STATISTICAL ANALYSIS OF SOME HEAVY METALS TOXICITY ON PLANTS USED IN PHYTOREMEDIATION

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Abstract. *Statistical analysis of data resulted from laboratory tests of three heavy metals (Cd, Pb, Ni) toxicity for a plant form Brassicaceae family, namely Brassica rapa (rape) was performed, using Minitab 18 software, as well. This analysis is justified since plants can be used in soil, water bioremediation by phytoremediation, when it is necessary to know the ability of plants to bioabsorb heavy metals and to face their toxicity. The collected data were processed by regression analysis to determine how the response variable changes when the predictor varies. In this case, the input variables are the concentration of heavy metals (Cd(II), Pb(II) and Ni(II), expressed in mg/L) and dry biomass of plant (g), and the response variable can be the length of roots or stems of rape. The analysis showed that the variables are moderately correlated and the influence of plant biomass on roots and stems growth can be neglected.*

Keywords: phytotoxicity, statistical analysis, root, stem, biomass

1. Introduction

1.1. Heavy metals in the environment

Heavy metals are among stable pollutants that are not subject to degradation processes, resulting in their concentration exceeding normal levels in soil, water and sediment, due mainly to massive industrialization and other related human activities [1].

Heavy metal pollution of living environments is due to both anthropogenic and natural activities. These pollutants are considered a "hazard to environmental health" and are included in the priority list of dangerous substances in the top 10 positions by the Agency for Toxic Substances and Diseases (ATSDR). According to information provided by IARC, most heavy metals are included in the list of substances classified according to their potential to cause cancer. Cadmium and

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hexavalent chromium are included in the first group - Carcinogenic for humans, lead, cobalt and nickel are part of group 2B - Possible carcinogenic for humans, and trivalent chromium and Group 3 mercury - Not classified as carcinogens [2]. Also, the toxicity of these persistent environmental pollutants depends on the detected concentrations (maximum admissible limits ranging from metal to metal) as well as their ability to bind to the thiol group in proteins, thus modifying the biochemical life cycle when they enter the cells of the organisms [1, 3]. The types of metals emitted as well as their quantities by sectors of activity are presented in Table 1.

Table 1. Emissions of heavy metals in the industrial sector registered in Romania at the level of 2015 [4]

Industrial sector	***The amount of metal emitted in water (kg)***						
	Cd	***Cr***	***Cu***	***Hg***	***Ni***	***Pb***	***Zn***
Energy production	22	321	578	1.8	243	284	280
Production and processing of metals	10	145		-	159	332	7470
Waste and wastewater management	340	3390	5060	-	3220	2220	31400
Ore processing			3940	-			3410
Chemical industry				32.4	65.4		155
Total	**372**	**3760**	**9580**	**34.2**	**3680.4**	**2830**	**42705**

Currently, there are many sites contaminated with different inorganic pollutants with different toxic and persistent characteristics at the global, regional and local level. The LUCAS database on the situation of heavy metals in European Union soils is very useful in deciding on soil protection strategies as well as mitigating the risks of bioaccumulation of metals along trophic chains (https://ec.europa.eu/eurostat/statistics-explained/index.php/LUCAS_-_Land_use_and_land_cover_survey). The minimum and maximum values detected at European level for these pollutants are presented in Table 2.

All these data call for detailed assessments and measures to reduce the amount of metal ions, especially in areas where heavy metal concentrations exceed the alert thresholds. All these measures must be in line with environmental standards and in line with current legislation requirements. Therefore, remediation of polluted sites has become a priority for society because of the increase in quality of life standards and environmental awareness. Currently, there are a variety of remedies for both solid and liquid media, which are classified into three major classes: physical, chemical and biological methods.

Table 2. Values of concentrations of heavy metals detected in soil at the level of the European Union [5]

Heavy metal	***Detected concentration (mg/kg)***		
	Minimum	***Maximum***	***Average***
Cd	0.02	3.17	0.09
As	0.46	252.53	3.72
Co	0.32	91.89	6.35
Cr	1.57	273.94	21.72
Cu	0.91	159.07	13.01
Hg	0	1.59	0.04
Mn	9.62	2285.23	373.05
Ni	0.36	466.48	16.36
Pb	1.63	151.12	15.3
Sb	0.01	10.91	0.25

1.2. Heavy metals removal by bioremediation using plants

Biological methods have gained particular attention from researchers in the field being viewed as feasible alternatives to physicochemical methods because they involve a number of environmentally friendly and cost-effective processes without the generation of toxic waste and can ensure eg remediation and restoration of the natural state of the soil [6-10].

Remediation of environmental components using plants, micro-organisms or other biological systems (bioremediation) capable of immobilizing / mobilizing / eliminating contaminants in the environment under controlled conditions (at a level below or / and to the extent that they become harmless) has a remarkable importance from the scientific, technological, socio-economic and cultural point of view [9, 11-15].

The mechanism of accumulation of heavy metals by plants is based on the root takeover and the transport of heavy metals into the plant. Radical take-over involves the following:

- roots can reduce metal ions bound to soil particles by metal-related reductases linked to the plasma membrane;
- plant roots can solubilize heavy metals by acidifying the soil with extruded protons in the roots;
- all these processes can be improved with mycorrhizal fungi and bacteria that colonize plant roots;
- solubilized metal ions can penetrate the roots through extracellular or intracellular pathways;
- non-essential heavy metals can compete effectively for the same transmembrane conveyor used by heavy metals.

The transport of heavy metals into the plant encompasses the following aspects:
- once penetrated into the root, metal ions can be stored or exported to the stem;
- metal transport takes place via xylem, but they can be redistributed in the stem by floe;
- in order to penetrate the ileum into the ileum, it must cross the intracellular symplastic barrier (intracellular penetration), and this can be the step of limiting the rate of translocation of the metal into the stem.

Although natural and / or controlled bioremediation processes can be used effectively in reducing environmental contamination and in preventing and controlling pollution, there are some difficulties with this approach. The effectiveness of plants and / or microorganisms in the bioremediation process is still limited by some shortcomings caused by the toxicity of target contaminants and the limited ability of living organisms involved in bioremediation to cope with the contaminated environment [16-19]. Excessive accumulation of contaminants in soil or water may have adverse effects on plants, called phytotoxic effects, which are manifested by inhibition of growth, photosynthesis disorders, decreased biomass, nutrient absorption deficit etc. [18-20].

Therefore, the tolerance of plants used in the bioremediation of various persistent pollutants must be thoroughly investigated and elucidated.

In this paper, some data resulted during plant tolerance tests against the toxicity of three heavy metals were processed and examined by statistical analysis, to demonstrate the correlation among heavy metal concentrations and plant biomass - on one hand, and the root and stem lengths as indicators of heavy metals effect on plants growth - on the other hand.

2. Materials and method

In this general context, for performing experiments on plant for environmental bioremediation, we have selected plant species suitable for laboratory investigations in toxicity tests, considering the rapid growth and ability to bioaccumulate heavy metals. The results discussed in this paper addresses the tolerance of a selected species belonging to the Brassicaceae family, namely *Brassica rapa* (rape).

The effects observed in terrestrial plant phytotoxicity tests can be grouped into two categories, namely:

- quantitative effects, in which results are obtained by measurements or counts. This category may include the following effects: seed germination number; the number of plants sprouted; germination or sprouting time; percentage of survival; stem height; root length; number of leaves; dry biomass produced by the above-ground plant parts and dry biomass of the roots;

- semi-quantitative effects, when results are obtained by observations. This category includes observations on abnormal changes in the growth, color, appearance of plants as compared to plants in the control samples. The semi-quantitative effect (in percent of the control) is appreciated and the results are then processed by statistical methods.

The results of the toxicity tests were recorded as Tables showing the observed effect at certain time intervals for each experimental variant and for each replicate. The basic action in the processing of the toxicity test results is the plotting of dose-effect or concentration-effect diagrams. To this end, the results recorded in the Tables are used for all dose and / or concentration-related experimental variants and for the control variant, considered as a zero dose or concentration. Depending on the test protocol, the results recorded at time t, which represents the duration of the test considered relevant to the test, are used in analysis. Also, depending on the number of replicates used in the test, the mean value of the effect is used to plot the dose-effect curve or concentration-effect curve. In addition, they are converted to percentages, which is a first statistical processing of the results, the average values having different intervals between the maximum and minimum values.

We should mention that the data obtained in the experimental trials are not shown in this paper, but only the results of statistical analysis.

The collected data were processed by regression analysis to determine how the response variable changes when the predictor varies. In this case, the input variables are the concentration of heavy metals (Cd(II), Pb(II) and Ni(II), expressed in mg/L) and dry biomass of plant (g), and the response variable can be the length of roots or stems of rape.

Parameter settings for regression analysis were as follows: confidence interval = 95, confidence interval type = both sides of the average, sum of squares for adjusted tests (type III), λ (for Box-Cox transformation) = 0.5 square).

3. Results and discussion

In the first stage, we have considered the response variables - the length of rape roots (L_{rr}) and the length of rape stems (L_{tr}), and as input variables - the concentration of heavy metals (C_{Cd}, C_{Pb}șiC_{Ni}) and dry rape biomass (root and stem) (B_{rr}, B_{tr}).

In the second step we performed the regression analysis using the Minitab 17 software (https://www.minitab.com/uploadedFiles/Documents/getting-started/Minitab17_GettingStarted-en.pdf), in which the input data were introduced and we obtained a regression equation (5), which represents an algebraic representation of the regression line and describes the relation of response and predictor variables:

$$Response^{0.5} = constant + coefficient \times predictor + \ldots + coefficient \times predictor \quad (5)$$

The length of rape roots (L_{rr}) was considered as a response variable (L_{rr}= f(C_{Cd}, B_{rr})). After entering the data in the Minitab software, Eq. (6) for Cd (II) toxicity was obtained:

$$L_{rr}{}^{0.5} = 2.056 - 0.01155 \times C_{Cd} + 421 \times B_{rr} \quad (6)$$

Eq. (7) was obtained when the length of rape stems (L_{tr}) was considered as response variable (L_{tr}= f(C_{Cd}, B_{tr})).

$$L_{tr}{}^{0.5} = 2.779 - 0.00644 \times C_{Cd} + 20.9 \times B_{tr} \quad (7)$$

When the input variables were considered as the concentration of Pb(II) (C_{Pb}) and the rape root biomass (B_{rr}), and the length of rape roots L_{rr}= f(C_{Pb}, B_{rr}) was considered as the response variable, the regression equation (Eq. 8) was obtained:

$$L_{rr}{}^{0.5} = 4.402 - 0.01137 \times C_{Pb} + 510 \times B_{rr} \quad (8)$$

Eq. (9) resulted by considering the length of rape stems (L_{tr}) as response variable (L_{tr}= f(C_{Pb}, B_{tr})).

$$L_{tr}{}^{0.5} = 2.637 - 0.00051 \times C_{Pb} + 63.1 \times B_{tr} \quad (9)$$

Also, the concentration of Ni(II) (C_{Ni}) and rape roots biomass (B_{rr}) were considered as input variables, and the length of rape roots(L_{rr}= f(C_{Ni}, B_{rr}) was considered as the response variable, the regression equation (Eq. 10) was obtained:

$$L_{rr}{}^{0.5} = 3.589 - 0.01317 \times C_{Ni} + 49 \times B_{rr} \quad (10)$$

Eq. (11) resulted by considering the lenght of rape stems (L_{tr}) as a response variable (L_{tr}= f(C_{Ni}, B_{tr})).

$$L_{tr}{}^{0.5}=4.051-0.01022\times C_{Ni}-0.5\times B_{tr} \qquad (11)$$

The model for the transformed response was analyzed: adjusted S, R^2 and R^2 adjusted were calculated, giving information on the extent to which the models represent the experimental data. S is the standard distance in which data values deviate from the regression line: the equation predicts the response much better if S has a lower value. R-Sq or R^2 quantitatively describes the variance of the observed response values that is explained by the predictors: if R^2 is close to 100, the results are better. R^2 adjusted (R^2(adj)) is a modified R^2 that has been adjusted for the number of terms in the model. This indicator is useful when comparing models with a different number of predictors. R^2 predicted (R^2(pred)) is a measure on how the model predicts the answer, if there are large differences between this R^2 (pred) and the other two statistics (R^2 and R^2(adj)) it results that the model is outdated. In this study the following values of the statistical parameters were obtained:

- when the lenght of rape roots (L_{rr}) was considered as response variable:

- L_{rr}= f(C_{Cd}, B_{rr}), S=0.85, R^2 = 68.49%, R^2 (adj)= 65.49%, R^2(pred) = 57.44%;
- L_{rr}= f(C_{Pb}, B_{rr}), S=0.64, R^2 = **88.99**%, R^2 (adj)= **87.94**%, R^2(pred) = **83.65**%;
- L_{rr}= f(C_{Ni}, B_{rr}), S=1.07, R^2 = 63.77%, R^2 (adj)= 60.32%, R^2(pred) = 49.74%;

- when the lenght of rape stems (L_{rr}) was considered as response variable:

- L_{tr}= f(C_{Cd}, B_{tr}), S=0.56, R^2 = 64.67%, R^2 (adj)= 61.31%, R^2(pred) = 52.40%;
- L_{tr}= f(C_{Pb}, B_{tr}), S=0.47, R^2 = 11.45%, R^2 (adj)= 3.01%, R^2(pred) = 0.00%;
- L_{tr}= f(C_{Ni}, B_{tr}), S=0.65, R^2 = **74.49**%, R^2 (adj)= 72.06%, R^2(pred) = 68.75%;

The results showed that R^2 indicator has the highest value for rape root length (output variable) influenced by input variables C_{Pb}, B_{rr}, value close to 100, which means that the results obtained are good. In the case of L_{rr}= f(C_{Cd}, B_{rr}) and

L_{rr}= f(C_{Ni}, B_{rr}) it can be said that together, the two predictors represent 68.49% and 63.77% respectively of the variation of the length of the rape roots. Also, the R^2 indicator has the highest value for the length of rape stems (output variable) influenced by input variables C_{Ni}, B_{rr}, R^2 = 74.49%. In the case of L_{tr}= f(C_{Cd}, B_{tr}) șiL_{tr}= f(C_{Pb}, B_{tr}) it can be said that together, the two predictors represent 64.67% and 11.45% respectively from the variation in the length of the rape stems. The low predicted values of R^2 indicator (0.00%) suggests that the model will not anticipate new observations as well as the sample data studied. Typically, generalizations beyond sample data will not be generalized when the model has a low prediction level of R^2.

Table 3 presents the variance analysis for the transformed response, which shows the magnitude of the variation in the given response, explained by the predictors. The most important results to be considered are the p values. For the interpretation of the p value, a confidence threshold α level is used which is usually 0.05. If for regression the p value is 0.000, it indicates that at least one of the regression coefficients is significantly different from zero.

Table 3. Variance analysis for the transformed response in the context of experiments on the toxicity of Cd(II), Pb(II), Ni(II) metal ions on root and stems lengths of rape

L_{rr}= f(C_{Cd}, B_{rr})					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Regression	2	33.299	16.6494	22.82	**0.000**
C_{Cd}	1	30.712	30.7119	42.10	**0.000**
B_{rr}	1	9.814	9.8144	13.45	**0.001**
Error	21	15.319	0.7295		
Total	23	48.618			
L_{tr}= f(C_{Cd}, B_{tr})					
Regression	2	12.3026	6.1513	19.22	**0.000**
C_{Cd}	1	6.9480	6.9480	21.71	**0.000**
B_{tr}	1	0.1375	0.1375	0.43	0.519
Error	21	6.7205	0.3200		
Total	23	19.0230			
L_{rr}= f(C_{Pb}, B_{rr})					
Regression	2	71.551	35.7753	84.89	**0.000**
C_{Pb}	1	15.007	15.007	35.61	**0.000**
B_{rr}	1	5.176	5.175	12.28	**0.002**
Error	21	8.850	0.4214		
Total	23	80.400			
L_{tr}= f(C_{Pb}, B_{tr})					
Regression	2	0.60089	0.30045	1.36	0.279
C_{Pb}	1	0.05581	0.05581	0.25	0.621

B_{tr}	1	0.59817	0.59817	2.7	0.115
Error	21	4.64915	0.22139		
Total	23	5.25004			
L_{rr}= f(C_{Ni}, B_{rr})					
Regression	2	42.329	21.164	18.48	**0.000**
C_{Ni}	1	38.31	38.31	33.45	**0.000**
B_{rr}	1	0.0668	0.0668	0.06	0.812
Error	21	24.0527	1.1454		
Total	23	66.38			
L_{tr}= f(C_{Ni}, B_{tr})					
Regression	2	26.13	13.06	30.67	**0.000**
C_{Ni}	1	21.70	21.70	50.91	**0.000**
B_{tr}	1	0.0003	0.0003	0.0	0.979
Error	21	8.9504	0.4262		
Total	23	35.09			

In the case of $Lrr = f(C_{Cd}, B_{rr})$ it can be observed that C_{Cd}, B_{rr} are significant factors for analysis, and when $Ltr = f(C_{Cd}, B_{tr})$,C_{Cd} is a significant factor for analysis (p = 0.000) and $B_{tr}$is an insignificant factor factor (p<0.05). C_{Pb}şiB_{rr} are significant factors for the $L_{tr} = f(C_{Pb}, B_{tr})$ analysis, while C_Pb and $B_{tr}$are insignificant factors for the $L_{tr} = f(C_{Pb}, B_{tr})$ analysis. For the influence of Ni(II) concentrations and biomass on the length of roots and stems of rape it can say that C_{Ni} is a significant factor for analysis, instead B_{rr}şiBtr are insignificant factors (p>0.05) according to Table 1.

Therefore, the toxicity analysis of heavy metal ions, performed in terms of the length of rape roots and stems on metal ion concentration, will lead to significant results, and biomass dependence can be ignored.

In the regression analysis, the variation of the Inflation Factor (VIF) shows how large the magnitude of a coefficient variance is. If VIF values approach 1, this indicates that predictors are not correlated; when 1<VIF<5 predictions are moderately correlated; if the values are greater than 5-10 (the predictors are very well correlated), this suggests that the regression coefficients are poorly estimated. Table 4 shows that VIF values are between 1 and 2.16, resulting that predictors (independent variables) are being moderately correlated.

A graphical technique for evaluating the distribution of the data set is the *normal probability graph.* From this graph it can be seen if the dataset is distributed approximately normally (i.e., the normal statistical distribution parameters can be estimated). When the points on this graph form an almost linear pattern, it shows that the normal distribution is a good model for the evaluated data. From Fig. 1 it can be seen that the points are very close to the linear model.

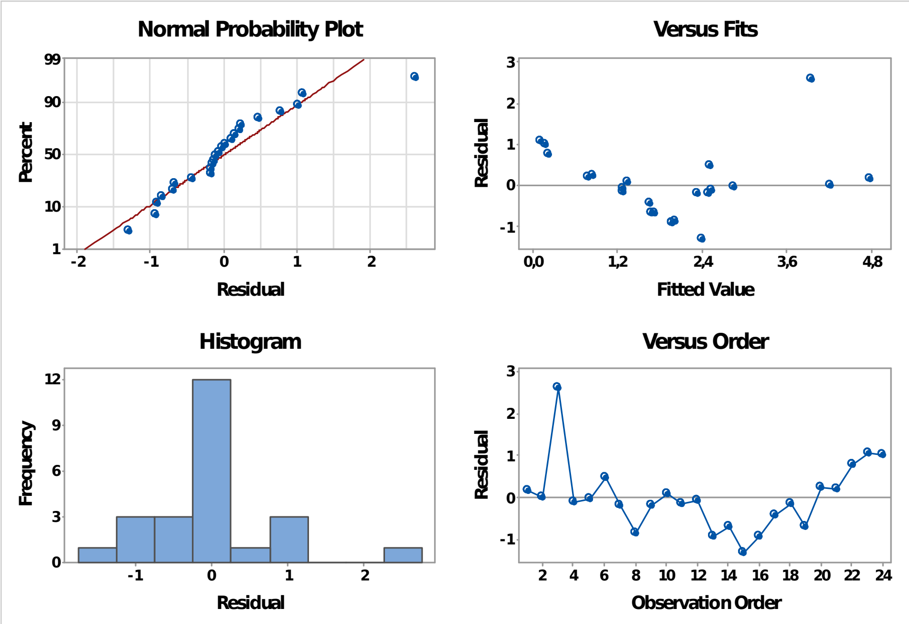
The distribution of a univariate data set can be seen in a histogram-like graphical representation. From Fig. 1a it can be seen that the variability is between -2 and

+3, with the possibility of an outlier (a point that is placed outside the range generated by most points), while Fig. 1b shows that variability is between -1 and 0.75 (without the possibility of an outlier).

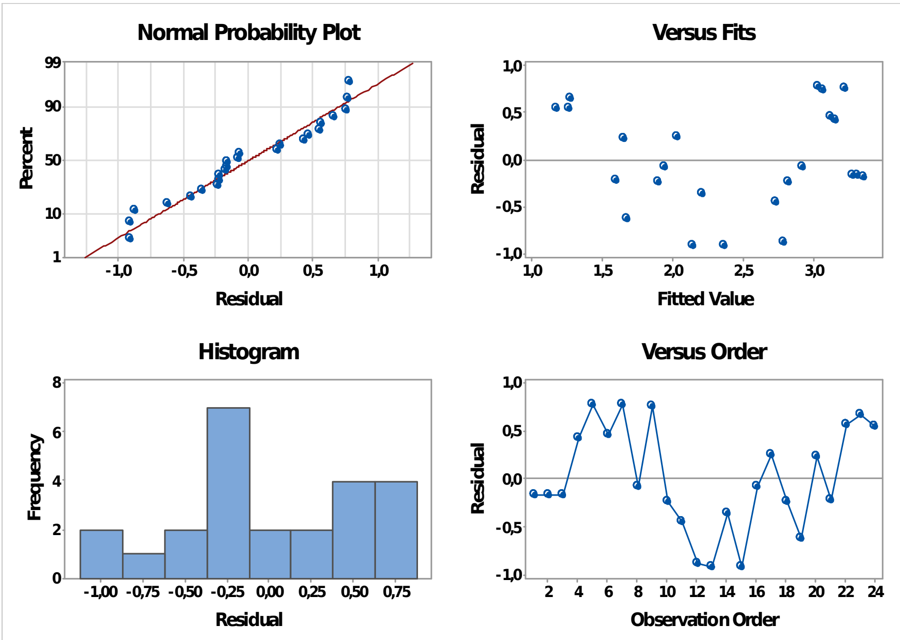
From Fig. 2a it can be seen that the histogram is bimodal, it has the center at zero and the variability is between -1.2 and 1.2. In the case of the histogram shown in Fig. 2b we will see that the variability is between -1.25 and 0.50 with the possibility of existence of outliers. Also, from Fig. 3a it can be seen that the variability is between -1 and 3 (with a rather large possibility of existence of outliers). The histogram shown in Fig. 3b is bimodal with zero center and variability between -1 and 1.

Table 4. Coefficients for the transformed response, in VIF values, in the context of experiments on the toxicity of Cd(II), Pb(II), Ni(II) metal ions on the length of roots and stems of rape

L_{rr}= f(C_{Cd}, B_{rr})		
Term	**P-Value**	**VIF**
Constant	**0.000**	
C_{Cd}	**0.000**	1.09
B_{rr}	**0.001**	1.09
L_{tr}= f(C_{Cd}, B_{tr})		
Constant	**0.000**	
C_{Cd}	**0.000**	1.5
B_{tr}	0.519	1.5
L_{rr}= f(C_{Pb}, B_{rr})		
Constant	**0.000**	
C_{Pb}	**0.000**	2.16
B_{rr}	**0.002**	2.16
L_{tr}= f(C_{Pb}, B_{tr})		
Constant	**0.008**	
C_{Pb}	0.621	1.16
B_{tr}	0.115	1.16
L_{rr}= f(C_{Ni}, B_{rr})		
Constant	**0.000**	
C_{Ni}	**0.000**	1.14
B_{rr}	0.812	1.14
L_{tr}= f(C_{Ni}, B_{tr})		
Constant	**0.000**	
C_{Ni}	**0.000**	1.21
B_{tr}	0.979	1.21

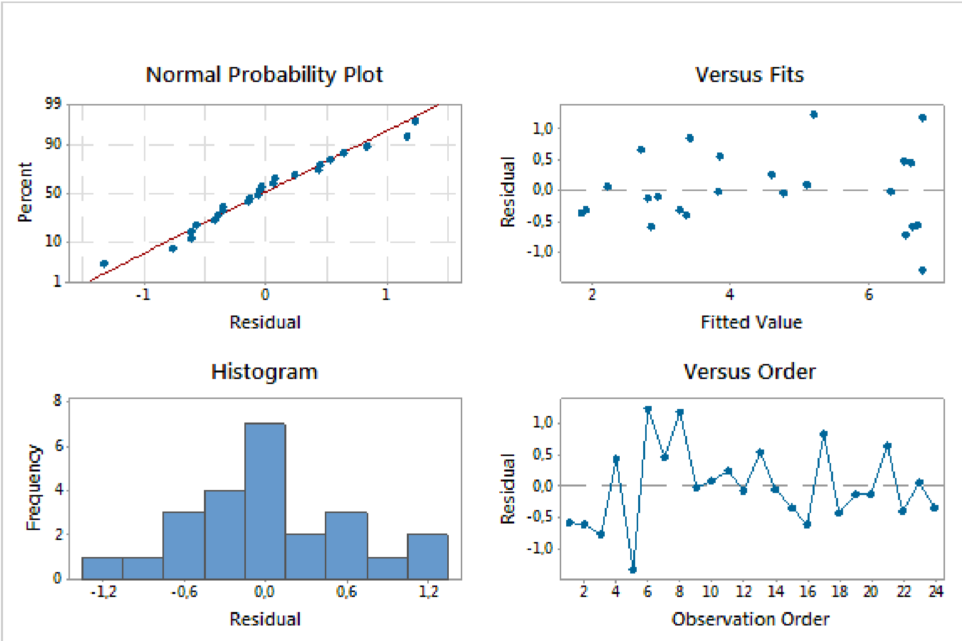


a)

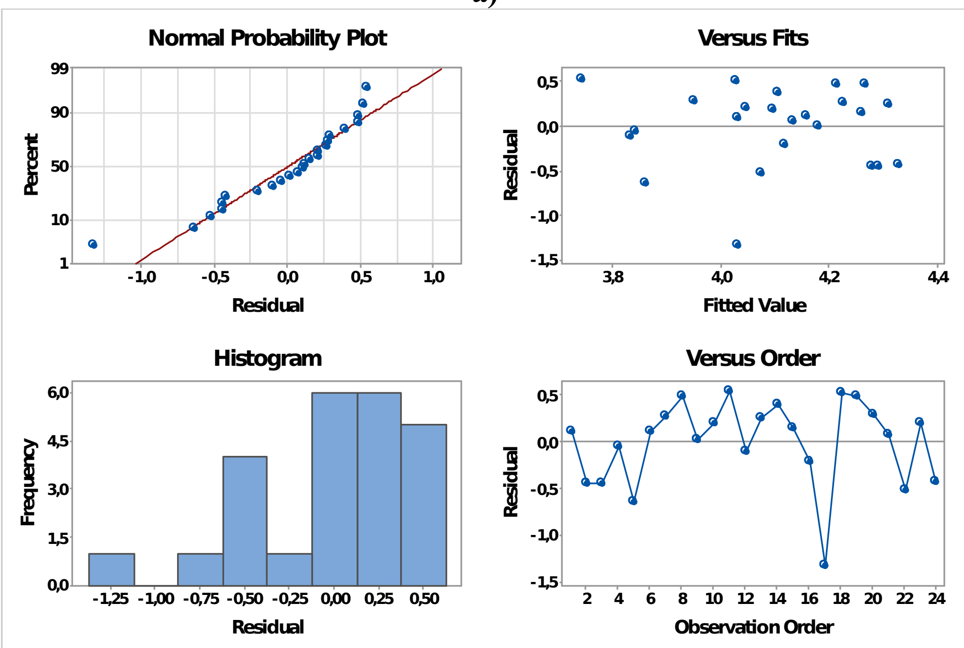


b)

Fig. 1. Residual charts for: a) L_{rr}= f(C_{Cd}, B_{rr}) b) L_{tr}= f(C_{Cd}, B_{tr})

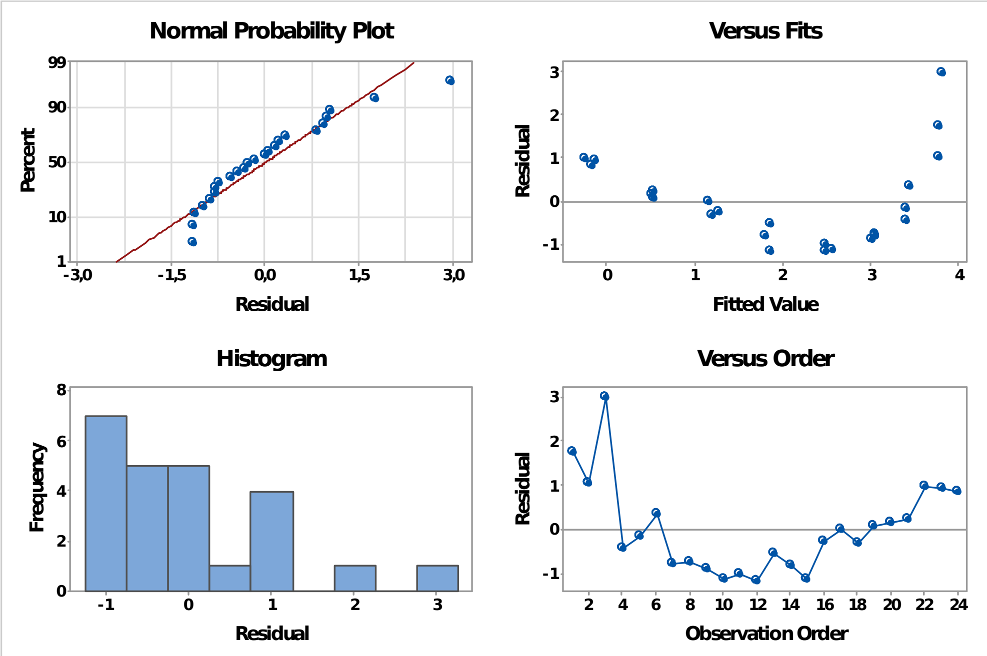


a)

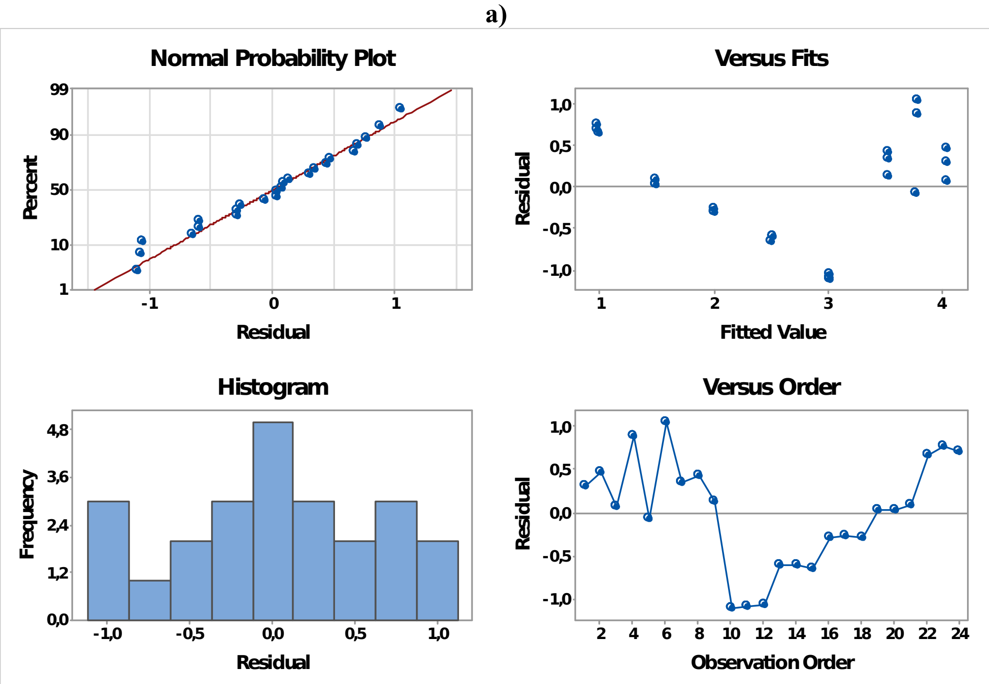


b)

Fig. 2. Residual charts for: a) L_{rr}= f(C_{Pb}, B_{rr}) b) L_{tr}= f(C_{Pb}, B_{tr})



a)



b)

Fig. 3. Residual charts for: a) L_{rr}= f(C_{Ni}, B_{rr}) b) L_{tr}= f(C_{Ni}, B_{tr})

Conclusions

The statistical analysis applied to evaluate the data resulted from laboratory tests of three heavy metals (Cd, Pb, Ni) toxicity on rape revealed the importance of the input and output variables.

The results showed that R^2 indicator has the highest value for rape root length (output variable) close to 100, influenced by input variables, namely concentration of lead, and rape biomass (C_{Pb}, B_{rr}), value which means that the results obtained are good. The low predicted values of R^2 indicator (0.00%) suggests that the model will not anticipate new observations as well as the sample data studied. Typically, generalizations beyond sample data will not be generalized when the model has a low prediction level of R^2. For the influence of Ni(II) concentrations and biomass on the length of roots and stems of rape it can say that C_{Ni} is a significant factor for analysis, instead B_{rr}șiBtr are insignificant factors ($p>0.05$). Therefore, the toxicity analysis of heavy metal ions performed in terms of the length of rape roots and stems on metal ion concentration, led to significant results, and showed that biomass dependence can be ignored.

Variation of the Inflation Factor (VIF) shows how large the magnitude of a coefficient variance is. Data shows that VIF values are between 1 and 2.16, resulting that predictors (independent variables) are being moderately correlated.

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