi [2], Jungers et al [7], Kalembsa [8], Tersic and Atlagic [9], Denoroy [10], Dorel and Chubey [11], Augustynowicz et al [12] and Sawicka [13], ash composition depends on habitat-related and agricultural factors, such as the date of the plantation set-up, plant density, cultivation and plant protection procedures, level of mineral fertilisation and harvest date. Irrespective of their effect, genetic factors (*ie* species and cultivar) have a great effect on phenotypic variation [1, 7, 8, 14–17]. Their effect on ash composition has not been properly explored. The aim of this study was to determine the amount of raw ash, produced by burning biomass of two cultivars of Jerusalem artichoke and assessment of the chemical composition of ash proper (separated from raw ash) in regard to its potential use in agriculture.

## Materials and methods

A field experiment was conducted at the Field Experimental Station in Parczew in 2003–2005, on light soil with a granulometric composition of clayey sand and good rye complex. Albik and Rubik cultivars were used as experimental factors. They were cultivated on a full dose of manure ( $30 \text{ Mg} \cdot \text{ha}^{-1}$ ) with constant mineral fertilisation at the doses:  $100 \text{ kg N} \cdot \text{ha}^{-1}$ ,  $44 \text{ kg P} \cdot \text{ha}^{-1}$  and  $125 \text{ kg K} \cdot \text{ha}^{-1}$ . The experiment was set up in mid-April, with a density of  $40\,000 \cdot \text{ha}^{-1}$ , using the method of randomised sub-blocks, in triplicate. The plot for harvesting had an area of  $25 \text{ m}^2$ . All the cultivation procedures were performed in accordance with good agricultural practice. The biomass was harvested at the end of October. Samples of overground parts of 10 plants from each plot were taken for chemical analyses. The feedstock moisture content was determined by drying and weighing (the ground biomass was dried at  $105 \text{ }^\circ\text{C}$  until a constant weight was achieved). Samples of Jerusalem artichoke tubers were dry-oxidised at a temperature of about  $500 \text{ }^\circ\text{C}$  and the amount of raw ash was subsequently determined gravimetrically. Ash proper (pure) was obtained from raw ash by separating the silica and decomposition of carbonates. To this end, an excess of  $6 \text{ mol HCl} \cdot \text{dm}^{-3}$  was added until the carbonates decomposed completely. Cations formed during the mineralisation process of the plant organic matter were transformed into chlorides. Subsequently, the excess of hydrochloric acid was evaporated on a sand bath and the silica was simultaneously deposited. The deposit remaining in the evaporator was dissolved in  $10 \text{ cm}^3$  of 5 % hydrochloric acid and transferred to a measuring flask, with silica being separated on hard filter paper. The deposit on a filter was washed three times with  $5 \text{ cm}^3$  of hydrochloric acid, and subsequently three times with  $10 \text{ cm}^3$  of deionised water. The solution in the measuring flask was made up to the specific volume, yielding the basic stock solution in which the contents of selected macro- and micro-elements and trace elements, including heavy metals, were

determined with an Optima 3200 RL atomic absorption spectrometer with inductively coupled plasma (ICP-AES), manufactured by Perkin Elmer.

Soil analyses were performed on 20 primary samples, comprising one bulk sample with a mass of about 0.5 kg. Dried soil was sifted through a 1 mm mesh sieve. The following were determined in the soil: granulometric composition by the areometric method, pH in 1 mole  $\text{dm}^{-3}$  KCl solution, organic carbon content ( $C_{\text{org}}$ ) – by the Tiurin method, chromium, cadmium, lead, copper and nickel – in accordance with the methodology adopted at chemical-agricultural stations. The results of the analyses were evaluated in accordance with the limiting values, developed by IUNG [18].

The results were worked out statistically by means of analysis of variance and polynomial regression. The significance of sources of variation was tested by the Fischer-Snedecor “F” test, while the LSD of 0.05 was evaluated by Tukey’s test. In order to determine the contribution of each source of variation and their interactions in the total variation of the attributes under study, the variation components were evaluated, using the following notation:  $\sigma_e^2$  – evaluation of environmental variation, associated with repeating observation or measurement over many years;  $\sigma_G^2$  – evaluation of genotypic (cultivar-related) variation;  $\sigma_p^2$  – evaluation of phenotypic (total) variation. Variation coefficients were also calculated for each feature of chemical composition of overground parts of the plant from the formula:

$$V = \frac{s}{x} \cdot 100 \%,$$

where:  $s$  – standard deviation,  $x$  – arithmetic average.

The weather in the years of study varied (Table 1). In 2003, the first half of the vegetation period was humid and warm, June, August and September were droughty, while October (which was decisive for the autumn growth of the overground parts) was wet. In 2004, the beginning of the vegetation period (April–May) was humid and cold, June and July were – dry, the weather in August and October was average and there was extreme drought in September. In 2005, the May–June period was wet and cold, while the other months – except August – were dry or droughty and warm [19].

Table 1

Sielianinov’s coefficient values ( $k$ ) according to meteorological station in Uhnin

Years	Months							Mean
	IV	V	VI	VII	VIII	IX	X	
2003	2.2	1.4	0.9	1.3	0.6	0.5	2.4	1.3
2004	4.1	1.3	0.9	0.5	1.1	0.2	1.3	1.4
2005	0.7	1.9	1.9	0.7	1.2	0.3	0.2	1.0

$k \leq 0.50$  – strong drought;  $0.50 \leq k \leq 0.69$  – drought;  $0.70 \leq k \leq 0.99$  – slight drought;  $k \geq 1$  – no drought, according to Luterbacher et al [19].

## Experiment results

The experiment was carried out on soils whose surface humic layers consisted of light sandy formations – clayey sands with acidic or slightly acidic pH (5.1–5.9) and medium level organic carbon content (Table 2). The content of available phosphorus and potassium in the soil was high to very high, whereas that of available magnesium was low to high [18]. The average content of heavy metals in the soil was equal to: Cd – 0.71; Co – 0.82; Cr – 1.21; Ni – 2.71; Pb – 30 mg · kg<sup>-1</sup> of dry soil.

Table 2

Characterization of soils according to agronomic categories

Agronomic category of soil	Year	Percentage content of fraction in diameter [mm]			Soil texture (acc. PTG)	pH <sub>KCl</sub>	C <sub>org</sub> [g · kg <sup>-1</sup> ]
		1–0.1	0.1–0.02	< 0.02			
Light	2003	57	24	19	pg	5.5	8.07
	2004	62	25	13	pg	5.9	9.22
	2005	66	21	13	pg	5.1	7.19

The main biomass safety indexes of Jerusalem artichoke biomass in renewable energy production or in agricultural use (of the ash produced from it) were the content of ash and selected elements, because it should emit as few gaseous substances (NO<sub>x</sub>, SO<sub>x</sub>, Cl) as possible.

The Albik cultivar of Jerusalem artichoke contained significantly more macro-elements (N, K, P, Ca, Cl) in its overground parts than the Rubik cultivar. The Albik cultivar was found to contain higher concentrations of magnesium and sodium and the content of sulphur was not significantly cultivar-dependent. The value of the variation index (V), which is a measure of how dispersed the results are, was low, which is a sign of stability of the analysed features of the chemical composition of ash produced from the overground parts of the plant under study. The potassium, phosphorus, calcium and chlorine content in the Rubik cultivar was more stable than in the Albik cultivar, in which, in turn, the nitrogen and magnesium content was more stable. The potassium content proved to be the most stable feature, whereas calcium was the element with the least stable content (Table 3). The structure of the variance components indicates different contribution of the cultivars in the total variation of individual macro-elements content. Cultivars had the decisive contribution in the overall variation of the content of potassium, magnesium, nitrogen and phosphorus. The dominant role in the overall variation of chlorine content was played by environmental variation, whereas for sodium and sulphur – cultivars and years interaction (Table 4).

In regard to micro-elements, the Albik cultivar contained larger amounts of manganese, while the Rubik cultivar – larger amounts of aluminium. The content of molybdenum and iron was not determined by the genetic properties of Jerusalem artichoke cultivars. Concentrations of molybdenum and iron in the Albik cultivar were more stable, while those of manganese and aluminium were more stable in the Rubik

Table 3

Effect of cultivars and years on the content of selected elements in dry mass of aboveground parts of Jerusalem artichoke [ $\text{g} \cdot \text{kg}^{-1}$  d.m.] (mean for 2003–2005)

Elements	Cultivars				LSD <sub>0.05</sub>
	Albik		Rubik		
	mean	V <sup>a</sup>	mean	V	
[ $\text{g} \cdot \text{kg}^{-1}$ ]					
Ash	44.34	12.70	38.99	10.77	1.40
Nitrogen	27.56	3.66	26.15	9.97	1.00
Potassium	33.14	10.84	31.66	10.96	9.63
Phosphorus	2.42	18.45	2.38	22.27	0.08
Calcium	12.90	43.85	12.12	36.72	0.43
Magnesium	4.50	12.12	4.61	8.23	0.16
Sodium	1.73	9.60	1.95	14.78	0.07
Sulphur	1.10	12.41	1.05	16.32	n <sup>b</sup>
Chlorine	0.29	41.50	0.27	45.18	0.01
[ $\text{mg} \cdot \text{kg}^{-1}$ ]					
Manganese	140.45	22.42	135.22	21.64	5.71
Molybdenum	0.16	37.77	0.18	42.11	n
Iron	229.27	23.76	227.57	26.42	n
Aluminium	628.05	28.72	850.19	20.94	25.80
[ $\text{mg} \cdot \text{kg}^{-1}$ ]					
Zinc	46.53	14.01	42.76	11.49	1.56
Chrome	3.12	19.04	3.15	20.16	n
Cadmium	0.29	19.43	0.31	27.32	0.01
Cobalt	0.22	8.59	0.23	13.03	n
Cuprum	7.88	11.24	8.33	11.33	0.30
Nickel	3.52	15.79	3.66	25.84	0.11
Lead	5.44	10.54	5.21	11.91	0.18

<sup>a</sup> Coefficient of variability; <sup>b</sup> non significance at the level  $\alpha \leq 0.05$ .

cultivar (Table 3). Cultivar-related properties proved to be the dominant source of variation in manganese content, while the interaction of years and cultivars was dominant in molybdenum, iron and aluminium content (Table 4).

In regard to heavy metals, overground parts of the Albik cultivar of *Helianthus tuberosus* contained more zinc and lead, while those of the Rubik cultivar contained more cadmium, copper and nickel. The content of chromium and cobalt was not significantly cultivar-dependent. The most variable features included the content of zinc, while that of nickel was the most stable. The content of chromium, cadmium,

Table 4

Effect of cultivars and years on the content of selected elements in dry matter of aboveground parts of Jerusalem artichoke and their percentage of the total variance

Elements	Impact significance			The percentage of variance in total variance		
	cultivars	years	cultivars × years	cultivars	years	cultivars × years
[g · kg <sup>-1</sup> ]						
Ash	*	**	**	47.3	24.9	25.3
Nitrogen	**	**	**	51.6	27.1	5.6
Potassium	**	**	**	56.9	34.5	7.9
Phosphorus	**	**	**	50.8	4.9	36.2
Calcium	*	**	**	9.8	41.0	47.7
Magnesium	**	**	n	55.5	37.8	3.4
Sodium	*	**	**	36.9	4.9	50.8
Sulphur	n	**	**	4.7	22.3	69.7
Chlorine	*	**	n	6.3	82.2	4.5
[mg · kg <sup>-1</sup> ]						
Manganese	**	**	**	55.1	21.2	21.7
Molybdenum	n	**	**	2.1	37.9	60.8
Iron	n	**	**	4.5	18.6	72.3
Aluminium	*	**	**	26.5	8.5	62.4
[mg · kg <sup>-1</sup> ]						
Zinc	*	**	**	5.7	62.7	30.1
Chrome	n	**	**	1.9	34.8	57.6
Cadmium	*	**	n	26.5	62.3	1.6
Cobalt	n	**	**	0.8	68.5	30.1
Cuprum	**	**	*	20.9	50.8	23.6
Nickel	**	**	**	9.4	41.9	47.7
Lead	**	**	**	10.2	38.4	50.2

\* Significance at the level  $\alpha \leq 0.05$ ; \*\* significance at the level  $\alpha \leq 0.01$ ; n – non significance at the level  $\alpha \leq 0.05$ .

cobalt, copper and nickel was more stable in Albik than in Rubik cultivar; the Rubik cultivar was found to contain zinc at more stabilised concentrations (Table 3). The structure of the variance components of the content of heavy metals was complex. Years of study played the dominant role in the variation of the content of zinc, cadmium, cobalt and copper. Different weather conditions in the years of study should be regarded as the main cause of the differences in the contents of elements. The ears 2003 and 2004 were humid, whereas 2005 was droughty. The interaction of cultivars

and years proved to be the predominant source of variation in chromium and lead content, which shows that the cultivars reacted differently to different weather conditions (Table 4).

## Discussion

Being a solid fuel, and apart from reducing carbon dioxide emission to the atmosphere (closed cycle), biomass should emit minimum amounts of nitrogen and sulphur oxides, chlorine and particulate matter to the atmosphere during the process of conversion into energy. Ash from certain types of biomass melts at a burning temperature and clogs grates, which seriously hampers the entire process. The content of raw ash in the dry matter of Jerusalem artichoke, determined in the study, was equal to  $41.7 \text{ g} \cdot \text{kg}^{-1}$  on average. Similar values ( $33\text{--}40 \text{ g} \cdot \text{kg}^{-1}$  for air-dry mass of stems of Jerusalem artichoke) have been reported by Kalembasa [8], Nemeth and Izsaki [20]. The content is much higher than that given by the popular quality standard of feedstock for renewable energy production – DIN 51731 [21]. According to Harmankaya et al [16], the content of ash in the air-dry matter of the overground parts of Jerusalem artichoke is about  $56 \text{ g} \cdot \text{kg}^{-1}$  and the melting point of the ash is low ( $960 \text{ }^\circ\text{C}$ ). This may make ash components deposit more intensely on the heating surface of boilers, thereby disturbing heat exchange. There is a weak, negative correlation between ash content and the calorific value of the feedstock, because an increase in ash content in biomass is accompanied by decrease in its calorific value. The problem can be solved by applying hybrid combustion or co-combustion systems and subsequently using the resultant ash as fertiliser.

The usability of the overground parts of *Helianthus tuberosus* as feedstock for renewable energy production is determined by various factors, including *eg* mineral composition of ash. The average content of macro-elements in ash was ( $\text{g} \cdot \text{kg}^{-1}$  of dry matter): nitrogen 26.8, potassium – 32.4, phosphorus – 2.40, calcium – 13.7, magnesium – 4.56, sodium – 1.84, sulphur – 1.07, chlorine – 0.28. The chlorine content lay within the limits specified by the DIN 53731 standard [20], while that of nitrogen and sulphur slightly exceeded them. Sunab et al [22] have shown that nitrogen fertilisation at the dose of up to  $150 \text{ kg N} \cdot \text{ha}^{-1}$  results in an increase in mineral nitrogen content by 1 to  $3 \text{ g} \cdot \text{kg}^{-1}$ , and – consequently – in nitrogen oxides emission during the plant combustion process. Chlorine content in combustion feedstock is a negative factor due to the formation of hydrochloric acid or alkaline metal chlorides, which reduces the ash melting point. Chlorine in biomass is found mainly in inorganic compounds, usually in salts: NaCl and KCl [11]. A high chlorine content in biomass being burned, in combination with water and high temperature during combustion or co-combustion with coal, forms a vapour mixture of hydrochloric acid, which corrodes low-temperature boilers. According to Batorek-Giesa and Jagustyn [23], the high concentration of the element is responsible for the formation of highly toxic dioxines. Nemeth and Izsaki [20] found Jerusalem artichoke to contain  $47 \text{ g Cl} \cdot \text{kg}^{-1}$ , whereas the value found in a study by those authors was below the limits specified in the DIN 53731 standard [21]. The content of emission-significant substances in the energy plants studied by Scholz

and Ellerbrock [5] lay within a wide range ( $\text{g} \cdot \text{kg}^{-1}$  of dry matter), *eg* nitrogen – 32, potassium – 2–19, sulphur – 0.4–3.3 and chlorine – 0.1–1.6. A similar content of those substances in non-tree energy plants was found by Batorek-Giesa and Jagustyn [23] – the content of ash and sulphur lay within a wider range, while the nitrogen content was 23–53  $\text{g} \cdot \text{kg}^{-1}$  of dry matter.

The average content of heavy metals in dry matter of the overground parts of Jerusalem artichoke was equal to ( $\text{mg} \cdot \text{kg}^{-1}$ ): cadmium – 0.30, cobalt – 0.225, chromium – 3.17, copper – 8.11, nickel – 3.59, lead – 5.33. The toxic effect of heavy metals becomes manifest when their concentrations in a plant environment becomes too high [8]. Lead, chromium, cadmium and nickel are included in the list of ten main environmental pollutants in Poland [24].

According to Barta and Patkai [25], Jasiewicz and Antonkiewicz [26] heavy metals, contained in biomass, play an important role during combustion because their condensation leads to the formation of submicron particles of fly ashes (aerosols). Such particles are difficult to remove in dust filters and they pose an environmental and health hazard. The following are the contents of those elements in biofuels which ensure their problem-free use, according to the DIN 51731 standard ( $\text{mg} \cdot \text{kg}^{-1}$  of dry matter): cadmium –  $< 0.5$  of cadmium, chromium –  $< 8$ , copper –  $< 5$ , lead –  $< 10$ , zinc –  $< 100$  [21]. According to Jasiewicz and Antonkiewicz [26], the average content of heavy metals in the overground parts of *Helianthus tuberosus* increased with increasing pollution of soil with heavy metals. The content of those metals lay within the ranges ( $\text{mg} \cdot \text{kg}^{-1}$  of dry matter): Cd 0.19–29.84; Pb 2.17–19.12; Ni 1.58–40.59; Cu 4.11–25.90; Zn 15.9–222.1. This study found that the overground parts of Jerusalem artichoke contained smaller amounts of cadmium, lead and zinc than the highest acceptable concentrations (as per the DIN 51731 standard). Sat [27] evaluated the effect of some metals, such as: iron ( $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ ), cobalt ( $\text{Co}^{2+}$ ), strontium ( $\text{Sr}^{2+}$ ), zinc ( $\text{Zn}^{2+}$ ), mercury ( $\text{Hg}^{2+}$ ), nickel ( $\text{Ni}^{2+}$ ), aluminium ( $\text{Al}^{3+}$ ) and lead ( $\text{Pb}^{2+}$ ) on peroxidases in *Helianthus tuberosus*. Those are a group of oxidoreductase enzymes which catalyse oxidation of different organic and inorganic substrates with hydrogen peroxide. He found Hg, Pb, Ni, Sr, Al and Zn to significantly inhibit the production of peroxidase by Jerusalem artichoke plants. The relationship between heavy metals concentration and genetic features of plants has been confirmed by Seiler and Campbell [28] as well as Sat [27].

According to Sawicka [13], genotypic variation contributes to 46.7–98.3 % of the overall variation of chemical composition. In terms of each feature, phenotypic variation of the cultivars and wild forms of Jerusalem artichoke is the combined effect of genetic and environmental variation. Seiler and Campbell [14], Wangsomunk et al [15], MacLaurin [29] have proven that the size of the genotypic variance components of Jerusalem artichoke is very high for nitrogen, calcium and potassium and that there is considerable potential for their improvement by selection. On the other hand, the contribution of genotypic variation was very low for heavy metals, which may suggest that improvement of those features by selection will be difficult. Long et al [30], Zhang et al [31], Khan et al [32] have shown that the differences between cultivars in taking up heavy metals by Jerusalem artichoke plants stem from the diverse physiological

reactions of the plants of different sensitivity to drought caused by polyethylene glycol (PEG). The activity of antioxidative enzymes, characterised by cell membrane lipid peroxidation, has proven to be the key. The cultivars with relatively higher water status than drought-sensitive ones contained lower levels of malondialdehyde (MDA) and higher levels of free proline. Moreover, the activities of catalase (CAT) and superoxide dismutase (SOD) were higher after a period of drought. Seiler and Campbell [14] have shown the genetic variation of the main components of chemical composition of the overground parts of Jerusalem artichoke. In their opinion, high variation within the population will allow for selection of individual elements in the chemical composition, except for phosphorus.

## Conclusions

1. Considering the use of biomass of Jerusalem artichoke for energy purposes and for direct combustion, one has to take into account the need to conduct regular analyses which also include determination of heavy metals.

2. The biomass of the overground parts of Jerusalem artichoke examined in this study contained lower levels of cadmium, lead and zinc and higher levels of copper than the highest acceptable concentrations specified in the German standard DIN 51731.

3. Cultivars contributed to most part of the variation of the levels of potassium, magnesium, nitrogen, phosphorus and manganese. The dominant role in the overall variation of chlorine content was played by environmental variation, whereas for sodium, sulphur molybdenum, iron, aluminium, chromium and lead it was cultivar and year interaction.

4. In terms of each feature, the phenotypic variation of the cultivars of Jerusalem artichoke (*Helianthus tuberosus* L.) is the combined effect of genetic and environmental variation.

5. Genetic variation of the cultivars will provide the possibility of development of a strategy for improvement of the yield of Jerusalem artichoke which can be used for energy purposes.

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#### OCENA SKŁADU CHEMICZNEGO SŁONECZNIKA BULWIASTEGO (*Helianthus tuberosus* L.) JAKO SUROWCA ENERGETYCZNEGO

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**Abstrakt:** Praca dotyczy analizy zmienności składu chemicznego popiołu części nadziemnych słonecznika bulwiastego, jako odnawialnego źródła energii. Czynnikiem eksperymentu były odmiany *Helianthus tuberosus*: Albik i Rubik, uprawiane na glebie lekkiej, kompleksu żyniego dobrego, w warunkach stałego nawożenia mineralnego i pełnej dawki obornika. W popiele tych roślin oznaczano: zawartość makro- i mikroelementów oraz wybranych metali ciężkich. Zmienność fenotypowa odmian słonecznika bulwiastego, pod względem każdej cechy, była łącznym efektem zmienności genetycznej i środowiskowej. Decydujący udział odmian, w wariancji całkowitej, miała zawartość: potasu, magnezu, azotu, fosforu i manganu. Dominującą rolę w zmienności ogólnej chloru, cynku, kadmu, kobaltu i miedzi odgrywała zmienność środowiskowa, zaś w przypadku sodu, siarki, molibdenu, żelaza, glinu, chromu i ołowiu – współdziałanie odmian i lat. Biomasa części nadziemnych słonecznika bulwiastego zawierała mniejsze ilości kadmu, ołowiu i cynku niż wartości dopuszczalne, określone w niemieckiej normie DIN 51731.

**Słowa kluczowe:** słonecznik bulwiasty, odmiany, bezpieczeństwo biomasy, makroelementy, mikroelementy, metale ciężkie



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## THE APPLICATION OF EFFECTIVE MICROORGANISMS PREPARATION IN GROWING OF ZONAL PELARGONIUM (*Pelargonium zonale*)

### ZASTOSOWANIE PREPARATU EFEKTYWNE MIKROORGANIZMY W UPRAWIE PELARGONII RABATOWEJ (*Pelargonium zonale*)

**Abstract:** The aim of the conducted experiment was to evaluate dehydrogenase activity in the substrate and to determine the effect of Effective Microorganisms (EM) on the growth and flowering of zonal pelargonium. Plants were grown in pots in a greenhouse in two types of substrate. One substrate was deacidified highmoor peat and the other was highmoor peat with an addition of loam (at a 4:1 volumetric ratio). Plants were treated once with an EM preparation diluted in water at different proportions of 1:10, 1:50 and 1:100. Inoculum was administered in foliar treatment by spraying plants at 10 cm<sup>3</sup> solution per plant and to the soil at 50 cm<sup>3</sup> solution per pot. Foliar and soil application of EM had a positive effect of plant flowering. In contrast, it did not have an effect on plant height and the number of leaves.

The SPAD index and chlorophyll content in leaves depended on the method of application, concentration of applied inoculum as well as the type of substrate, in which pelargonium was grown.

Irrespective of the concentration of EM and the type of substrate inoculation of both soil and plants contributed to an increased activity of dehydrogenases.

**Keywords:** Effective Microorganism, dehydrogenases, SPAD, chlorophyll

For many years now zonal pelargonium has ranked as a leading plant used in the decoration of balconies, terraces and flower beds.

Modern horticulture in the era of strict ecological regulations strives for the limitation of chemical agents and mineral fertilisers. At the same time consumers demand purchased products to be of high quality. In case of ornamental plants only those plants are willingly purchased which are characterised by abundant flowering,

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appropriate habit and health. In pelargonium growing considerable problems are connected with the incidence of fungal and bacterial diseases. For this reason particularly such biological preparations are considered advantageous, which would have a positive effect not only on the health condition of plants, but also traits indicating their high quality. An example of such a preparation, which has attracted considerable interest, is the microbiological inoculum of EM (*Effective Microorganisms*), containing *eg* lactic acid bacteria, photosynthesising bacteria, yeasts, moulds as well as Actinomycetes [1]. As it was reported by Stielow [2], the application of EM provides several positive effects, such as *eg* reduction of putrefaction processes, better plant rooting, enhanced drought resistance, an increase in the photosynthetic effect, inhibition of pathogen development as well as more abundant flowering.

In literature information on EM concerns mainly the application of this inoculum in growing of vegetable and pomiculture crops, as well as cereals [3–6]. The primary role in the mineralisation of organic matter in a given substrate and ensuring nutrient availability for plants are attributed to microorganisms. Decomposition of organic matter in the substrate occurs at the participation of exo- and endogenous enzymes produced by microorganisms.

Dehydrogenases are endogenous enzymes, classified to the group of oxidoreductases, which oxidise organic matter by cleavage of hydrogen protons and electrons.

These enzymes are considered to be indicators of microbial activity in a given environment [7].

To date no studies have been conducted on the effect of EM on the growth and flowering of bed plants, thus the aim of the experiment was to evaluate dehydrogenase activity in the substrate as well as determine the effect of this inoculum on growth and flowering of zonal pelargonium.

## Material and methods

Rooted seedlings of zonal pelargonium (*Pelargonium zonale*) cv. 'Andria' were planted to pots of 12 cm in diameter and grown in a greenhouse. In terms of the type of substrate plants were divided into two groups. The first comprised plants grown in a peat substrate, while the other group was grown in a peat substrate with an addition of loam (at a volumetric ratio of 4:1). Schema of experiments show Table 1.

After planting plants were treated once with an EM preparation diluted in water at different proportions of 1:10, 1:50 and 1:100. The control comprised plants not treated with the inoculum. The microbial inoculum was administered in a foliar application by spraying plants with a dose of 10 cm<sup>3</sup> solution per plant as well as applied to the soil at 50 cm<sup>3</sup> solution per pot.

When the first flowers appeared on plants, measurements were taken concerning the following traits: plant height, the number of leaves, the SPAD index using an N-Tester apparatus, as well as the number of inflorescences buds and inflorescences and the length of the peduncle.

Substrate samples for the evaluation of dehydrogenase activity were collected at three dates: after seedling planting, during vegetative growth and during flowering.

Table 1

## Schema of experiments

Substrate	Combinations	Application of EM
Peat	K	0
	I	watering 1:10
	II	watering 1:50
	III	watering 1:100
	IV	spraying 1:10
	V	spraying 1:50
Peat with loam	VI	spraying 1:100
	K1	0
	I1	watering 1:10
	II1	watering 1:50
	III1	watering 1:100
	IV1	spraying 1:10
	V1	spraying 1:50
VII	spraying 1:100	

Dehydrogenase activity was determined by spectrophotometry using 1 % TTC (triphenyltetrazolium chloride) as a medium, after 5-h incubation at a temperature of 30 °C, at a wavelength of 485 nm. Enzyme activity was expressed in  $\mu\text{mol TOF} \cdot \text{g}^{-1} \cdot \text{d.m. substrate} \cdot \text{h}^{-1}$  [8]. The statistical analysis applied in this experiment was performed using the Statistica 8.0 programme [9].

After the measurements had been taken, leaf samples were collected in order to determine chlorophyll content according to a modified method by Shoaf and Lium [10] with extraction of dimethyl sulfoxide (DMSO). Absorbance of the extract was measured at wavelengths of 645, 652 and 663 nm and next chlorophyll content was measured using the Arnon formula [11].

The experiment comprised 14 combinations (type of substrate  $\times$  method of EM application) with 10 replications, with one plant constituting a replication.

Results were subjected to the analysis of variance and means were grouped using the Duncan test at the significance level  $\alpha = 0.05$ .

## Results and discussion

On the basis of the conducted analyses, irrespective of the method of application and EM concentration, or the type of substrate, no significant effect was found on such traits as plant height, length of peduncle or the number of leaves. In contrast, an advantageous effect of effective microorganisms was observed on flowering of pelargonium (Table 2). In case of plants grown in the peat substrate the highest number of inflorescence buds in relation to the control was recorded for plants, which were watered and sprayed with an EM solution at a concentration of 1:50 and 1:100. In turn, in the substrate composed of peat and loam only plants sprayed with an EM solution at

Table 2

## Effect of EM on morphological traits

Treatments of EM	Plant height [cm]		Length of the inflorescens peduncle		Number of inflorescences buds		Number of inflorescences	
	peat	peat + loam	peat	peat + loam	peat	peat + loam	peat	peat + loam
Control	16.5 a	16.5 a	17.8 ab	20.1 c	0.9 a	1.3 b	2.9 a	2.8 a
Watering 1:10	17.5 a	18.1 a	20.5 ab	21.9 d	1.1 a	1.0 a	2.7 a	3.9 c
Watering 1:50	14.2 a	17.9 a	20.8 b	19.3 b	1.6 c	1.0 a	4.2 c	3.5 b
Watering 1:100	15.1 a	16.5 a	17.5 a	18.1 a	1.5 c	1.2 b	2.9 a	3.0 a
Spraying 1:10	17.6 a	17.4 a	20.6 ab	19.6 b	1.1 a	1.5 c	3.3 b	2.3 a
Spraying 1:50	17.0 a	17.5 a	19.7 ab	19.2 b	1.2 b	0.9 a	3.4 b	3.5 b
Spraying 1:100	17.0 a	18.0 a	20.2 ab	20.3 c	1.5 c	1.3 b	4.2 c	3.0 a

Means followed by the same letter do not differ significantly at  $\alpha = 0.05$ .

a concentration of 1:10 formed more inflorescence buds. In the combinations, in which plants were watered and sprayed with a solution at a concentration of 1:100, statistical analysis did not show significant differences between EM-treated plants and the control. In the other combinations the application of the microbial inoculum had a disadvantageous effect on the analysed trait.

The EM microbial inoculum had a significant effect on the number of formed inflorescences. Plants grown in the peat substrate watered with an EM solution at a concentration of 1:50 and sprayed with a solution at a concentration of 1:100 formed by 44 % more inflorescences. Significant differences were not observed only in the combinations, in which the inoculum was applied to the soil at a concentration of 1:10 and 1:100.

Plants grown in the substrate with an addition of loam formed more inflorescences thanks to the soil application of EM at a concentration of 1:10 and 1:50 and spraying with a solution at a concentration of 1:50.

Literature contains little information on the effect of EM on flowering in ornamental plants. The positive effect of effective microorganisms applied to the soil on the formation of shoots and diameter of flowers in roses and the number of inflorescences in gerberas is confirmed by studies by Gorski and Kleiber [12]. Those authors also observed a positive effect of EM after foliar application on the diameter of flowers in rose and the number of formed inflorescences and leaves in gerberas. Moreover, studies were also conducted on the effect of EM in the growing of saffron (*Crocus sativus*), from which it results that the use of inoculum increases the number of forming bulbs as well as fresh and dry weight of stigmas [13]. A favourable impact of microbiological inoculums on the flowering of scarlet sage was reported by Wolna-Maruwka et al [14]. As it was reported by Abd et al [15], EM treatment of dateplum persimmon (*Diospyros kaki*) applied to the soil and leaves markedly increases yields as well as fruit weight in comparison to control plants.

Effective microorganisms had a varied effect on the SPAD index and chlorophyll content in leaves (Table 3).

Table 3

Effect of EM on number of leaves, greening index of leaves (SPAD) and chlorophyll content

Treatments of EM	Number of leaves		Greening index of leaves (SPAD)		Chlorophyll content <i>a + b</i>	
	peat	peat + loam	peat	peat + loam	peat	peat + loam
Control	32.6 b	30.0 a	47.4 b	46.7 b	2.5 a	2.9 b
Watering 1:10	31.2 ab	28.3 a	46.1 ab	44.9 ab	2.9 b	2.3 a
Watering 1:50	30.5 ab	29.2 a	43.2 ab	48.0 c	2.6 a	2.4 a
Watering:100	27.7 a	29.4 a	47.8 b	48.7 c	2.5 a	2.6 a
Spraying 1:10	31.2 ab	28.6 a	47.5 b	45.1 b	3.0 b	1.6 a
Spraying 1:50	28.7 a	29.4 a	47.9 b	42.0 a	3.2 b	2.2 a
Spraying 1:100	29.5 ab	29.8 a	44.3 a	41.0 a	2.6 a	2.6 a

Means followed by the same letter do not differ significantly at  $\alpha = 0.05$ .

In the conducted experiment the SPAD index was not a reflection of chlorophyll content in leaves. The statistical analysis of chlorophyll content in leaves showed significant differences between control plants grown in peat and watered with an EM solution at a concentration of 1:10 and sprayed with a solution at a concentration of 1:10 and 1:50. In the other combinations no effect of EM was observed on the content of this form of chlorophyll in leaves.

In turn, an analysis of the SPAD index did not show differences between control plants and plants treated with EM.

The microbial inoculum when applied on plants grown in the peat substrate with an addition of loam had a different effect on the investigated traits. In this case the SPAD index was higher in plants watered with an EM solution at a concentration of 1:50 and 1:100. This was not confirmed by analyses of chlorophyll content in leaves. After inoculation plants were characterised by a lower content of this pigment in comparison to the control plants. A higher chlorophyll content after treatment of plants with the EM inoculum in comparison to plants not subjected to such treatment was confirmed by studies by Khan et al [16]. Also studies by Xu [17], Wang et al [18] and Mridha et al [19] show that the application of EM increases the activity and efficiency of photosynthesis and thus it leads to improvement of plant quality and higher yields.

When analysing changes in the level of dehydrogenase activity in the successive development phases of pelargonium (Fig. 1) it was stated that irrespective of the method and concentration of the applied inoculum the activity of dehydrogenases increased gradually in the course of the experiment, reaching maximum values at the phase of plant flowering (date III). The above phenomenon was most probably connected with the presence of root exudates rich in proteins, sugars, amino acids, vitamins, etc. In the opinion of Wielgosz and Szember [20], at the plant flowering phase the composition of root exudates changes; additionally, their amount excreted to the substrate increases as well.

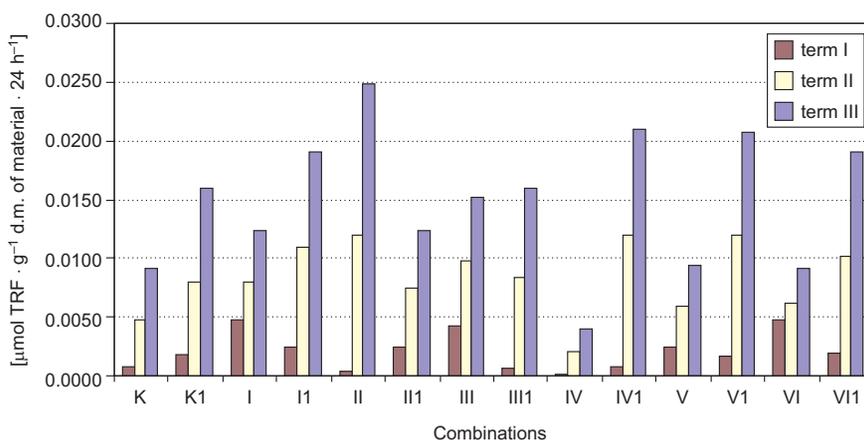


Fig. 1. The activity of dehydrogenases:  $LSD_{0.05} = 0.004$  (for term I);  $LSD_{0.05} = 0.006$  (for term II);  $LSD_{0.05} = 0.008$  (for term III)

On the basis of obtained results of analyses it was also stated that irrespective of the dose of the applied EM preparation, inoculation of both soil and plants contributed to an increase in dehydrogenase activity. However, the conducted two-way analysis of variance showed that in many cases differences in the activity of the discussed enzymes between the control and treated plants were statistically non-significant.

In turn, it results from a study by Wielgosz et al [21] that after two months of the experiment duration the level of dehydrogenase activity in soil inoculated with an EM preparation in wheat culture remained at the level very close to that in the control soil – with no EM inoculum added.

On the basis of the investigations conducted by the author of this study it was stated that the strongest effect on an increase in the activity of the discussed oxidoreductases was recorded for the inoculation of peat substrate with an EM preparation at a 1:50 dose. In turn, the weakest effect on the biochemical activity of the substrate was found for the spraying of pelargoniums grown in the peat substrate using an EM inoculum at a dose of 1:10.

## Conclusions

Foliar and soil application of EM had a positive effect on plant flowering. The EM preparation did not have an effect on plant height or the number of leaves. The SPAD index and chlorophyll content in leaves depended on the method of application, concentration of the applied inoculum as well as the type of substrate, in which pelargonium was grown. Irrespective of EM concentration and the type of substrate, inoculation of both soil and plants contributed to an increased dehydrogenase activity. The highest dehydrogenase activity was reached in the flowering phase of pelargonium.

## Acknowledgements

Our study was supported by the Ministry of Science and Higher Education, grant No. N N310444938.

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## ZASTOSOWANIE PREPARATU EFEKTYWNE MIKROORGANIZMY W UPRAWIE PELARGONII RABATOWEJ (*Pelargonium zonale*)

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**Abstrakt:** Celem przeprowadzonego doświadczenia była ocena aktywności dehydrogenazy w podłożu, a także określenie wpływu Efektywnych Mikroorganizmów (EM) na wzrost i kwitnienie pelargonii rabatowej. Rośliny uprawiano w doniczkach, w szklarni w dwóch rodzajach podłoża. Pierwszym podłożem był odkwaszony torf wysoki, a drugim – torf wysoki z dodatkiem glinki (w stosunku objętościowym 4:1). Rośliny jednorazowo potraktowano preparatem EM rozcieńczonym w wodzie w różnych proporcjach 1:10, 1:50 i 1:100. Szczepionkę aplikowano dolistnie, opryskując rośliny dawką 10 cm<sup>3</sup> roztworu na roślinę i doglebowo w dawce 50 cm<sup>3</sup> roztworu na doniczkę. Dolistna i doglebowa aplikacja EM wywarła pozytywny wpływ na kwitnienie roślin. Nie miała natomiast wpływu na wysokość roślin i liczbę liści.

Indeks zazielenienia liści oraz zawartość chlorofilu w liściach zależała od sposobu aplikacji, stężenia zastosowanej szczepionki a także od rodzaju podłoża, w którym uprawiana była pelargonie.

Niezależnie od stężenia EM i rodzaju podłoża inokulacja zarówno gleby, jak i roślin przyczyniła się do zwiększenia poziomu aktywności dehydrogenaz.

**Słowa kluczowe:** Efektywne Mikroorganizmy, dehydrogenaza, SPAD, chlorofil

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## EFFECT OF STRESS CONDITIONS ON THE ANTIOXIDANT PROPERTIES OF GERMINATING RADISH SEEDS

### WPLYW WARUNKÓW STRESU NA WŁAŚCIWOŚCI ANTYOKSYDACYJNE KIELKUJĄCYCH NASION RZODKIEWKI

**Abstract:** During their growth and development plants are exposed to various types of stress, including drought, temperatures that are too high or too low, or air pollution. This study evaluated the antioxidant properties of extracts from seeds germinating in stress conditions. Radish seeds (*Raphanus sativus* var. *sativus*) of the variety Szkarłatna were used for the study. This is a popular radish, whose edible part is round and red with a white tip. The experiment was conducted in three variants: radish seeds were subjected to heat shock, salt stress and stress induced by acrolein. The antioxidant activity of the aqueous extracts from the radish seeds or sprouts was measured using the ABTS and DPPH methods. The effect of temperature shock was manifested as a decrease in total antioxidant capacity. Salt stress in the seedlings was manifested as a decrease in total antioxidant capacity with respect to the control only in the case of the highest NaCl concentration applied (200 mM). Where 50 mM and 100 mM concentrations of the NaCl solution were applied, an increase in total antioxidant capacity was observed. The changes in total antioxidant capacity induced by acrolein in the germinating radish seeds were less pronounced than in the case of the salt or temperature stress.

**Keywords:** total antioxidant capacity, radish, heat shock, salt stress, stress induced by acrolein

## Introduction

During their growth and development plants are exposed to various types of stress, such as drought, temperatures that are too high or too low, ultraviolet radiation, air pollution, or pathogens. According to the definition proposed by Lichtenthaler, stress in plants refers to unfavourable conditions or substances that affect or block the growth, development or metabolism of the plants [1]. Stress factors need not have lethal effects on the organism, but they induce a defence reaction. During their growth and

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development most plants suffer physiological and biochemical damage due to stress. Stress conditions can lead to the generation of *reactive oxygen species* (ROS), which cause damage to proteins, lipids, carbohydrates, and DNA. Over the course of their evolution plants, like other organisms, have developed defence mechanisms which protect them against ROS. Enzyme antioxidant protection in plants involves the activity of such enzymes as superoxide dismutase, catalases, ascorbate peroxidase, glutathione reductase, glutathione peroxidase, glutathione S-transferase, and others. Also taking part in antioxidant protection is a large group of antioxidants (ascorbic acid, ASH, glutathione, GSH, phenolic compounds, alkaloids, non-protein amino acids and tocopherol). The enzyme and non-enzyme systems of antioxidant defence work together to protect the cell against oxidative damage [2].

The literature provides many examples of research on various types of stress in plants, particularly in their adult forms. Investigating stress in germinating seeds opens up many new research possibilities. Dormant seeds, protected by their seed coats, are exceptionally resistant to stress factors that can be lethal for adult plants. Seeds in the dry state have been shown to produce reactive oxygen species [3]. ROS can react in a non-specific manner with all of the types of molecules from which seed structures are built. In seeds, as in adult plants, antioxidant enzyme activity has been detected. We also know that oxidation of proteins in seeds can also facilitate the onset of germination. The concentration of superoxide anion radical and hydrogen peroxide has been shown to increase as soon as maturity was attained in the sunflower embryo. Accumulation of ROS was accompanied by an increase in lipid peroxidation and in the concentration of carbonyl groups in specific proteins [4]. Thus ROS, particularly  $H_2O_2$ , may play an important role in cell signalling.

A study on ageing in onion seeds confirmed the occurrence of oxidative stress; a decrease in the activity of the enzymes SOD and CAT was observed, as well as an increase in lipid peroxidation and accelerated ageing [5]. In wheat *Triticum aestivum* seeds the level of antioxidant enzymes (catalase, peroxidase, dehydrogenase) was found to decrease with storage time. The same effect was induced by conditions causing accelerated ageing in the seeds [6].

Seed germination is a complex process during which a high metabolism rate is rapidly restored; the biochemical and structural integrity of the cells is restored, enabling the seedlings to grow. Even the seed swelling process entails the possibility of stress caused by the considerable increase in turgor pressure in the cell. The processes of repair and replacement of organelles, *eg* mitochondria, during germination have been described [7].

Reactive oxygen species are known to take part in regulating the metabolism of seeds during various stages of their development [8]. Oxidative signalling is a term referring to the functions of ROS in cells, which depending on their concentration can either be signalling molecules determining successive physiological stages in the seeds or exert harmful effects [3].

Increased hydrolytic enzyme activity, increased water intake, activation of reserve substances accumulated in the seeds, and the interruption of dormancy are the consequence of metabolic conversions induced by free radicals [9]. Seeds can be

subjected to various types of stress in order to interrupt their dormancy and accelerate germination. Laser light stimulates earlier germination, growth and development of plants, which is explained as the effect of free radicals [10]. There are many ways to measure the intensity of the response of plants to stress. This study proposes two methods for measuring total antioxidant capacity to assess the response of germinating seeds to selected stress factors.

The aim of the study was to evaluate the antioxidant properties of extracts of radish seeds germinating in conditions of salt stress, stress induced by acrolein, and stress induced by high temperature.

## Materials and methods

### Materials

#### Chemicals

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (*ABTS*), 1,1-diphenyl-2-picrylhydrazyl (*DPPH*) were purchased from Sigma; trolox from Aldrich; potassium persulfate ( $K_2S_2O_8$ ) from Sigma-Aldrich; and ethanol from POCH. All chemicals and reagents were analytical grade or purest quality.

#### Plant material and extract preparation

Radish seeds *Raphanus sativus* var. *sativus* of the variety Szkarłatna were used for the study. This is a popular radish with a good flavour, whose edible part is round and red with a white tip. The experiment was conducted in three variants: the radish seeds were subjected to thermal shock, salt stress and stress induced by acrolein. The seeds to be subjected to thermal stress were first rinsed in sterile water and then soaked for 3 hours. Then they were dried on filter paper and placed on 15-cm-diameter Petri dishes lined with filter paper, in the amount of 1 gram each. The seeds were placed in the dark for 4, 8 or 12 hours at 45 °C. After 4, 8 and 12 hours they were watered and left at room temperature in natural light conditions. The remaining seeds were placed on 15-cm-diameter Petri dishes lined with filter paper, in the amount of 1 g each. Solutions containing different concentrations of sodium chloride or acrolein were poured in identical amounts on some of the seeds. The aqueous sodium chloride solution was applied at concentrations of 50 mM, 100 mM and 200 mM, and the aqueous acrolein solution at 2.5 mM, 5 mM and 10 mM. A control sample was prepared at the same time. The seeds germinated in natural light conditions at room temperature and were watered with identical amounts of water each day. Material was collected for analysis 72 hours after the beginning of the experiment.

To prepare the extracts, 1 gram of seedlings without seed coats was used for each extract. To determine the initial (control) antioxidant potential of the seeds, extracts were prepared from the seeds. The seedlings or seeds were homogenized with 10 cm<sup>3</sup> chilled distilled water (4 °C), and the homogenate was centrifuged. All of the extracts were prepared in three replications.

## Methods

### Determination of total antioxidant capacity

The total antioxidant capacity of the radish seedling extracts was determined by two spectrophotometric methods – the method proposed by Brand-Williams et al [11] using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, and the method by Re et al., with modifications by Bartosz, using the ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation [12, 13].

The ABTS method for determining antioxidant activity is based on the reaction of ABTS<sup>+</sup> with antioxidants present in the extract, which is accompanied by a decrease in the intensity of the colour of the solution. A solution of ABTS radical cation was prepared by means of potassium persulfate oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt. The assay was performed by adding ABTS radical cation solution to 50 cm<sup>3</sup> of an aqueous solution of radish seedlings diluted 10-fold. Absorbance was measured 30 minutes after the reaction was initiated at 414 nm. A control sample was prepared by adding water to ABTS radical cation solution.

In the DPPH method, the stable DPPH radical captures an electron from the antioxidant. As a consequence of this reaction the ethanol solution of DPPH loses its intense violet colour. The method is based on measurement of the decrease in absorbance at 517 nm. The DPPH solution was prepared by dissolving DPPH in an amount of ethanol resulting in absorbance of about 1.0. The assay was performed by adding 1,500 cm<sup>3</sup> ethanol solution of DPPH to each 50 cm<sup>3</sup> sample of seedling extract. The decrease in absorbance was measured with respect to the control (DPPH + water) 30 minutes after the reaction was initiated.

All determinations were made in at least three independent replications. For both methods antioxidant content was expressed as μM of trolox per 1 g dry weight.

## Results and discussion

The antioxidant activity of the aqueous extracts from the radish seeds or sprouts was measured by the ABTS and DPPH methods. Because these methods use specific indicators, the results of the measurements differ, despite the fact that antioxidant content is expressed as trolox equivalent (Table 1). Low antioxidant content was observed in the extracts prepared from the seeds. During the germination process the total antioxidant capacity in the control seedlings increased nearly 4-fold in the ABTS method and over 14-fold in the DPPH method in comparison with the total antioxidant capacity of the dry seeds. The seedlings for further analysis were collected 72 hours after sowing. This growth time was selected because optimum properties for consumption are attained at this time, according to some producers of seeds for sprouts. Other authors also recommend consuming radish sprouts 3 to 5 days after sowing [14].

Table 1

Total antioxidant capacity measured by the ABTS and DPPH methods

Experimental conditions	ABTS [ $\mu\text{M} \cdot \text{l g}^{-1} \text{ d.m.}$ ]	DPPH [ $\mu\text{M} \cdot \text{l g}^{-1} \text{ d.m.}$ ]
Seeds	67.8 $\pm$ 1.14	1.23 $\pm$ 0.04
Control seedlings	256.93 $\pm$ 31.56	17.56 $\pm$ 2.44
NaCl 50 mM	329.47 $\pm$ 28.31	22.32 $\pm$ 1.76
NaCl 100 mM	275.95 $\pm$ 13.71	18.04 $\pm$ 0.74
NaCl 200 mM	209.00 $\pm$ 2.48	15.47 $\pm$ 7.01
Acrolein 2.5 mM	289.07 $\pm$ 13.73	16.41 $\pm$ 0.69
Acrolein 5 mM	277.44 $\pm$ 18.00	16.95 $\pm$ 0.62
Acrolein 10 mM	264.62 $\pm$ 25.68	16.49 $\pm$ 2.41
4 hours of thermal shock	242.22 $\pm$ 12.70	18.04 $\pm$ 0.48
8 hours of thermal shock	239.76 $\pm$ 13.69	17.09 $\pm$ 1.96
12 hours of thermal shock	220.45 $\pm$ 3.77	15.81 $\pm$ 0.62

Seed sprouts contain greater quantities of vitamins, minerals and antioxidants than seeds [15]. Germination has been found to increase the nutritional value of broccoli seeds. Ascorbic acid content in broccoli seeds was relatively low (18.6  $\mu\text{g/g}$ ), but following germination it increased 40 times in the sprouts (754.5  $\mu\text{g/g}$ ) converted to dry weight [16]. Extracts from red cabbage and white mustard sprouts were confirmed to be a rich source of antioxidants, while the extract from cardamine sprouts had the lowest antioxidant properties [17]. Radish seedlings, like those of other plants of the Cruciferae family, are highly recommended as a source of natural antioxidant compounds providing protection against lifestyle diseases [18]. They contain typical antioxidants as well as secondary metabolites with antioxidant properties: glucosinolates, quercetin, isothiocyanates, and organic selenium compounds [19, 20].

In the present study the effect of temperature stress (Table 1) was manifested as a decrease in total antioxidant capacity of about 6, 7 and 14 % as measured by the ABTS method (4, 8 and 12 hours of thermal shock) and about 3 and 10 % in the DPPH method (for 8 and 12 hours of thermal shock). After 4-hour temperature stress the total antioxidant capacity measured by the DPPH method was virtually unchanged.

The total antioxidant content in the extract decreases, but the concentration of particular antioxidant compounds can decrease or increase, *ie* vitamin content increases [21]. Antioxidants belong to different classes of chemical compounds, and their ability to react with radicals varies as well. This has been confirmed experimentally [22] in a comparison of the ability of identical amounts of 1 mM antioxidant solutions to react with DPPH. Ascorbic acid and ascorbyl palmitate had similar properties, glutathione had lower antioxidant capacity than vitamin C, while the amino acid L-cysteine had the lowest capacity to react with the DPPH radical.

Other authors emphasize that young alfalfa, broccoli and radish sprouts have high phenolic compound content and high antioxidant properties that decrease sharply with growth time [23]. The response of the antioxidant system in plants depends on the type

and level of the stress they are subjected to. High levels of irradiation or cooling increase the total content of phenolic compounds and antioxidant capacity in cruciferous plant seedlings in comparison to control samples [24]. Laser light exerts a similarly positive effect on seeds, but this reaction too depends on the type of seeds. Content of ascorbic acid and phenolic compounds has been found to decrease in broccoli sprouts subjected to electron beam and gamma radiation. The authors suggest that this is caused by the reaction of the sprouts to radiation-induced stress [24].

The salt stress to which the seedlings were exposed was manifested as decreased total antioxidant capacity in relation to the control seedlings only in the case of the highest of the concentrations applied (Table 1). Total antioxidant capacity decreased by nearly 19 % as measured by the ABTS method, and by about 12 % in the DPPH method. For the 50 mM and 100 mM concentrations of the NaCl solution, an increase in total antioxidant capacity was observed. The reaction of plants to salinity is an individual trait. Resistance to salinity varies both among species and among varieties. Although salinity negatively affects the growth and development of plants, other authors have demonstrated that relatively low concentrations of NaCl can stimulate plant growth. Matuszak et al found that low concentrations of NaCl increased the fresh and dry weight of the above-ground part and roots of wheat seedlings of the Almari variety and stimulated the growth of the above-ground part and roots of barley seedlings of the Sigra variety [25, 26]. As the concentration of NaCl in the medium increases, germination capacity is reduced and the biomass of the plants increases more slowly in comparison with the control. These authors also observed that low concentrations of NaCl in the medium stimulated germination capacity and growth in wheat seedlings of the Roma variety with respect to the control solution [27]. Moreover, according to literature data, salinity in the soil can sometimes positively affect yield, yield quality, and the plant's resistance to disease [28].

In general, salinity reduces seed germination by inhibiting water intake and through the toxic effects of excess  $\text{Na}^+$  and  $\text{Cl}^-$  ions. An excessive concentration of salt in the soil leads to physiological drought – a state in which plants, despite the presence of moisture in the soil, are unable to take up water. Furthermore, salinity reduces the photosynthesis rate and may negatively affect the content of photosynthetic pigments (chlorophylls and carotenoids). Salinity also induces changes in metabolism in plants; for example, protein is accumulated. Changes also take place in mineral metabolism and antioxidant enzyme activity. Changes induced by salt stress can lead to reductions in yield size and quality.

Plants are sensitive in varying degrees to soil salinity. Most susceptible are the youngest plants; as they grow, their resistance to salt in the soil increases. Most ornamental plants and fruit-bearing bushes are sensitive to salinity, while among vegetables, the strongest reactions are noted in radish, lettuce, celery, bean, and broad bean.

Most utilitarian plant species are sensitive even to relatively low salinity, which reduces the accessibility of water, lowering its potential in the soil solution. Moreover, an excess of ions, particularly  $\text{Na}^+$  and  $\text{Cl}^-$ , interferes with cellular ion metabolism and can induce oxidative stress [29]. Salinity reduces the dry weight of plants and decreases the height and number of leaves even at a concentration of 35 mM NaCl [30].

Acrolein [31] is an aldehyde formed as the result of heat treatment of plant and animal fats, carbohydrates, and amino acids. Its cytotoxic activity mainly involves conjugation with glutathione, one of the main cellular antioxidants. The changes in total antioxidant capacity induced by acrolein in the germinating radish seeds were not as pronounced as in the case of salt or temperature stress (Table 1). If the total antioxidant capacity of the control seedling extract is taken to be 100 %, then the total antioxidant capacity of the radish seedling extracts exposed to acrolein was 113, 108 and 103 % for concentrations of 2.5, 5 and 10 mM in the ABTS method, and 93, 97 and 94 % for concentrations of 2.5, 5 and 10 mM in the DPPH method (Table 1).

Acrolein reduces the germination capacity of wheat seeds [32]. Germination of wheat and the length of its embryo decreased following exposure to acrolein [33]. Research on the effect of acrolein on seed germination has a practical aspect, because it is used as a pesticide during seed storage.

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## WPLYW WARUNKÓW STRESU NA WŁAŚCIWOŚCI ANTYOKSYDACYJNE KIEŁKUJĄCYCH NASION RZODKIEWKI

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**Abstrakt:** Podczas procesu wzrostu i rozwoju rośliny narażone są na różnego rodzaju stresi m.in.: suszę, zbyt wysoką lub za niską temperaturę czy zanieczyszczenie powietrza. W prezentowanej pracy oceniono właściwości antyoksydacyjne ekstraktów z nasion kiełkujących w warunkach stresu. Do badań zastosowano nasioną rzodkiewki (*Raphanus sativus* var. *sativus*) odmiana Szkarłatna z białym końcem. Doświadczenie wykonano w trzech wariantach, nasioną rzodkiewki poddano szokowi termicznemu, stresowi solnemu oraz stresowi wywołanemu akroleiną. Aktywność przeciwutleniającą wodnych ekstraktów z nasion lub z kiełków rzodkiewki mierzono metodą ABTS i DPPH. Wpływ stresu temperaturowego przejawiał się spadkiem całkowitej zdolności antyoksydacyjnej. Stres solny, któremu poddano siewki, przejawiał się obniżeniem

całkowitej zdolności antyoksydacyjnej względem siewek kontrolnych jedynie przy najwyższym z zastosowanych stężeń NaCl (200 mM). W przypadku zastosowania 50 mM i 100 mM stężenia roztworu NaCl zaobserwowano wzrost całkowitej zdolności antyoksydacyjnej. Podczas działania akroleiny na kiełkujące nasiona rzodkiewki nie stwierdzono tak znaczących zmian całkowitej zdolności antyoksydacyjnej, jak w przypadku stresu solnego czy temperaturowego.

**Słowa kluczowe:** całkowita zdolność antyoksydacyjna, rzodkiewka, szok termiczny, stres solny, stres wywołany akroleiną



Stanislaw Z. LABUDA<sup>1</sup>

## CONSIDERATIONS REGARDING OF THE STATISTICAL CALCULATIONS WITH THE pH INDEX IN SCIENTIFIC RESEARCHES

### ROZWAŻANIA DOTYCZĄCE STATYSTYCZNYCH OBLICZEŃ ZE WSKAŹNIKIEM pH W BADANIACH NAUKOWYCH

**Abstract:** Considerations were conducted in connection with incorrect way of statistical calculations of experimental data with the pH index as well as with the improper interpretation of results with the pH index in numerous scientific publications which were published in different serious and well-known scientific publishing houses. Statistical analysis of experimental data can be done when the values of the data are expressed in the units of the uniform scale.

**Keywords:** pH index, statistical analysis

Considerations were based on simple thesis that the general way of the statistical calculation of experimental data of values pH index as well as the presentation of results the pH index in many scientific publications were sometimes completely incorrect. It this was shown based on numerous examples of different well-known scientific publishing houses. The problem with incorrect statistical calculations of pH values as experimental data, was noticed many years ago and it was published [1, 2], and next, reminded about this that as before the statistical calculations with values of the pH index as experimental data were counted and interpreted incorrectly [3]. The pH index is based on traditional and modern scientific views which very well define on mathematical and chemical of the understanding of the problem as methods of calculations and interpretations [4]. However, there are simple and accepted ideas on the pH index, but any simplified assumption should be made, so a general assumption let will be that  $\text{H}_2\text{O}$  dissociation will be expressed as  $\text{H}_2\text{O} + \text{H}_2\text{O} = [\text{H}_3\text{O}^+] + [\text{OH}^-]$ , and the pH index defined as the  $\text{pH} = -\log_{10} [\text{H}_3\text{O}^+] \text{ dm}^{-3}$  for  $[\text{H}_3\text{O}^+] = \text{mol} [\text{H}_3\text{O}^+] \text{ dm}^{-3}$ , and only the acceptable simplification are accepted, where  $[\text{H}_3\text{O}^+]$  may be expressed as

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$[H^+]$ , then definition of the and notation it is  $pH = -\log_{10} [H^+]$ , and  $[H^+] = \text{mol } [H^+] \text{ dm}^{-3}$ , and  $[H^+] = 10^{-pH}$ .

## Considerations and reflections

In the meantime just still published were works with inappropriate statistical calculations with the pH index and this can be shown on numerous publications in well-known scientific publish house. Further studies and observations indicated however that for many years the number of the publication with statistical miscounts the pH index considerably had increased, therefore submits this essay under the consideration to all whom this may concern (Table 1).

Table 1

Example-publications with inappropriate statistical calculations with the pH index

Journal	Author according to year issue	Characteristic*
Soil Sci.	Carter [5]	AVt, Cor, REq
Plant and Soil	Bertrand et al [7]	Men, StD, $\Delta$
Can. J. Soil Sci.	Zebarth et al [18]	AVt
New Zeal. J. Agric. Res.	McDowell and Monaghan [19]	Men, CIn, AVt
Science	Jackson et al [20]	Men, StD
Soil Till. Res.	Sharma et al [21]	AVt
Agron. J.	Staggenborg et al. [22]	Men, StD, REq
Europ. J. Soil Sci.	Viscarra Rossel et al [23]	Men, Med, StD
Soil Biol. Biochem.	Aciego Pietri and Brookes [24]	REq
J. Environ. Qual.	Larney et al [25]	Men, StD, AVt, REq
Commun. Soil Sci. Plant Anal.	Unger et al [26]	Men, Med, Per
Catena	Gomez et al [27]	PCA
Ecological Indicators	Lagomarsino et al [28]	Men, StD, AVt
Appl. Geochem.	Pelfrene et al [29]	Men, Med
Soil Sci. Soc. Am. J.	Rabenhorst [6]	REq
Agric. Ecosys. Environ.	Tanaka et al [30]	CIn, AVt
Applied Soil Ecology	Fernandez-Calvino et al [31]	Men, StD, Cor
Geoderma	Harmand et al [14]	REq
European J. Soil Biol.	Fterich et al [32]	AVt
J. Hydrol.	Lebron et al [33]	Men, StD
Pedosphere	Xian-Li et al [34]	Men, Med

\* Statistical analysis: Men – Mean, Med Median, StD – Standard deviation, AVt – Analysis of variance and significance test, CIn – Confidence interval, REq – Regression equation, Cvr – Coefficient of variation, Cor – Correlation coefficient, Per – Pearson correlation coefficient, PCA – Principal component analysis.

In the statistical analysis is lots of methods of the calculation of experimental data, but all statistical methods based are on basic characteristics as sample size, arithmetic mean, and variance, but, however a most important rule in the statistical analysis this is,

that distribution of experimental data it was the normal distribution. Whereas enough common are applied inadequate calculations with the pH index, because often it is unremembered that the pH index is after all in the logarithmic scale. It is easy to see how are differences between the data in the pH index in the logarithmic scale, and converted data and expressed in  $[H^+]$  as  $\mu\text{mol } [H^+] \text{ dm}^{-3}$  in the uniform scale (Table 2).

Table 2

The comparison of arithmetic mean in commonly used numerical scales

Feature	Numerical value											Mean n = 11
Uniform scale	0	1	2	3	4	5	6	7	8	9	10	5
Logarithmic scale	$10^0$	$10^1$	$10^2$	$10^3$	$10^4$	$10^5$	$10^6$	$10^7$	$10^8$	$10^9$	$10^{10}$	1010101010
pH in logarithmic scale	0	1	2	3	4	5	6	7	8	9	10	0.99563519
$[H^+]$ in uniform scale	$10^{-0}$	$10^{-1}$	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$	$10^{-7}$	$10^{-8}$	$10^{-9}$	$10^{-10}$	0.10101010

In paper of Carter [5] were given the results of pH index for 6 treatments in the range pH 5.5 – pH 7.9, and also value the LSD as 0.1. This results may be good example in considerations of statistical calculations and interpretation because this is of course challenging, namely, what does this indicate the LSD 0.1 as value of the pH index. And it is even sure, that value the 0.1 it is not value the pH 0.1. Because values the pH 5.5 and the pH 7.9 is greater really 251 times, and it is not 1.4 times. And this can also be noticed for example that value the pH 4 it is  $0.0001 \text{ mol } [H^+] \text{ dm}^{-3}$ , and the value the pH 8 is  $0.0000001 \text{ mol } [H^+] \text{ dm}^{-3}$ , so the difference between pH 4 and pH 8 is just the 10000 times, but not 4 times, indeed. As well as it is sure that for data of value pH 4 and pH 8 the mean value is the pH 4.3. So, therefore this is sure, that cannot be any constant value differences between values of pH index in the statistical considerations with pH index. Since the mean value of the pH index by conversion values from the pH index to  $[H^+]$  but this anyway one cannot at all reasonably express as value the LSD in the pH index, it is sure (Table 3).

Table 3

The comparison of statistical calculations of number data and data as the pH index

Item	Value as pH index after [5]	Value of pH index	Number value on base pH index $\text{mol } [H^+] \text{ dm}^{-3}$	Number value on base pH index $\mu\text{mol } [H^+] \text{ dm}^{-3}$
Data 1	5.5	pH 5.5	0.0000031623	3.1623
Data 2	5.9	pH 5.9	0.0000012589	1.2589
Data 3	6.2	pH 6.2	0.0000006310	0.6310
Data 4	7.1	pH 7.1	0.0000000794	0.0794
Data 5	7.8	pH 7.8	0.0000000158	0.0158
Data 6	7.9	pH 7.9	0.0000000126	0.0126
pH index mean	—	pH 6.07	0.0000008600	0.8600
LSD	0.1	—	—	—

In two series there are values of the pH index, first the pH 3.9, pH 4 and 4.1, and the second the pH 7.9, pH 8, and the pH 8.1, but in both of these series the pH 4 and 8 there are not the mean values, but it is not means that the differences between the values of pH index in every of these series it is value the pH 0.1, but the only may to indicate that in the first is range pH 3.9 – pH 4.1, and the second the range is pH 7.9 – pH 8.1. Values of the pH index can be convert into values the  $[H^+]$  and expressed in the mol  $[H^+] \text{ dm}^{-3}$ . So, the difference between values pH 3.9 and pH 4.0 converted into  $[H^+]$  is  $25.89254118 \mu\text{mol } [H^+] \text{ dm}^{-3}$ , while the difference between pH 4 and pH 4.1 it is  $20.56717653 \mu\text{mol } [H^+] \text{ dm}^{-3}$ . But otherwise it is in comparison of values pH 7.9 and pH 8.0, because then this difference is  $0.0025893 \mu\text{mol } [H^+] \text{ dm}^{-3}$ , and between values pH 8 and pH 8.1 it is value  $0.0020567 \mu\text{mol } [H^+] \text{ dm}^{-3}$ , well, this not the end, because value of differences expressed in the  $[H^+]$  one cannot convert again into the pH index, since value  $25.89254118 \mu\text{mol } [H^+] \text{ dm}^{-3}$  it is value the pH 4.5, and the value  $0.0025893 \mu\text{mol } [H^+] \text{ dm}^{-3}$  is value the pH 8.5, because then to be sure in both cases this would be the nonsense. One can also notice specific contrast between values pH 3.9 and pH 4 as well as between pH 7.9 and pH 8, where conversion from value the pH index into values the  $[H^+]$  gives the difference 10000 times. Now then, statistical calculations of experimental data with the pH index as well as the interpretation of results should conform with the essence of pH index which is in the logarithmic scale (Table 4).

Table 4

Example-calculations for numerical data in the uniform and the logarithmic scale

Item	Data in the uniform scale	Data in the logarithmic scale	Value on base pH mol $[H^+] \text{ dm}^{-3}$	Different value $\mu\text{mol } [H^+] \text{ dm}^{-3}$
Data 1	3.9	pH 3.9	0.000125892541	25.89254118
Data 2	4.0	pH 4.0	0.0001	
Data 3	4.1	pH 4.1	0.000079432823	20.56717653
Data 4	7.9	pH 7.9	0.000000012589	0.0025893
Data 5	8.0	pH 8.0	0.00000001	
Data 6	8.1	pH 8.1	0.000000007943	0.0020567
Mean as results	6.0	pH 4.30	0.000050892650	—

In the equation elaborated by the NTCCHS [6] the numbers 4, 5, 6 and 7 there are as the x features and really perform the equation  $y = -60x + 595$  (Fig. 1), but values the pH index which are in the logarithmic scale the all data value of pH index should be converted into the uniform scale, at this juncture the pH 4 it is value  $10^{-4} \text{ mol } [H^+] \text{ dm}^{-3}$ , pH 5 it is value  $10^{-5} \text{ mol } [H^+] \text{ dm}^{-3}$ , pH 6 it is value  $10^{-6} \text{ mol } [H^+] \text{ dm}^{-3}$ , and pH 7 it is value  $10^{-7} \text{ mol } [H^+] \text{ dm}^{-3}$ , and then the correct mathematical relation  $f(x)$  expressed is in the logarithmic equation  $y = 26.058 \ln(x) + 235$  (Fig. 2). And one can easily indicate on simple examples for numbers 4, 5, 6 and 7 the mean it is 5.5, and for values the pH 4, pH 5, pH 6, and pH 7, the mean it is the pH 4.56, and for numbers 4 and 7 the mean values is 5.5 too, but for values pH 4 and pH 7 the mean value is pH 4.3, and next,

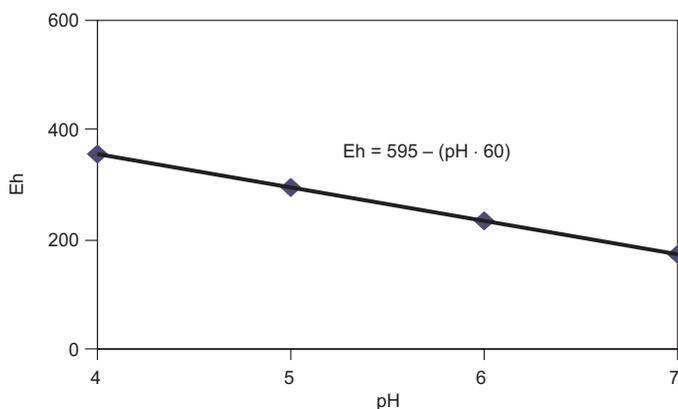


Fig. 1. Fig. 1. The dependence between the pH index and Eh (in mV) [6]

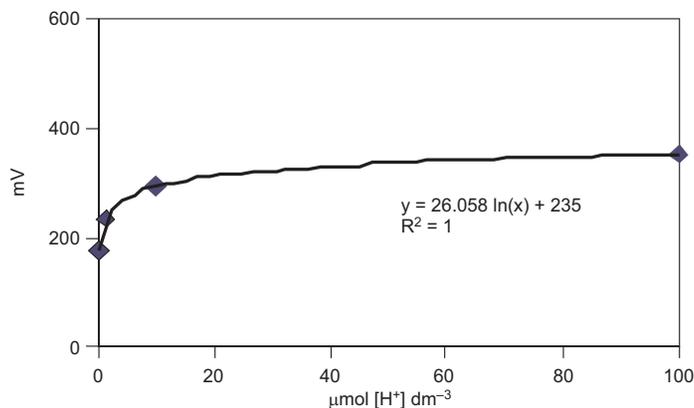


Fig. 2. The relationship between  $[\text{H}^+]$  and redox potential calculated on the basis of data from Fig. 1

the difference for pH 4 and pH 5 in the uniform scale it is the 10 times, for pH 5 and pH 6 is also 10 times, and for pH 6 and pH 7 is 10 times too, while the difference for values the pH 4 and pH 6 it is the 100 times, but it is not 2 times, and at last the difference for the pH 4 and pH 7 in the uniform scale it is the 1000 times, but it is not 3 times. Then this considerations can be finished so unsophisticatedly that pH 4 this is not the number 4, pH 5 it is not 5, the pH 6 it is not 6, and at last pH 7 it is not 7, and on this it is the end of the argument and the evidence.

It can be introduced as considerations on basis of the theoretical example where dependences were counted for integers 4, 5, 6, and 7 in the range 4–7 as the x feature, and data of the y feature as value 123, 204, 284 and 365 mV, at this juncture the calculated equation was  $y = -80.6x + 687.3$ , and for mean the x value = 5.5 the results was  $y = 244$  (Table 5, Fig. 3). And next counted also the theoretical example for pH 4, pH 5, pH 5, and pH 7 the range of three spaces values of pH index for the range pH 4 –

Table 5

Example-calculations based on the data converted into the units of uniform scale

Range	Value	Mean	Equation	Result
4-7	4-7	$x = 5.5$	$y = -80.6x + 687.3$	$y = 244$
pH 4 – pH 7	$100-0.1 \mu\text{mol} [\text{H}^+] \text{dm}^{-3}$	$x = 27.755$	$y = 35.004 \ln(x) + 203.7$	$y = 320$
5-6	5-6	$x = 5.5$	$y = -282x + 1795$	$y = 244$
pH 5 – pH 6	$10-1 \mu\text{mol} [\text{H}^+] \text{dm}^{-3}$	$x = 4.052$	$y = 122.47 \ln(x) + 103$	$y = 274$

pH 7, but just the x feature value which were counted on the basis of pH index were 0.1, 1, 10, and 100  $\mu\text{mol} [\text{H}^+] \text{dm}^{-3}$ , and then calculated logarithmic equation  $y = 35.004 \ln(x) + 203.7$ , and for mean value of the pH 4.56 calculated mean of value  $x = 27.775 \mu\text{mol} [\text{H}^+] \text{dm}^{-3}$ , and then the result value  $y = 320 \text{ mV}$  (Table 5, Fig. 4).

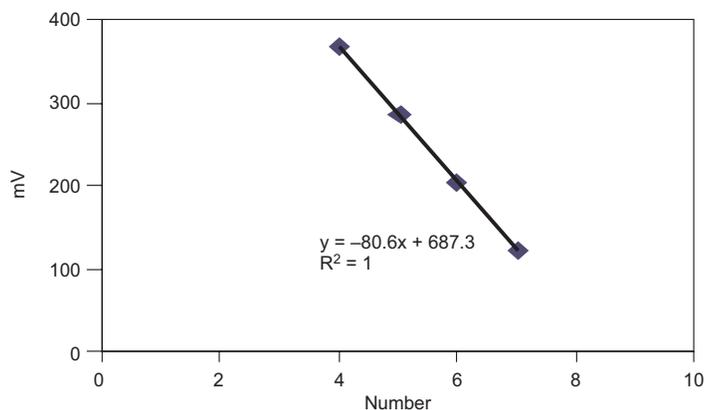


Fig. 3. The example-dependence where the x axis value there are the numbers 4, 5, 6, and 7, it means in the range 4-7

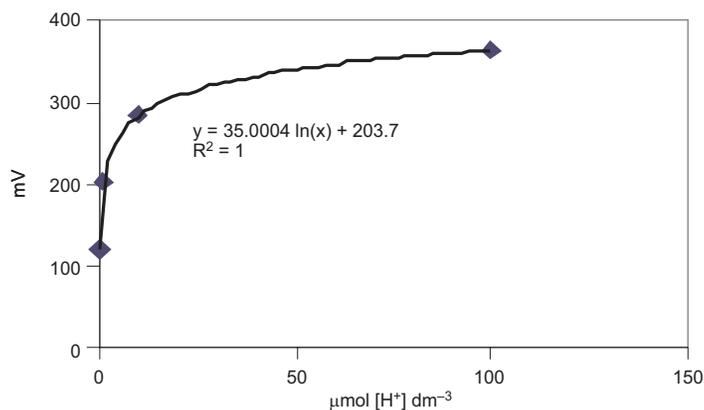


Fig. 4. The relationship calculated on the assumption that the x axis there are values of  $[\text{H}^+]$  data converted from the value of the pH index, it means of the three spaces the pH 4, pH 5, pH 6, and pH 7, where on the Fig. 3 this were the numerical values

Next considerations however are similar but with the attention, that data the x features in this case as numbers 5.1, 5.3, 5.7, and 5.9 in the range of one integer, that is in the range 5–6, and data the y feature were values 123, 204, 284, and 365 mV, then counted the equation  $y = -282x + 1795$ , where for mean value  $x = 5.5$ , the result was  $y = 244$  mV (Table 5, Fig. 5). But if the number values 5.1, 5.3, 5.7, and 5.9 accept as values pH 5.1, pH 5.3, pH 5.7, in the range pH 5 – pH 6, it is in the one space pH index, but to statistical calculation it should be convert into values in regular scale, that are data the x feature as 1.26, 2.00, 5.01 and 7.99  $\mu\text{mol} [\text{H}^+] \text{dm}^{-3}$  in the range 1–10  $\mu\text{mol} [\text{H}^+] \text{dm}^{-3}$ . Values the y feature were 123, 204, 284, and 365 mV. At this juncture on the basis of the dependence between the x feature and with the y feature counted the logarithmic equation  $y = 122.47 \ln(x) + 103$ , so for the mean data value  $x = 4.052 \mu\text{mol} [\text{H}^+] \text{dm}^{-3}$ , the result was  $y = 274$  mV (Table 5, Fig. 6).

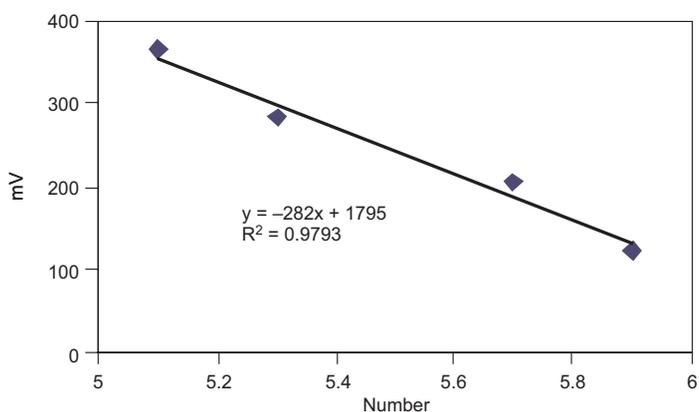


Fig. 5. The example-dependence where the x axis value there are the numbers 5.1, 5.3, 5.7, and 5.9, it means in the range 5–6

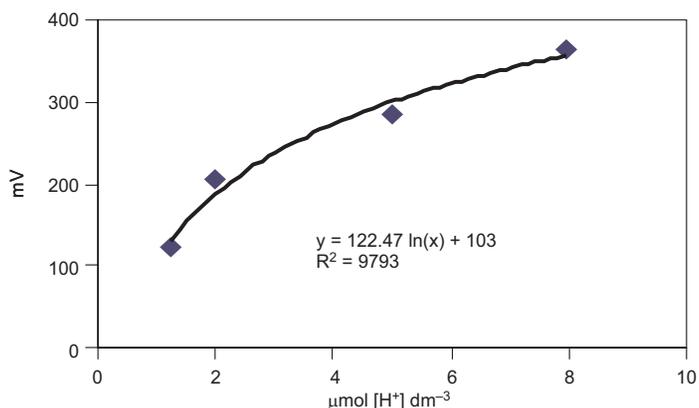


Fig. 6. The relationship calculated on the assumption that the x axis there are values of the  $[\text{H}^+]$  data converted from the range of one space of the pH index, it means the pH 5.1, pH 5.3, pH 5.7, and pH 5.9, where on the Fig. 5 this were the numerical values

Let be the example carried out research in which considered were of the dependence between the cation saturation state index (CSS) and values the pH index for the sample size  $n = 25$ . Values of the CSS index, which as the x feature were in the range 0.110–5.340, and values of the pH index that is y feature in the range pH 3.8 – pH 6.2, just it can be seen on the graph (Fig. 7). But the regression equation was counted when values of pH index were converted from the pH index in the logarithmic scale into the uniform scale, which were in the range 0.631–159  $\mu\text{mol} [\text{H}^+] \text{dm}^{-3}$ , and then the equation was  $y = 17.174/x$ , and this is just correct way of the calculation of data, when in the experimental data were done measurements the pH index, and then the experimental data were correctly statistically counted (Fig. 8).

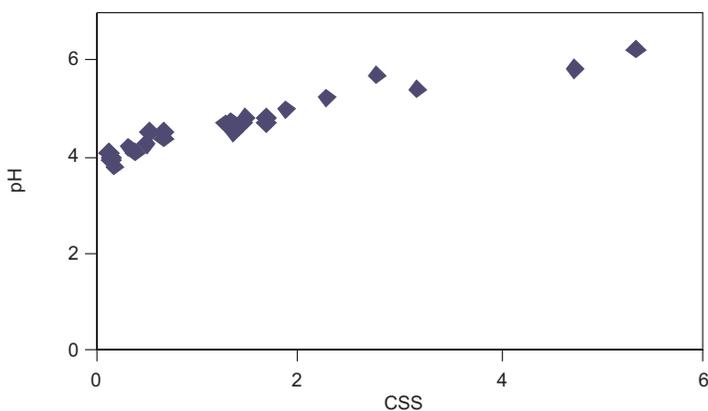


Fig. 7. Relationship between the CSS index and measured values of the pH index in 0.01 mol  $\text{CaCl}_2$  [1], and the CSS index [35]

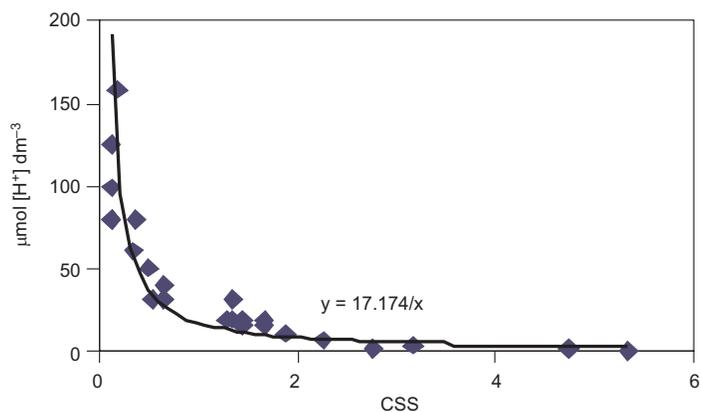


Fig. 8. Relationship between the CSS index and  $[\text{H}^+]$  calculated on the basis measurements of the pH index (Figure 7) for  $n = 25$  [1]

Let be other example from the real experience too, where values of the pH index and were it data the x features in the one space of the pH index, that was between pH 5 and

pH 6, and this was series of the pH value as the pH 5.25, pH 5.3, pH 5.41, pH 5.4, pH 5.65, pH 5.8, pH 5.71, and pH 5.93, and data the y feature as series 520, 450, 450, 410, 370, 360, 350, and 320 mV. The dependences between the x feature as values the pH index and the y feature as the Eh values were express in the mV graphically, where easily can be seen, that data of the y features as the Eh values were in the range 300 – 600 mV and this dependences could be the only show (Fig. 9). Counted too the regression equation between investigated features, but it made on the basis of values the pH index converted into unit in the uniform scale in this case the  $\mu\text{mol } [\text{H}^+] \text{ dm}^{-3}$ , and values of the y feature were in the range 1–6  $\mu\text{mol } [\text{H}^+] \text{ dm}^{-3}$ . Then calculated regression equation  $y = 38.188x + 282.24$  and expressed authentic dependence between the x feature and the y feature as well as conformed with principles calculations of statistical experimental data (Fig. 10).

A serious problem related to the pH index is the usage of incorrect term as the ‘pH unit’, so this is in many papers [7–12], where is used notion the ‘pH unit’, however such

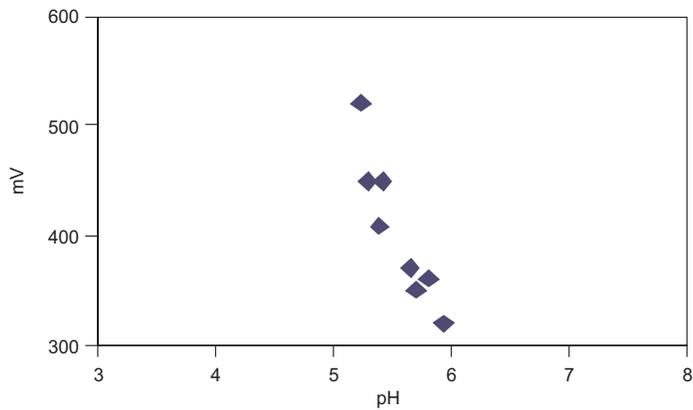


Fig. 9. Relationship between values of the pH index and redox potential in soil solution

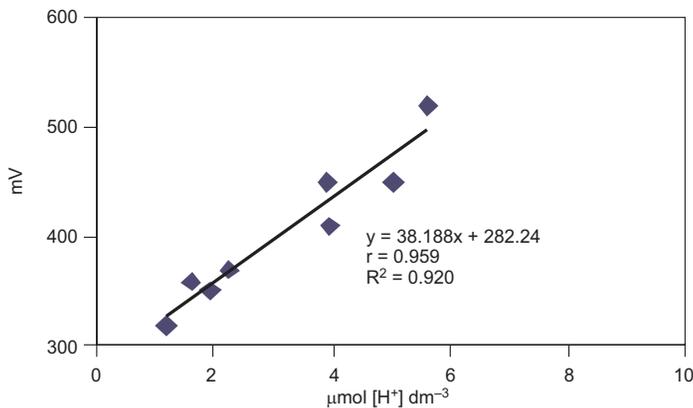


Fig. 10. Relationship between values  $[\text{H}^+]$  calculated on the basis measurements of the pH index (Fig. 9) and redox potential for  $n = 8$  [2]

unit at all does not exist, indeed, or more appropriately explanation the pH index stated as 'no dimension' [13]. Then too the strange idea of ' $\Delta$  pH' as the simple difference between values of the pH index [7, 14], which in the reality cannot exist never and ever, and no evidence here is needed, because simple example explains the case, well, easily it can be seen that value the pH 0.1 is greater than values the pH 7.1 just 10000000 times. However, the best example, but the most affecting case is the scientific elaboration, where presented are improper results of statistical calculations with the pH index [15].

Presented in the paper numerous examples with the statistical calculation the pH index may underlie to the careful statement that scientific publications with incorrect statistical calculations of data pH index have also some good evidence that reviewers sometimes did not prevent to the publication of papers with incorrect calculations and the wrong interpretation of results with the pH index. Maybe also with the pleasure one can to read the article on the problems of scientists education which how it seems, that at all did not pass and are always current and commonly needed [16]. However, and it maybe too, that happen especially and exceptionally careful rules and contribution in the scientific publishing house [17].

And the end the conclusions last but not least to scientists as well as to students attention. Every statistical calculations of experimental data, as well as the calculations of variance and regression equations may be made when the data values there are in units, and only in the uniform scale, and no indices in the logarithmic scale, and also and especially value of pH index as data directly cannot be statistically calculated. Also it is the most important, the alls and without exception, that as every experimental data must to have the normal distribution of data to use of statistical analysis methods, because all the statistical methods based on the mean values, and it cannot be perform in this case with the pH index.

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## ROZWAŻANIA DOTYCZĄCE STATYSTYCZNYCH OBLICZEŃ ZE WSKAŹNIKIEM pH W BADANIACH NAUKOWYCH

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**Abstrakt:** Rozważania były prowadzone w związku z niepoprawnym sposobem obliczeń statystycznych danych doświadczalnych ze wskaźnikiem pH, jak również z niewłaściwą interpretacją wyników badań ze wskaźnikiem pH w licznych naukowych publikacjach, które były opublikowane w wielu poważnych i dobrze znanych wydawnictwach naukowych. Analiza statystyczna danych doświadczalnych może zostać zrobiona, gdy wartości danych są wyrażone w jednostkach skali jednolitej.

**Sowa kluczowe:** wskaźnik pH, analiza statystyczna

Joanna LACH<sup>1\*</sup> and Ewa OCIEPA<sup>1</sup>

## EFFECT OF THE PROCESS OF WG-12 ACTIVATED CARBON MODIFICATION ON THE SORPTION OF CHROMIUM

### WPLYW PROWADZENIA PROCESU MODYFIKACJI WĘGLA AKTYWNEGO WG-12 NA SORPCJĘ CHROMU

**Abstract:** The article presents the results of activated carbon modification with the usage of Joule heat. The modification was carried out in the reactor (h = 25 cm and d = 5,5 cm) filled with activated carbon. The reactor was equipped with two electrodes located on both sides and connected with a direct current generator. The flow of the current through the activated carbon bed was accompanied by gradual increase in activated carbon temperature. The modification took place during the flow of carbon dioxide while heating up and/or cooling down the bed. The effects of modification were evaluated on the basis of Cr(III) and Cr(VI) adsorption isotherms each time on three parts of activated carbon sampled from different heights of the bed. The presented method of modification of the WG-12 carbon for removal of Cr(III) and Cr(VI) cations from aqueous solutions improved sorption capacities of the investigated sorbents towards both ions (WG/400EII/40AIRs, WG/400EI/80AIRs). In the majority of cases, modification increased the efficiency of removing one ion while reducing the other.

**Keywords:** sorption, activated carbon, modification, chromium

In some cases removal of heavy metals from water can be efficiently performed with activated carbons that also show potential for removing organic compounds from water [1–5]. The phenomenon of removal of ions on activated carbons is more complex than that of organic compounds. Ions can be effectively removed on carbons that have functional groups, of either acidic or basic character, on their surface. The most common mechanism behind adsorption of cations is ion exchange, though the ion removal can also take place through the formation of surface complexes, and is also associated with the possibility of occurrence of reduction and oxidation processes as well as the precipitation of insoluble compounds (*eg* hydroxides, carbonates, *etc.*) in pores [4].

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Another issue is the occurrence of different forms of particular ions (or co-existence of different ions), depending on the pH of solution, in which the process is initiated. Thus, we observe the occurrence of *eg* Cr(III) in the form of cations of a different valence number and a dynamic radius (due to the formation of aquo-complexes) and, at high water pH values, in the form of an anion. In the case of Cr(VI), the following anions can be present in water solutions depending on their pH:  $\text{Cr}_2\text{O}_7^{2-}$ ,  $\text{HCrO}_4^-$ ,  $\text{CrO}_4^{2-}$ .

Activated carbons, which are usually manufactured with the aim of removing organic compounds from water, have relatively few oxygen groupings taking part in the sorption of *eg*, heavy metals. Therefore, different studies are being conducted that aimed at increasing the amount of oxygen on the surface of activated carbon [5–7]. Oxidation can be done using either oxidizing gases or oxidizing liquids [4, 8]. Liquid-phase oxidation (particularly where nitric acid is used) results in the highest increase in the amount of accumulated oxygen. By oxidizing carbons in gaseous phase, the amount of oxygen can be increased up to 15 % while in liquid phase – up to 25 %.

One of the metals that is effectively removed on activated carbons is chromium(III) and (VI) [9–11]. The aim of the work presented in this paper is to assess different conditions of modification of the WG-12 carbon in terms of removal of chromium(III) and (VI) from water. Modification was carried out on an innovative testing stand where active carbon was heated up by utilizing its electric conduction (SEOW). As a result of the electric current flowing through it, the carbon bed was heated up (by released Joule heat). The presented modification was made with the use of carbon dioxide and air.

## Materials and methods

Activated carbon (grade WG-12, manufactured in Poland from hard coal) was used for tests. Prior to measurements activated carbon was washed with distilled water several times. The characteristics of the investigated carbon are provided in Tables 1 and 2. Then, the carbon was dried at 145 °C. After washing and drying carbon was subjected to modification at temperature of 400 °C on the testing stand (SEOW) that utilized the electric conduction of carbon to heat up the carbon bed [12]. The modification was conducted in a reactor with height of  $h = 25$  cm and diameter of  $d = 5.5$  cm filled with activated carbon. Electrodes placed on both sides of the reactor were connected to a direct current source. The flow of electric current through the bed resulted in an increase in activated carbon temperature. The modification took place as air flowed during cooling of that bed.

Table 1

Physical and chemical properties of activated carbon WG-12

Index	WG-12
Bulk density [ $\text{g}/\text{dm}^3$ ]	420
pH of water extract	10.1
Methylene blue number [LM]	30
Iodine adsorption [ $\text{mg}/\text{g}$ ]	1050

Table 2

The distribution of capillaries volumes in activated carbon WG-12

Carbon symbol	Capillaries radius [nm]					$\Sigma\Delta V$ [cm <sup>3</sup> /g]	Specific surface [m <sup>2</sup> /g]
	< 1.5	1.5–15	15–150	150–1500	1500–7500		
	Capillaries volume $\Delta V$ [cm <sup>3</sup> /g]						
WG-12	0.4213	0.1049	0.0648	0.2731	0.1478	1.0114	1005

The modification on the Joule heat-utilizing test stand was carried out in two phases: heating up the bed to temperature of 400 °C with and without gas flow (duration of approx. 6–8 min), and cooling down with flowing air down to temperature of 100 °C (with gas flow duration during cooling of about 20 min). The measurement of temperatures was taken at three different heights of the reactor. The carbon bed sampled from the reactor was divided into three parts: g – upper part, s – middle part, d – lower part.

The carbons modified on the Joule heat-utilizing test stand were designated with the letter “E”. Heating of the investigated carbon was performed with direct current of voltage of 48 V and 32 V, respectively. The determination of the modification conditions was made in accordance with the principle as shown on the example of the WG/400EI/40AIRg carbon – where WG is the abbreviation denoting active carbon; 400 – temperature up to which the carbon was heated; EI – one cycle of carbon heating and cooling on the SEOW stand; AIR – carbon air flow during cooling, in dm<sup>3</sup>/h; g – carbon batch originating from the upper part of the reactor (s – from the middle part of the reactor, d – from the lower part of the reactor).

The initial carbon and the modified carbon were used for the measurement of Cr(III) chromium sorption isotherms for initial concentrations ranging from 500 to 1500 mg/m<sup>3</sup>. The chromium(III) cation was obtained from chromium chloride (CrCl<sub>3</sub>). The measurements were carried out from the solutions with pH = 6. The pH correction was made with a diluted NaOH solution. At the examined value of pH solution chromium was present as Cr<sup>3+</sup>, Cr(OH)<sup>2+</sup> and Cr(OH)<sup>+</sup>, with its predominating forms being Cr(OH)<sup>2+</sup> (slightly above 60 %) and Cr(OH)<sub>2</sub><sup>+</sup> (more than 38 %) [9].

Sorption of chromium(VI) occurring in the form of an anion in the solution was also carried out. The isotherms of Cr(VI) were made for initial concentrations ranging from 600 to 2000 mg/m<sup>3</sup>. The solutions were prepared from potassium dichromate, and the sorption process was conducted from the solution with pH = 6. At this pH, HCrO<sub>4</sub><sup>-</sup> was predominating form of chromium (13a).

The tests were conducted under static conditions. 1 g of activated carbon was added to 250 cm<sup>3</sup> of the solution in selected concentration, and then the solution was stirred for 2 hours and left unstirred for another 22 hours. The contact time for the investigated carbons with the solutions (*ie* 24 hours) was established based on previous investigations [13].

## Results and discussion

In the first testing stage, carbon was modified by heating up on the SEOW to 400 °C and then cooling down with air flowing at a rate of 40 dm<sup>3</sup>/h. This modification was

carried out in a single heating-cooling cycle. The carbon bed in the reactor was divided into three parts (lower, middle and upper). Cr(III) sorption isotherm measurements were made separately on carbon sampled from different reactor heights and on the initial carbon (WG0) (Fig. 1).

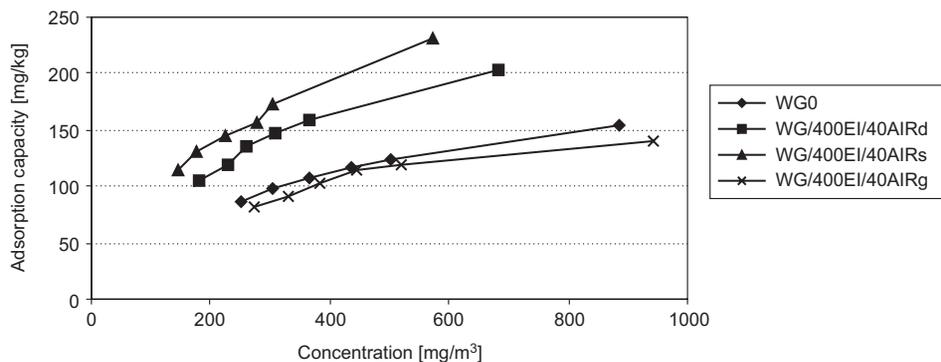


Fig. 1. Sorption isotherms of chromium(III) on modified activated carbon WG-12

The carbons sampled from the lower and middle reactor parts are characterized by higher capabilities for sorption of chromium (III) from the solution, compared with the initial carbon WG-12. The WG/400EI/40AIRs carbon (sampled from the middle part of the bed) turned out to be the most effective in absorbing the investigated cation. Most of air oxygen reached the lower bed part but at the same time this part of the bed was the most intensively cooled, as the inflowing gas was not heated up. The time of carbon dioxide contact with the active carbon, especially at high temperatures, was short (cooling of the bed from 400 °C to 300 °C for this layer took less than 2 minutes). The middle part of the bed was cooled the most slowly, because the air was already partially heated up after passing through the lower part of the bed, and also because it was the most efficiently isolated from the surroundings with activated carbon layers situated above and below. At the same time, however, slightly less oxygen reached this layer, as it was used in reactions with the surface of the lower layer of active carbon. The carbon taken from the upper part of the bed showed slightly lower sorption capabilities compared with the initial sorbent. Most probably, the processes of thermal decomposition of functional groups during heating up of the carbon predominated in this case over the formation of new ones during cooling. This is most likely due to the shortage of oxygen in the upper layers of the reactor.

At the subsequent stage of testing, measurements of Cr(III) sorption on carbons modified under the same conditions, but in two heating-cooling cycles, were carried out. The sorption isotherms obtained with the use of these carbons showed an unfavourable effect of the double heating-cooling cycle on the sorption capacity compared with the single cycle for removal of Cr(III) on such sorbents (Fig. 2). Only the carbon sampled from the middle part of the bed showed sorption capacity higher than that of the initial carbon WG0. Most probably, the functional groups formed in the first heating-cooling

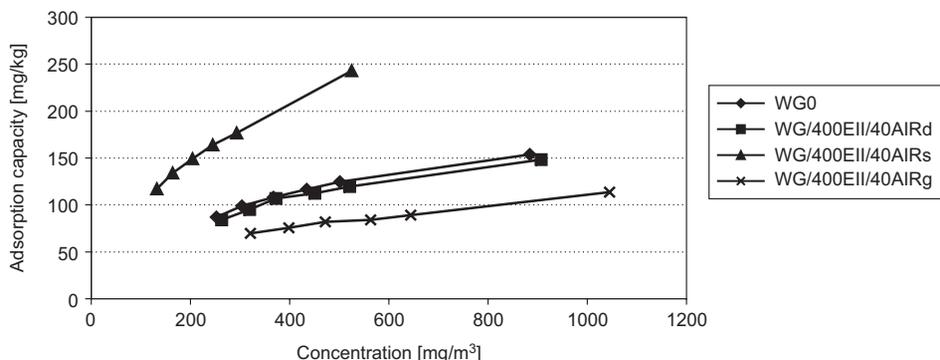


Fig. 2. Sorption isotherms of chromium(III) on modified activated carbon WG-12

cycle were largely decomposed in the subsequent heating phase. The second cooling no longer produced such promising results. This is most likely due to reduction of the reactivity of the carbon surface.

Measurements were also carried out for carbon modified in one cycle (Fig. 3) with air flow twice as high as during cooling ( $80\text{dm}^3/\text{h}$ ). In the case of carbons modified using air with a lower flow rate, the carbon sampled from the middle part of the reactor exhibited clearly enhanced Cr(III) sorption capabilities compared with WG0 but also higher compared with the sorbent WG/400EI/40AIRs. The carbon sampled from the lower part of the reactor has much higher sorption capacity of chromium cation compared with WG0 while Cr(III) sorption capacity similar to that of the carbon modified at lower air flow intensity. Regardless the modification method applied, sorbents sampled from the upper part of the bed sorbed less Cr(III) in the test conditions compared with unmodified carbon. The air flow at higher intensity causes, on the one hand, the inflow of larger amounts of oxidizing gas, but, on the other hand, faster bed cooling and shorter time of oxygen contact with the high temperature-heated carbon.

Modified carbons were also used for the removal from water of hexavalent chromium occurring in the form of an anion in the water solution. In the case of carbon

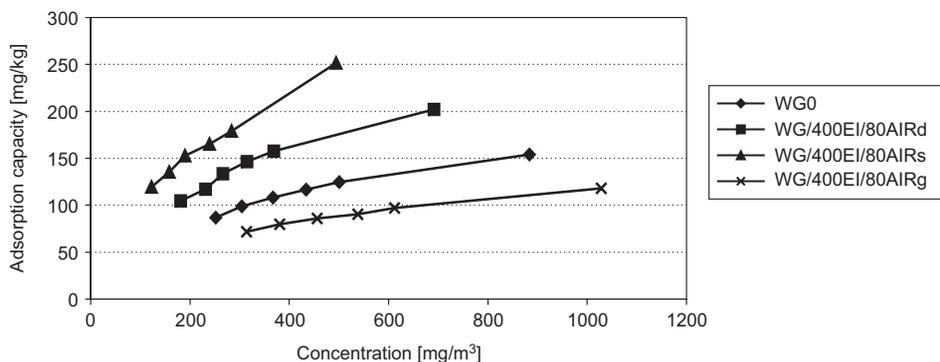


Fig. 3. Sorption isotherms of chromium(III) on modified activated carbon WG-12

modification with air flowing at a rate of  $40 \text{ dm}^3/\text{h}$  during cooling, only the carbon sampled from the upper part of the bed sorbed more investigated ions (Fig. 4). The carbons sampled from the lower and middle parts of the bed sorbed more Cr(III) cations compared with the initial WG-12 but less Cr(VI) anions.

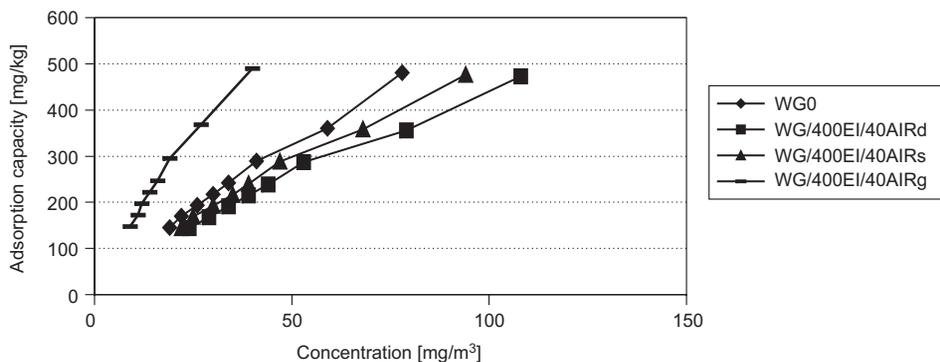


Fig. 4. Sorption isotherms of chromium(VI) on modified activated carbon WG-12

If modification was conducted in analogous conditions, except that in two heating-cooling cycles, the obtained sorbents, regardless the sampling height of the reactor, sorbed larger quantities of Cr(VI) than the initial carbon did (Fig. 5). Taking into consideration the sorption of both investigated cation and anion, the carbon modified with this procedure, but sampled from the middle part of the bed (WG/400EII/40AIRs), increased its sorption capacities towards both investigated chromium forms.

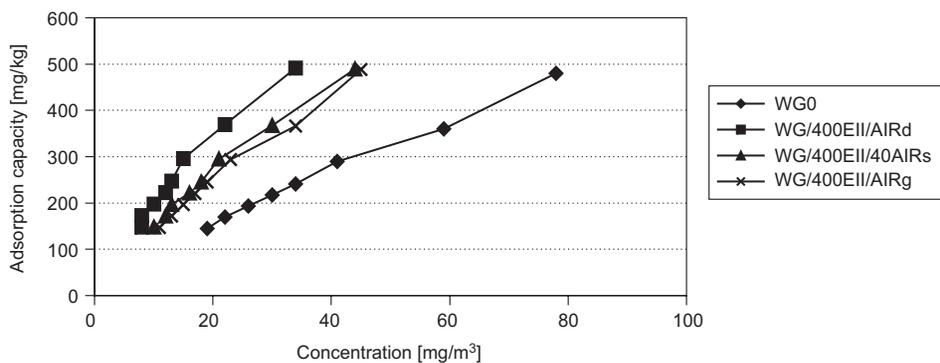


Fig. 5. Sorption isotherms of chromium(VI) on modified activated carbon WG-12

As a result of modification on the SEOW stand during air flow at a rate of  $80 \text{ dm}^3/\text{g}$ , a sorbent of increased Cr(VI) sorption capacity was obtained for carbon sampled from the middle part of the reactor (Fig. 6). This carbon also absorbed more Cr(III) compared with the initial WG-12. In the remaining cases, the obtained sorbents absorbed less Cr(VI) anions.

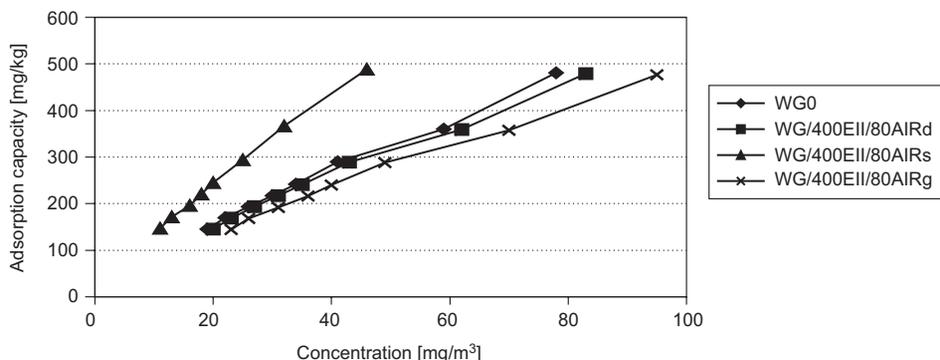


Fig. 6. Sorption isotherms of chromium(VI) on modified activated carbon WG-12

When compared the efficiency of Cr(III) and Cr(VI) removal under the test conditions, the Cr(VI) anion was much more effectively removed, both on the initial and the modified activated carbons (Table 3). The efficiency of Cr(III) cation removal on the initial activated carbon WG-12 reached 50 %. As the result of modification on the SEOW stand carbons that remove the cations with efficiency above 70 % (WG/400EII/40AIRs, WG/400EI/80AIRs) can be obtained. Also, in the majority of instances, sorbents of enhanced sorption capacity of the Cr(VI) anions were obtained as the result of modification. The efficiency increased in that case from 96.4 % to 98.4 %. The differences in efficiencies are not in this case significant, as the equilibrium concentrations, upon which sorption capacities depend, are very small.

Table 3

The degrees of Cr(III) removal on activated carbons from aqueous solutions ( $C_0 = 1000 \text{ mg/m}^3$ )

Carbon	Removal degree of Cr(III) [%]	
	Cr(III)	Cr(VI)
WG0	49.9	96.4
WG/400EI/40AIRd	63.6	95.6
WG/400EI/40AIRs	69.4	96.1
WG/400EI/40AIRg	47.9	98.4
WG/400EII/40AIRd	47.9	97.7
WG/400EII/40AIRs	70.7	98.2
WG/400EII/40AIRg	35.6	98.1
WG/400EI/80AIRd	63.1	96.5
WG/400EI/80AIRs	71.6	98.0
WG/400EI/80AIRg	38.8	96.0

Only in two cases modification produced sorbents of enhanced sorption capacities for both Cr(III) and Cr(VI) (WG/400EII/40AIRs, WG/400EI/80AIRs). For the remaining

modified carbons, increasing sorption capacity for the Cr(III) anion involved reduction of sorption capacity for the Cr(VI) anion, or vice versa. Most probably, a change in the nature of oxygen groupings already present on the surface also took place in this case alongside carbon surface oxidation during modification.

## Conclusion

The presented method of modification of the carbon WG-12 for removal of Cr(III) and Cr(VI) cations from aqueous solutions allows to improve sorption capacities of sorbents towards both investigated ions (WG/400EII/40AIRś, WG/400EI/80AIRś). In the majority of cases, modification increased the efficiency of removing one ion while reducing that of the other. Most probably, a change in the nature of oxygen groupings already present on the surface took also place alongside carbon surface oxidation during modification. Among the modification conditions investigated, the SEOW stand modification of carbon with heating it up to 400 °C and then cooling it down with air flowing at a rate of 80 dm<sup>3</sup>/h turned out to be the best, considering the sorption of Cr(III). In this case, the carbon sampled from the lower part of the reactor and the carbon sampled from the middle part of the reactor are both characterized by significantly increased Cr(III) removal efficiency compared with the unmodified carbon. In the case of sorption of the Cr(VI) anions, these anions were most effectively removed on carbons modified in two cycles of bed heating and cooling with air flowing at a rate of 40 dm<sup>3</sup>/h.

Modification results are most likely to be influenced by two opposite phenomena: decomposition of oxides during heating and their formation or change in their nature during cooling with air.

## Acknowledgement

The research was founded from the resources of BS/PB-401/301/12.

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### WPLYW PROWADZENIA PROCESU MODYFIKACJI WĘGLA AKTYWNEGO WG-12 NA SORPCJĘ CHROMU

Instytut Inżynierii Środowiska  
Politechnika Częstochowska

**Abstrakt:** W artykule przedstawiono wyniki modyfikacji węgla aktywnego z udziałem ciepła Joule'a. Modyfikację prowadzono w reaktorze o wysokości  $h = 25$  cm i średnicy  $d = 5,5$  cm wypełnionym węglem aktywnym. Z dwóch stron reaktora przyłożono elektrody podłączone do źródła prądu stałego. Przepływowi prądu przez złożę towarzyszyło podwyższenie temperatury węgla aktywnego. Modyfikacja następowała podczas przepływu dwutlenku węgla podczas nagrzewania i/lub studzenia tego złoża. Efekty modyfikacji oceniono na podstawie izoterm sorpcji Cr(III) i Cr(VI) każdorazowo na trzech partiach węgla aktywnego pobieranego z różnych wysokości złoża. Przedstawione w pracy sposoby modyfikacji węgla WG-12 pod kątem usuwania z roztworów wodnych kationu Cr(III) i Cr(VI) pozwalają na uzyskanie sorbentów o zwiększonych pojemnościach w stosunku do obydwu badanych jonów (WG/400EII/40AIRś, WG/400EI/80AIRś). W większości przypadków modyfikacja zwiększyła skuteczność usuwania jednego jonu, ale obniżyła drugiego.

**Słowa kluczowe:** sorpcja, węgiel aktywny, modyfikacja, chrom



# Varia



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The deadline for sending the Abstracts is **15th July 2013** and for the Extended Abstracts: **1st October 2013**. The actualized list (and the Abstracts) of the Conference contributions accepted for presentation by the Scientific Board, one can find (starting from **31st July 2013**) on the Conference website:

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