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OPTIMIZING THE USE OF THE PHYTOTOXKIT TEST TO ASSESS THE TOXICITY OF SOIL CONTAMINATED WITH CREOSOTE

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Abstract. Soil phytotoxicity studies were performed with different doses of creosote by means of the PHYTOTOXKIT test, using *Sinapis alba*, *Lepidium sativum*, *Sorghum saccharatum* as test plants. The obtained results indicate highly significant effect of the creosote dose, duration of soil incubation, type of test plant and period, after which the root length measurement was performed during the phytotoxicity index root test. The analysis of results indicates the highest sensitivity of *Sorghum saccharatum* to creosote and the highest correlation of results obtained with the aid of *Lepidium sativum* when measuring the root length after the first day the seeds are lined with the size of the dose. The proposed mathematical model makes it possible to predict the reaction of test plants on the size of creosote dose as well as to assess its amount in the soil based on the root phytotoxicity. These results allow for a significant simplification of the test and shorten its duration. This allows the modified test to be used for simple monitoring of not only the phytotoxicity but also the creosote residues during reclamation of contaminated soil.

Keywords: phytotoxicity, plants, creosote, mathematical model

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread pollutants of natural, but mainly anthropogenic environments (Abdel-Shafy and Mansour 2016). The most important of the latter are residential heating, coal gasification and liquefying plants, carbon black, coal-tar pitch, asphalt production, coke and aluminum production, catalytic cracking towers or motor vehicle exhaust. These compounds have toxic, mutagenic and carcinogenic properties and are widely distributed in the environment. This group of compounds also includes creosote, which is very often a soil pollutant. Such contamination causes serious consequences for the soil environment. It is manifested in toxicity and mutagenic effects on living organisms (Zemanek *et al*. 1997) as well as numerous changes in the composition of soil microflora (Kästner *et al*. 1994, Törneman *et al*. 2008). Therefore, research is being undertaken to reclaim the creosote-contaminated soils in order to restore biological activity and restore their use (Weir *et al*. 1995, Guerin 1999, Atagana *et al*. 2003, Atagana 2004a, Atagana 2004b, Rasmussen and Olsen 2004, Viñas *et al*. 2005, Abdel-Shafy and Mansour 2016).

During reclamation of contaminated soils, both in laboratory tests as well as in operations carried out in practice on a larger scale, it is necessary to continuously monitor the progress of biodegradation by determining the toxicity of treated soil and contents of creosote components. Chemical and instrumental methods for the quantitative determination of polycyclic aromatic hydrocarbons applied for this purpose (Weir *et al*. 1995, Guerin 1999, Atagana *et al*. 2003, Atagana 2004a, Atagana 2004b, Viñas *et al*. 2005, Abdel-Shafy and Mansour 2016) are labor intensive, complicated and long-lasting. Therefore, many researchers are referring to biological methods, in which biotests are used (Wieczorek *et al*. 2012, Wołejko *et al*. 2016). The most commonly used test is Microtox, in which the test organism is *Vibrio fischeri* bacterium, Ostracodtoxkit, containing shellfish *Heterocypris incongruens*, and PHYTOTOXKIT (Phytotoxkit 2004), in which the test organisms are *Sinapis alba*, *Lepidium sativum*, *Sorghum saccharatum* (Wieczorek *et al*. 2012, Wierzbicka *et al*. 2015, Wołejko *et al*. 2016, Steliga and Kluk 2017). These researchers confirm both the high suitability of biotests in controlling the course of reclamation as well as their simplicity and low costs.

In this work, an attempt was made to analyze the results of the PHYTOTOXKIT test in order to simplify it without losing values of the obtained results and the possibility of assessing the level of creosote residues in the soil with its use.

MATERIALS AND METHODS

The soil from Lipnik near Szczecin was used for the study. It was a loamy sand with a content of 3.67% organic matter and pH_{H2O} 6.59 and pH_{KCl} 6.36. The soil was applied with creosote in doses of 1 $g \cdot kg^{-1}$, 5 $g \cdot kg^{-1}$ and 25 $g \cdot kg^{-1}$. The control combination was the same soil without addition of creosote as well as reference soil. The soil was incubated for 14 days and on 1, 7 and 14 days after the creosote application, a PHYTOTOXKIT test was carried out using *Sinapis alba*, *Lepidium sativum*, *Sorghum saccharatum*. Measurement of the root length of test plants was carried out after 1, 2 and 3 days from seeding the soil with a help of ImageJ software. The measurement results were used to calculate the root phytotoxicity index (RFI) according to the formula:

$$RFI = \frac{(control\ soil - soil\ with\ creosote)}{control\ soil} \times 100$$

The STATISTICA program was used for statistical analysis of results. Achieved results were analyzed by means of variance analysis (ANOVA), correlations were calculated and approximation of the obtained data was performed to establish the nature of the relationship between the creosote dose and the root phytotoxicity index.

RESULTS AND DISCUSSION

All factors used in the experiment exerted a considerable impact on the soil inhibition index (Table 1). Thus, values of this index were significantly different in soils containing various doses of creosote (0.1, 0.5 and 2.5%) and also those obtained with the aid of various test plants (*Sinapis alba*, *Lepidium sativum*, *Sorghum saccharatum*). The index values were different in particular research dates: 1, 7 and 14 days. Furthermore, significant differences also occurred depending on the duration of the test, i.e. measurement of root length after 1, 2 or 3 days of covering the soil.

Table 1. Multifactor analysis of variance for experimental results (ANOVA)

Effect	Sum of square	Degree of freedom	Mean of square	*F*-test	*p*-value
Total	**860431.4**	**1**	**860431.4**	**22784.28**	**0.000000**
Term	**3277.2**	**2**	**1638.6**	**43.39**	**0.000000**
Day	**1311.2**	**2**	**655.6**	**17.36**	**0.000001**
Dose	**21702.8**	**2**	**10851.4**	**287.35**	**0.000000**
Plant	**2949.5**	**2**	**1474.8**	**39.05**	**0.000000**
Term*Day	209.1	4	52.3	1.38	0.246787
Term*Dose	**9086.7**	**4**	**2271.7**	**60.15**	**0.000000**
Day*Dose	362.2	4	90.6	2.40	0.056872
Term*Plant	**973.5**	**4**	**243.4**	**6.44**	**0.000149**
Day*Plant	**15834.9**	**4**	**3958.7**	**104.83**	**0.000000**

Effect	Sum of square	Degree of freedom	Mean of square	*F*-test	*p*-value
Dose*Plant	**927.9**	**4**	**232.0**	**6.14**	**0.000228**
Term*Day*Dose	395.8	8	49.5	1.31	0.250333
Term*Day*Plant	354.2	8	44.3	1.17	0.325857
Term*Dose*Plant	**676.7**	**8**	**84.6**	**2.24**	**0.032569**
Day*Dose*Plant	**1234.0**	**8**	**154.3**	**4.08**	**0.000399**
Term*Day*Dose*Plant	562.8	16	35.2	0.93	0.537447
Error	3058.9	81	37.8		

Occurrence of the above differences makes it possible to optimize the way the test is performed, while simplifying and shortening its duration. An analysis of the obtained results was carried out by comparing the test results with the use of individual plants, the time, after which the root length was measured and use of the reference soil and soil from Lipnik as the control.

Soil phytotoxicity indices obtained with both types of controls have almost identical mean values, and a very similar spread (Fig. 1). The mean phytotoxicity indices of the tested soil from Lipnik obtained using the reference soil and soil from Lipnik amount to 71.90 and 72.88, respectively at the standard deviations of 20.45 and 19.77. This shows the almost identical structure of both sets. Matching the results obtained with both methods was also very good (Fig. 2). The correlation coefficient between them is close to unity (0.9535) and very highly statistically significant. These data indicate that giving up the reference soil and replacing it with the test soil, the phytotoxicity of which is determined, does not cause significant inaccuracies in the results. Some literature data also indicate the possibility of omitting the reference soil. The tests were carried out in this way by Wieczorek *et al*. (2012) in diesel oil phytotoxicity studies, or by Sekutowski and Sadowski (2009) in phytotoxicity studies on herbicides.

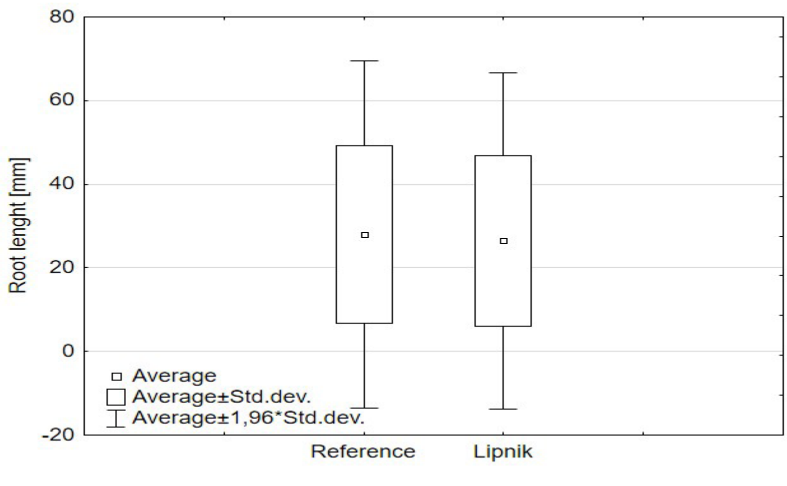

Fig. 1. Plot of mean and scatter of phytotoxicity indices obtained using the reference soil and soil from Lipnik

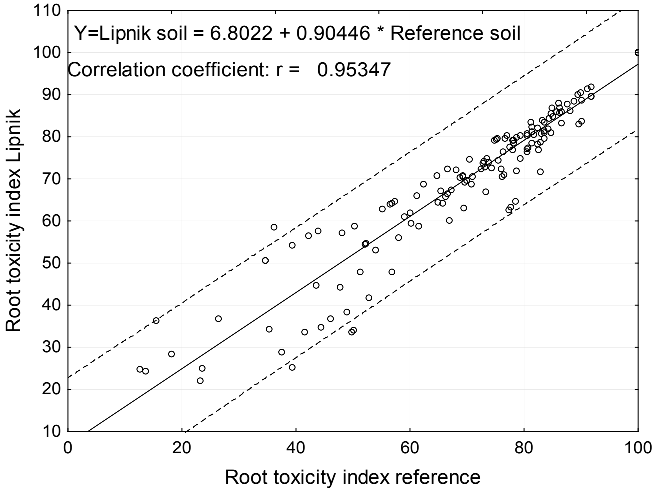

Fig. 2. Plot of root phytotoxicity index fitting obtained using the reference soil and soil from Lipnik

Table 2. Root phytotoxicity indices of soil treated with creosote

Incubation	Test day	Dose [g·kg^{-1}]	Root phytotoxicity index		
			Sinapis alba	*Lepidium sativum*	*Sorghum saccharatum*
1	1	1	59.94	58.39	100.00
1	1	5	64.09	70.56	100.00
1	1	25	71.51	100.00	100.00
1	2	1	72.31	75.41	43.09
1	2	5	77.03	81.41	61.59
1	2	25	85.36	87.42	75.70
1	3	1	79.43	79.16	60.16
1	3	5	86.22	82.14	75.70
1	3	25	90.99	89.70	85.79
7	1	1	46.95	61.76	0
7	1	5	52.43	74.14	0
7	1	25	53.55	79.39	0
7	2	1	57.69	69.36	53.39
7	2	5	70.94	77.07	62.37
7	2	25	82.35	90.18	70.69
7	3	1	70.64	63.58	70.08
7	3	5	77.08	67.49	72.30
7	3	25	84.02	83.34	83.44
14	1	1	28.07	31.22	79.32
14	1	5	56.32	81.04	100.00
14	1	25	67.43	100.00	100.00
14	2	1	26.43	35.83	33.52
14	2	5	69.09	77.31	68.95
14	2	25	81.57	100.00	74.36
14	3	1	30.78	33.80	34.84
14	3	5	77.44	74.40	79.71
14	3	25	87.49	100.00	86.93

Root phytotoxicity indices of applied creosote are presented in Table 2. The soil was incubated for 3 weeks and the PHYTOTOXKIT tests (incubation day) were performed at 7 days intervals. During the tests, root length measurement were made on days 1, 2 and 3 after soil seeding (test day). Mean value of the root phytotoxicity indices were almost the same for *Sinapsis alba* and *Sorghum saccharatum*, 66.93 and 65.63, respectively, while for *Lepidium sativum* it was about 12% higher and amounted to 74.97. This indicates the sensitivity of the latter plant as an indicator of phytotoxicity. Similarly, the highest sensitivity of *Lepidium sativum* was observed by Tarnawski and Baran (2018) in the toxicity test of bottom sediments containing heavy metals. Also Sundi (2015) reports analogous information, but not for all the studied soils. Values of root phytotoxicity indices obtained in these studies are in the range given by other authors (Wieczorek *et al*. 2012, Kanarbik *et al*. 2014, Wierzbicka *et al.* 2015, Steliga and Kluk 2017).

When considering the individual dates of the bio-testing, one can find the highest values of the root phytotoxicity index at the beginning of the incubation, on the first day after applying the creosote to the soil. In subsequent periods, these values are slightly smaller, which may be due to its reduced availability as a result of the immobilization occurring at the soil sorption complex or reduction in concentration as a result of degradation or volatilization. The average value of the root phytotoxicity index for the measurement of root length after the first day of seeding the soil for all test plants was 64.3. When measuring the root length of test plants, the index increased in each subsequent day by approximately 7%. However, each of the plants reacted differently. A gradual increase of 6–8% on the following days of the test was observed for *Sorghum saccharatum*. In the case of *Sinapis alba*, the lowest mean value of the root phytotoxicity index was observed when measured on the first day of the test (58.82), for the remaining two days, it was almost equal and higher by about 20% (71.88 and 71.68), whereas in the case of *Lepidium sativum*, the measurement of root length in the following days gave the results of the root phytotoxicity index very similar, with no upward trend. Thus, it seems that when using this test plant, reliable results can be obtained by measuring the root length just one day from the seed lining on the soil.

For each of the days when the root length of the test plants was measured and for mean values of measurements from those days, the dependence of the root amount of phytotoxicity index on the creosote dose in the soil was plotted. An approximation was also performed by plotting the exponential curve of the association of these two quantities (Fig. 3). The obtained logarithmic equations illustrating the dependence of the root phytotoxicity index on the creosote dose in the soil are shown in Table 3. The table also shows the correlation coefficients between the creosote dose in the soil and the corresponding root phytotoxicity index. All of these coefficients are highly statistically significant, except for *Sorghum saccharatum* root length measured after the first day the seed lining on the soil. The value of significant coefficients is high showing a strong correlation between both val-

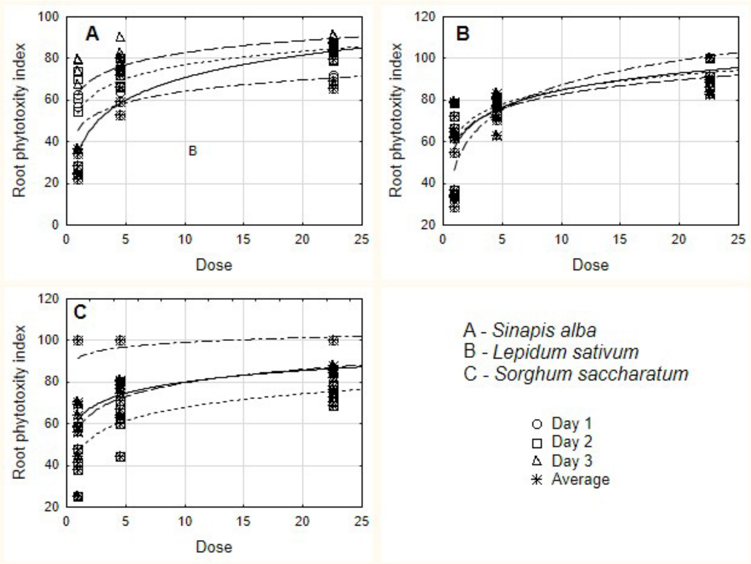

Fig. 3. Plots of dependence of soil phytotoxicity index determined with *Sinapis alba*, obtained at different time of seed exposure (test day), on the dose of creosote and logarithmic approximation curves

ues. The mean correlation coefficients are greatest for *Lepidium sativum*, and in descending order for *Sinapis alba* and *Sorghum saccharatum* (0.73055, 0.54743 and 0.42500, respectively), which indicates the high suitability of the first of these plants in the PHYTOTOXKIT test. This conclusion is also confirmed by high value of correlation coefficients for *Lepidium sativum* in the case of root length measurements on particular days, especially on the first day after seeding the soil.

Equations of exponential curves, which are presented in Table 3, can be used to calculate the value of the root phytotoxicity index for any dose of creosote in the soil. In order to verify the usefulness of this mathematical model, values of the root phytotoxicity index for the studied soil and applied creosote doses were calculated and then compared with values determined experimentally. As shown in Table 4, in which the correlation coefficients between root phytotoxicity indices determined experimentally and those calculated using mathematical modeling are presented, there is quite great compatibility. Correlation coefficients are highly significant (except for *Sorghum saccharatum* calculated when measuring the root length after one day from the seed lining to the soil) and have very high values, which indicates significant reliability of the mathematically obtained root phytotoxicity index. Although the PHYTOTOXKIT test manufacturer recommends the use of three plants and measurement of root length after 3 days, literature shows tests with different durations, up to 7 or even 11 days (Sekutowski and Sadowski 2009, Kanarbik *et al*. 2014, Wierzbicka *et al*. 2015). Some authors perform a test in their studies using different

Table 3. Logarithmic regression equations for the soil phytotoxicity index dependence on the creosote dose obtained for different exposure times of the seeds (test day) and correlation coefficients between the dose and the soil phytotoxicity index for test plants (statistically significant correlation coefficients indicated in bold)

Test plant	Test day	Matching equation	Correlation coefficient
Sinapis alba	1	Y = 59.7754 + 8.2784*log10(x)	**0.62659**
Sinapis alba	2	Y = 72.1348 + 9.3376*log10(x)	**0.63570**
Sinapis alba	3	Y = 80.1412 + 8.2701*log10(x)	**0.53818**
Sinapis alba	Average	Y = 70.6838 + 8.6287*log10(x)	**0.54743**
Lepidium sativum	1	Y = 56.8709 + 29.7674*log10(x)	**0.84635**
Lepidium sativum	2	Y = 75.8043 + 8.5868*log10(x)	**0.69890**
Lepidium sativum	3	Y = 78.7407 + 7.5397*log10(x)	**0.68629**
Lepidium sativum	Average	Y = 70.4720 +15.2980*log10(x)	**0.73055**
Sorghum saccharatum	1	Y = 100 + 3.8783-E-14*log10(x)	-
Sorghum saccharatum	2	Y = 44.8899 + 23.3268*log10(x)	**0.68025**
Sorghum saccharatum	3	Y = 61.9057 + 18.3354*log10(x)	**0.65989**
Sorghum saccharatum	Average	Y = 68.9319 + 13.8874*log10(x)	**0.42500**

amount of plants, or different sets of plants. And so Sekutowski and Sadowski (2009) used *Sinapis alba*, *Fagopyrum esculentum* and *Cucumis sativus* as test plants, Kopeć *et al*. (2017) and Ciesielczuk *et al.* (2018) used *Triticum* sp., *Sinapis alba* and *Lepidium sativum*, while Šestinová *et al*. (2012) – only *Sinapis alba* and Radziemska *et al.* (2017) – only *Lolium perenne*.

Table 4. Correlation coefficients between measured phytotoxicity indices and those calculated with use of model equations from Table 2 (statistically significant correlation coefficients indicated in bold)

Calculated for:	Calculated phytotoxicity index	
	With consideration of individual test day	With consideration of average for test days
Average for all plants and test day	**0.3624**	**0.5510**
Average for *Sinapis alba*	**0.3430**	**0.5420**
Average for *Lepidium sativum*	**0.4282**	**0.7478**
Average for *Sorghum saccharatum*	**0.3471**	**0.4242**
Sinapis alba test day 1	**0.9289**	
Sinapis alba test day 2	**0.9096**	
Sinapis alba test day 3	**0.9002**	
Lepidium sativum test day 1	**0.9656**	
Lepidium sativum test day 2	**0.9310**	
Lepidium sativum test day 3	**0.9570**	
Sorghum saccharatum test day 1	-	
Sorghum saccharatum test day 2	**0.9738**	
Sorghum saccharatum test day 3	**0.9669**	

Data analysis presented above prompts to consider the possibility of limiting the number of test plants to only one, namely as mentioned above the most useful *Lepidium sativum*, and to measure the length of the roots after the first day after laying them on the ground.

Table 5. Comparison of calculated and measured dose values and root phytotoxicity index (explanation in the text) – average values

Dose applied [g·kg^{-1}]	Root phytotoxicity index		Dose calculated [g·kg^{-1}]
	calculated	measured	
1	45.93	58.38	1.17
5	73.53	70.56	2.88
25	101.13	95.54	19.93

To verify the consistency of experimental and calculated data, a comparison was made, which is presented in Table 5. The first column contains the applied creosote doses. In the second column, the root phytotoxicity index is calculated using the variant of the equation for *Lepidium sativum* and the root length measurement on the first day after seed lining on the soil, which shows the best fit of the mathematical model to experimental values – correction factor of 0.84635 (RFI – root phytotoxicity index, c – creosote concentration in the soil).

$$RFI = 56.8709 + 29.7674 \times \log c$$

The third column lists values of root phytotoxicity indices measured experimentally and the fourth – creosote concentrations in the soil calculated on their basis. To calculate this, the equation has been transformed into a form that makes possible to calculate the concentration based on the value of the root phytotoxicity index.

$$c = 10^{(RFI - 56.8709) \div 29.7674}$$

The soil creosote concentration results obtained in the calculations were very similar to the real ones. The correlation coefficient between them was highly statistically significant and had high value of 0.9957. The scatter plot, in which both these values are presented, also indicates a good matching quality (Fig. 4). The line of relations between them runs almost exactly on the diagonal, and the points corresponding to individual doses are within the confidence interval of 0.05 (statistically significant).

Achieved results allow to simplify the methodology for determining the phytotoxicity of soils contaminated with creosote. Limiting the number of test plants to one and shortening the period of time, after which the root length measurement is carried out, does not affect the accuracy of the test. Applicability of the mathematical model allows to determine the amount of creosote in the soil with sufficient accuracy, e.g. for monitoring the course and progress of its reclamation.

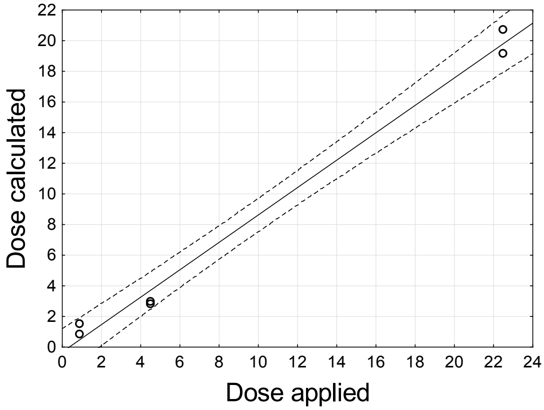

Fig. 4. Scatter plot indicating the fitting quality of the actual creosote dose in the soil and that calculated using the mathematical model

CONCLUSIONS

1. The results of PHYTOTOXKIT tests conducted with the use of soil from Lipnik allow obtaining equivalent values as with the use of reference soil, which enables correct interpretation of test results using soil from Lipnik.
2. Statistical analysis performed (ANOVA, correlation coefficients) indicates the unambiguous association of PHYTOTOXKIT test results to the level of creosote content in the soil.
3. The phytotoxicity interaction of creosote decreases gradually during the soil incubation, which is especially evident at its low doses.
4. The most sensitive to the presence of creosote in the soil is *Sorghum saccharatum* and then in descending order *Lepidium sativum* and *Sinapis alba*.
5. The highest correlation between the creosote dose in the soil and the root phytotoxicity index was obtained using *Lepidium sativum*, when measuring the root length after the first day of seeding the soil.
6. The use of the proposed mathematical modeling allows to predict the reaction of test plants on the size of the creosote dose as well as to assess its residue in the soil based on the root phytotoxicity index, which makes it possible to easily monitor the course of reclamation of contaminated soils.

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