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Experimental study and parameters optimization of heavy metals phytoremediation removal process by *Azolla pinnata* using response surface methodology

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ABSTRACT: Heavy metals contamination of industrial wastewater is a significant environmental concern due to the toxicity, persistence, and bio accumulative nature of metals such as cadmium (Cd), nickel (Ni), and lead (Pb).

Phytoremediation is efficient, eco-friendly and economic selected for the treatment using *Azolla pinnata* as quantic plant. The physical characterization of *Azolla pinnata* shows structural characteristic contributed significantly to the high removal efficiency.

This study investigated the potential of the aquatic *Azolla pinnata* as a low cost and environmentally sustainable phytoremediation agent for the removal of Cd, Ni, and Pb from contaminated water. Synthetic wastewater containing different initial metals concentrations (0.5, 1, 3, and 5 mg/L) was treated with *A. pinnata*, and changes in metal concentrations were monitored throughout the treatment period. Metals removal efficiency was evaluated based on the reduction in dissolved metal concentrations.

Response surface methodology (RSM) was applied to optimize the effects of biomass dosage, treatment time, and initial metal concentration for the high removal of these meals. Analysis of variance (ANOVA) was used to determine the significance of the experimental factors and their interactions. The results demonstrated that *Azolla pinnata* effectively removed the investigated heavy metals, with particularly high removal performance observed for Pb and Cd. The optimization analysis indicated that a treatment time of approximately 18.4 days and an *A. pinnata* biomass of 49.4 g provided the optimized conditions for achieving maximum simultaneous removal of Cd, Ni, and Pb. These findings demonstrate the potential of *Azolla pinnata* as an effective, economical, and environmentally friendly phytoremediation material for heavy metals contaminated wastewater. The use of plant biomass also offers potential for sustainable treatment systems by reducing dependence on conventional chemical-based remediation methods.

1. INTRODUCTION

Heavy metals are defined as metallic elements that have a relatively high density compared to water [1]. With the assumption that heaviness and toxicity are inter-related, heavy metals also include metalloids, such as arsenic, that are able to induce toxicity at low level of exposure. In recent years, there has been an increasing ecological and global public health concern associated with environmental contamination by these metals. Also, human exposure has risen dramatically as a result of an exponential increase of their use in several industrial, agricultural, domestic and technological applications. Reported sources of heavy metals in the environment include geogenic, industrial, agricultural, pharmaceutical, domestic effluents, and atmospheric sources. Environmental pollution is very prominent in point source areas such as mining, foundries and smelters, and other metal-based industrial operations [1, 2, 3, 4].

Heavy metals are defined as metallic elements that have a relatively high density compared to water, it has harmful effects on human health, and exposure to these metals has been increased by industrial and anthropogenic activities and modern industrialization. Contamination of water and air by toxic metals is an environmental concern and hundreds of millions of people are being affected around the world. Food contamination with heavy metals is another concern for human and animal health [4,5]. Heavy metals are released into the environment from various sources including mining, urbanization, chemical industry, sewage plants, pesticide plants, biomedical and unsafe agricultural practices [6].

Although heavy metals are naturally occurring elements that are found throughout the earth's crust, most environmental contamination and human exposure result from anthropogenic activities such as mining and smelting operations, industrial production and use, and domestic and agricultural use of metals and metal-containing compounds [5-8]. Environmental contamination can also occur through metal corrosion, atmospheric deposition, soil erosion of metal ions and leaching of heavy metals, sediment re-suspension and metal evaporation from water resources to soil and ground water [8]. Natural phenomena such as weathering and volcanic eruptions have also been reported to significantly contribute to heavy metal pollution [7-9]. Industrial sources include metal processing in refineries, coal burning in power plants, petroleum combustion, nuclear power stations and high-tension lines, plastics, textiles, microelectronics, wood preservation and paper processing plants [10,11].

Heavy metals are quintessential to maintain various biochemical and physiological functions in living organisms when in very low concentrations, however they become noxious when they exceed certain threshold concentrations. Although it is acknowledged that heavy metals have many adverse health effects and last for a long period of time, heavy metal exposure continues and is increasing in many parts of the world. Heavy metals are significant environmental pollutants and their toxicity is a problem of increasing significance for ecological, evolutionary, nutritional and environmental reasons [12].

Cadmium is the seventh most toxic heavy metal. It is a by-product of zinc production which humans or animals may get exposed to at work or in the environment. Once this metal gets absorbed by humans, it will accumulate inside the body throughout life. This metal was first used in World War I as a substitute for tin and in paint industries as a pigment. In today's scenario, it is also being used in rechargeable batteries, for special alloys production and also present in tobacco smoke. About three-fourths of cadmium is used in alkaline batteries as an electrode component, the remaining part is used in coatings, pigments and plating and as a plastic stabilizer. Humans may get exposed to this metal primarily by inhalation and ingestion and can suffer from acute and chronic intoxications. Cadmium distributed in the environment will remain in soils and sediments for several decades. Plants gradually take up these metals which get accumulated in them and concentrate along the food chain, reaching ultimately the human body.

Nickel is a transition element extensively distributed in the environment, air, water, and soil. It may derive from natural sources and anthropogenic activity. Although nickel is ubiquitous in the environment, its functional role as a trace element for animals and human beings has not been yet recognized. Environmental pollution from nickel may be due to industry, the use of liquid and solid fuels, as well as municipal and industrial waste. Nickel contact can cause a variety of side effects on human health, such as allergy, cardiovascular and kidney diseases, lung fibrosis, lung and nasal cancer.

Lead is a highly toxic metal whose widespread use has caused extensive environmental contamination and health problems in many parts of the world. Lead is a bright silvery metal, slightly bluish in a dry atmosphere. It begins to tarnish on contact with air, thereby forming a complex mixture of compounds, depending on the given conditions. The sources of lead exposure include mainly industrial processes, food and smoking, drinking water and domestic sources. The sources of lead were gasoline

and house paint, which has been extended to lead bullets, plumbing pipes, pewter pitchers, storage batteries, toys and faucets [13-17].

The recommended limits of heavy metals in drinking water based on Environmental Protection Agency (EPA) guidelines are 5.0, 100, and 15 ppb for Cd, Ni, and Pb, respectively, [18].

There are numerous methods available to remove heavy metals from the environment, including ion exchange, coagulation and precipitation with iron and aluminium salts, adsorption, electrocoagulation, membrane filtration, nanofiltration, electrolysis, reverse osmosis. These techniques, however, are expensive and negatively impact the impacted area. Consequently, novel alternative treatments utilizing inexpensive materials are required, like bioremediation, and phytoremediation [19-21].

Removal of heavy metals from the wastewater is difficult because they exist in different chemical forms. Most metals are not biodegradable, and they can easily pass through different trophic levels to persistently accumulate in the biota [22,23]. Various techniques used for the removal of heavy metals from wastewater such as reverse osmosis [24], ion exchange [25], chemical precipitation [26], adsorption [27] and phytoremediation [28]. Phytoremediation is based upon several processes such as phytodegradation, phytovolatilization, phytoaccumulation and phytoextraction. These methods are efficient, eco-friendly and economic [29]. Different aquatic plants have been used with considerable success in the wastewater phytoremediation process such as Duckweeds (*Lemna* sp.), Small water fern (*Azolla* sp.) and blue cabbage (*Pistia* sp.) have been used to remove heavy metals from wastewater [30, 31]. The *Lemna gibba* is a tiny, free-floating aquatic plant that is a member of the Lemnaceae family. Because they can develop quickly, are easier to harvest, and are more tolerant of cold than other species, duckweed species are used in water quality studies to assess heavy metals and are thought to be a superior alternative for wastewater treatment [32,34]. It was indicated that *Azolla pinnata* has the potential as an agent of phytoremediation especially for wastewater treatment [35,36].

Response Surface Methodology (RSM) used for the prediction of maximum heavy metals removal [37-39]. It has been discovered that response surface methodology (RSM) is a helpful technique for researching how two or more variables interact [40]. RSM is used to estimate an area that satisfies the operating parameters and determine the system's ideal operating conditions [41,42].

In this research study *Azolla pinnata* used as aquatic plant for phytoremediation of heavy metals from synthetic industrial waste water contaminated with varying percentages of Cd, Ni, and Pb at varying exposure durations was treated by this plant at varying weights. Through the use of response surface methodology (RSM), this study seeks to optimize the removal effectiveness of these materials from wastewater by optimizing exposure period, *Azolla pinnata* weight, and heavy metals concentration.

2. MATERIALS AND METHODS

2.1 Aquatic plant

Azolla pinnata (*Azolla* P.) was selected due to the high metal uptake capacity, rapid growth, environmental adaptability, and their ability to converted to biochar as adsorbent with enhanced physicochemical properties for heavy metal removal.

2.2 Synthesis of Cd, Ni, and Pb Wastewater

A series of combined solutions of Cd, Ni, and Pb (0.5, 1, 3, and 5 mg/L initial concentration) were prepared by diluted certified stock solution of heavy metals (1000 mg/L) Cd, Ni, and Pb, (Sigma-Aldrich, Germany) in distilled water to achieve the required concentrations in order to synthesis wastewater.

2.3 Statistical Analysis

The phytoremediation process parameters were studied with a standard response surface methodology (RSM) design called a central composite design (CCD). This method is suitable for fitting a quadratic surface and it helps to optimize the effective parameters with a minimum number of experiments, and also to analyze the interaction between the parameters. Generally, the CCD consists of a $2n$ factorial runs with $2n$ axial runs and n_c center runs (six replicates).

The phytoremediation removal of cadmium, nickel lead ions from synthesis industrial waste water was studied using (RSM). The input variables studied were (i) x_1 , initial concentration of heavy metals (Cd, Ni and Pb); (ii) x_2 , *Limna* G. weight and (iii)

x3, treatment time. These three variables together with their respective ranges were chosen based on the literature and preliminary studies. The number of experimental runs from the central composite design (CCD) for the three variables consists of eight factorial points, six axial points and six replicates at the centre points indicating that altogether 20 experiments were required, as calculated from the below equation:

$$N = 2n + 2n + n_c = 23 + 2 \times 3 + 6 = 20 \quad (1)$$

Where N is the total number of experiments required and n is the number of process variables.

The experimental sequence was randomized in order to minimize the effects of the uncontrolled factors. The response (Y1, Y2 and Y3) were cadmium, nickel and lead removals, respectively. The response was used to develop an empirical model which correlated the response to the three treatment process variables using a second-degree polynomial equation as given by below equation:

$$Y = b_o + \sum_{i=1}^n b_i x_i + \sum_{i=1}^n b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n b_{ij} x_i x_j \quad (2)$$

Where Y is the predicted ions removal response, the constant coefficient, b_i the linear coefficients, b_{ij} the interaction coefficients, b_{ii} the quadratic coefficients and x_i , x_j are the coded values of the phytoremediation ions removal variables. The parameters involved in the treatment were varied using the response surface methodology (RSM). The three variables studied were x1 is the initial concentration of heavy metals (Cd, Ni and Pb) in (mg/L), x2 is the Azolla P. weight in (g), and x3 is the phytoremediation treatment time in (day).

The design matrix consisted of three input variables (X1: concentration, X2: exposure time, X3: biomass) with 20 combinations, including six replicates at the center point to ensure reproducibility and statistical validity.

The responses were analyzed using analysis of variance (ANOVA) to test model adequacy, regression coefficients, and significance of interaction terms. The three dependent responses in this study were: Y1: Removal of Cd, Y2: Removal of Ni, and Y3: Removal of Pb.

The predicted error of experimental and predicted results for Cd, Ni and Pb removal calculated on the following equation:

$$\% \text{Prediction Error} = \frac{(\text{predicted} - \text{experimental})}{\text{predicted}} \times 100 \quad (3)$$

2.4 Phytoremediation experimental

The experiments performed using plastic containers. The Cd, Ni and Pb, phytoremediation efficiency of Azolla pinnata was assessed using 3L of synthesis wastewater with different initial concentrations over a period of 15 days. 30 g of plant used as active weight for the phytoremediation process. The synthesis wastewater samples collected for the purpose of determine Cd, Ni, and Pb concentrations before the experiments and, periodically at days 1, 5, 10, and 15. Atomic absorption spectrometer (Shimadzu, Model 7000, Japan) used for analyze concentrations. All experiments were performed in triplicate, and the results are reported as the mean. Metal removal efficiency calculates using equation (4):

$$\% \text{ (Removal)} = \frac{(C_o - C_e)}{C_o} \times 100 \quad (4)$$

where C_o and C_e (mg/L) are the concentrations of metals at initial and equilibrium, respectively

3. RESULTS AND DISCUSSION

3.1 Physical characterization of Azolla pinnata

3.1.1 Elemental composition (EDX)

Table 1, present the elemental composition of the selected plant, carbon contents was measured as 37.3 %, oxygen contents were 32.7 % and the minor elements, including Mn, Si, Cu, and S, were also detected in small amounts. These elements were

present because the plant fibres were not chemically treated or extensively washed prior to analysis, and were directly extracted from the parent biomass.

Table 1: Elemental composition of *Azolla pinnata*

Element	Atomic %	Atomic % Error	Weight %	Weight % Error
C	47.7	0.2	37.3	0.1
N	10.6	0.6	9.7	0.5
O	31.4	0.2	32.7	0.2
Na	3.8	0.0	5.7	0.0
Mg	0.9	0.0	1.5	0.0
Si	0.1	0.0	0.1	0.0
P	0.4	0.0	0.8	0.0
S	0.6	0.0	1.2	0.0
Cl	1.7	0.0	3.9	0.0
K	2.2	0.0	5.6	0.0
Ca	0.4	0.0	1.1	0.0
Cu	0.1	0.0	0.5	0.0

3.1.2 Morphology and microstructure analysis

Scanning Electron Microscopy (SEM) was used to observe the surface morphology and microstructure of the aquatic plant. SEM image was captured at 50× magnifications. Figures 1 show the SEM micrographs of *Azolla P.*, structure micrographs revealed a porous and irregular surface structure, providing numerous active sites for heavy metal binding. This structural characteristic contributed significantly to the high removal efficiency.

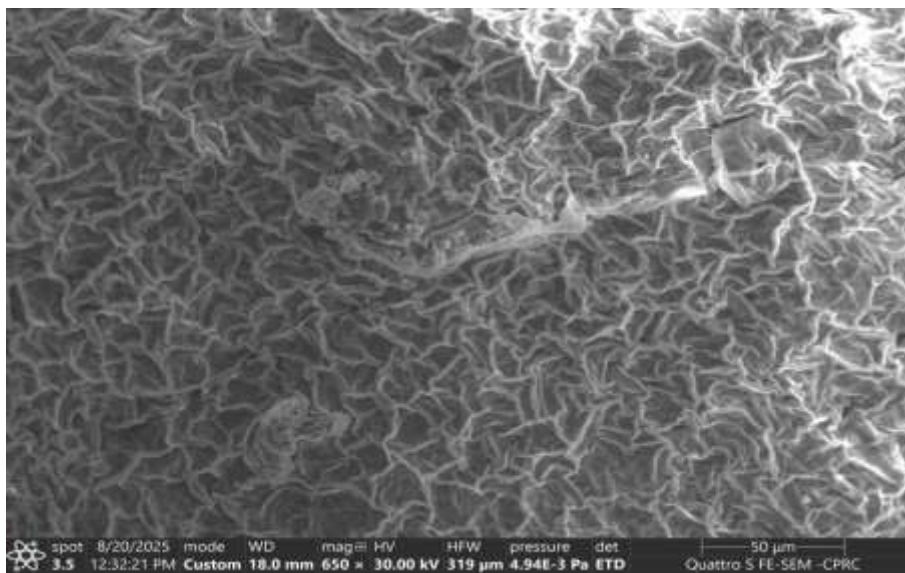


Figure 1: SEM image of *Azolla P.*

3.1.3 Analysis of plant surface chemistry

Figure 2 show the FTIR spectra of *Azolla P.*, the spectra exhibited distinct functional groups on the raw biomass. FTIR spectra also confirmed functional groups associated with metal binding. For example, *Azolla P.* exhibited strong peaks related to hydroxyl and carboxyl groups, making it suitable for Pb and Cu removal, with the potential for biomass reuse in multiple cycles.

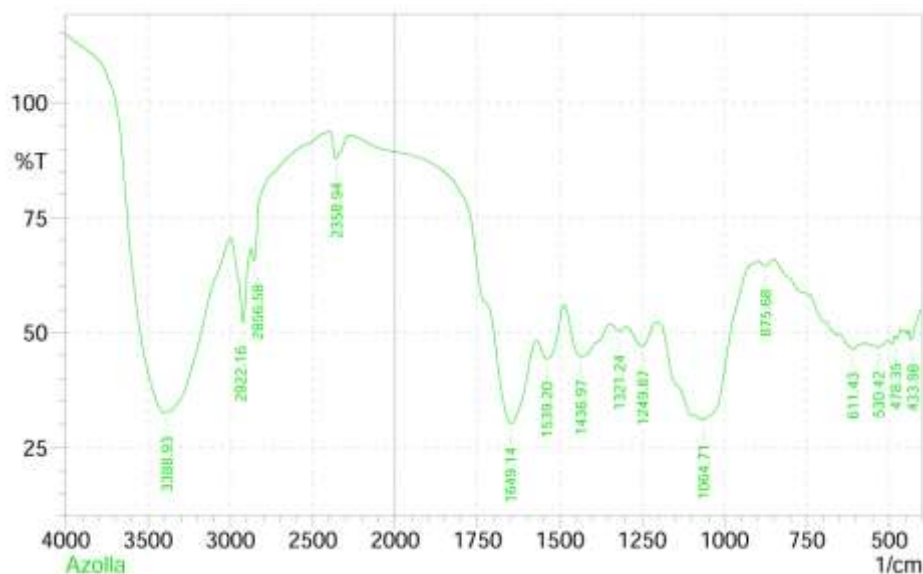


Figure 2: Fourier-transform infrared spectroscopy (FT-IR) spectra of *Azolla P.*

3.2 Phytoremediation removal matrix of Cd, Ni, and Pb using *Azolla P.*

Table 2, show the matrix of predicted experimental data for the interaction between input and output parameters for the removal of heavy metals from the synthesis wastewater by phytoremediation using *Azolla P.*, which shows predicted data and experimental results, indicating the relationship between input parameters (Cd, Ni, and Pb concentration, mg/L), (exposure time, day), and the (weight of *Azolla P.*, gm) and the response of heavy metals removal

Table 2: DOE matrix for Cd, Ni, and Pb removal using *Azolla P.*

Run	Initial Concentration, X1 (mg/L)	Exposure time, X2 (day)	<i>Azolla P</i> weight, X3 (g)	Cd removal, Y1 (%)	Ni removal, Y2 (%)	Pb removal, Y3 (%)
1	2.75	11.00	45.00	92	90	100
2	2.75	11.00	45.00	92	90	100
3	5.00	21.00	50.00	100	100	100
4	0.50	21.00	40.00	100	100	100
5	5.00	21.00	40.00	100	98	100
6	2.75	11.00	45.00	92	90	100
7	0.50	21.00	50.00	100	100	100
8	2.75	1.00	45.00	48	45	75
9	2.75	11.00	45.00	92	90	100
10	2.75	11.00	50.00	95	93	100
11	5.00	1.00	40.00	24	21	47
12	0.50	1.00	50.00	45	40	75
13	2.75	11.00	45.00	92	90	100
14	0.50	1.00	40.00	41	37	70
15	2.75	11.00	45.00	92	90	100
16	0.50	11.00	45.00	100	100	100
17	5.00	1.00	50.00	28	25	50
18	2.75	21.00	45.00	100	100	100
19	5.00	11.00	45.00	97	95	100
20	2.75	11.00	40.00	90	88	100

3.3 Response surface methodology predicted plots of Cd, Ni, and Pb phytoremediation using *Azolla P.*

The predicted responses obtained from the response surface methodology (RSM) were compared with the actual experimental responses to validate the predicted data. Based on the sequential model sum of squares, the models were selected according to the highest-order polynomial in which the additional terms were statistically significant and the models were not aliased. For metal removal, the quadratic model was recommended by the software and therefore selected. The final empirical models in terms of coded factors (parameters), after exclusion of insignificant terms, for cadmium removal (Y_1), nickel removal (Y_2), and lead removal (Y_3) are presented in Equations (5-7).

$$Y_1 = 94.00 - 3.70 x_1 + 31.40 x_2 + 1.3 x_3 + 1.50 x_{12} - 23.00 x_{22} - 4.50 x_{32} + 4.25 x_{1x2} + 0.00 x_{1x3} - 1.00 x_{2x3} \quad (5)$$

$$Y_2 = 92.24 - 3.80 x_1 + 33.00 x_2 + 1.40 x_3 + 1.91 x_{12} - 23.09 x_{22} - 5.09 x_{32} + 3.62 x_{1x2} + 0.38 x_{1x3} - 0.63 x_{2x3} \quad (6)$$

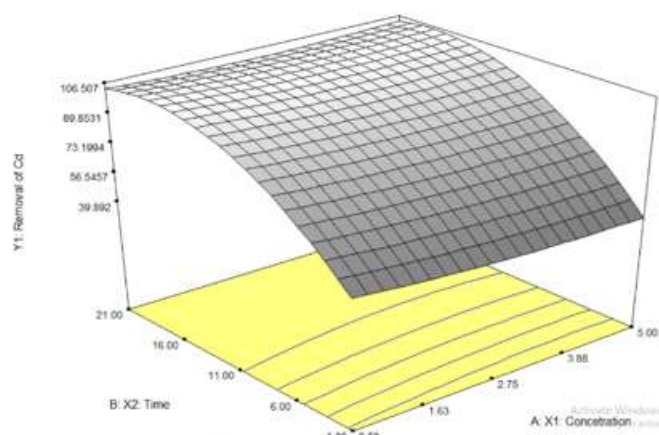
$$Y_3 = 101.05 - 4.80 x_1 + 18.3 x_2 + 0.80 x_3 - 2.64 x_{12} - 15.14 x_{22} - 2.64 x_{32} - 6.00 x_{1x2} - 0.25 x_{1x3} - 1.00 x_{2x3} \quad (7)$$

Three-dimensional surface plots illustrated the interactions among variables and their effects on metal removal efficiency. Regression analysis indicated good agreement between predicted and experimental values, confirming the adequacy of the quadratic model. These findings are consistent with previous studies that reported *Azolla pinnata* as an efficient accumulator of Cd, Ni, and Pb, owing to its fast growth and high bioconcentration factor.

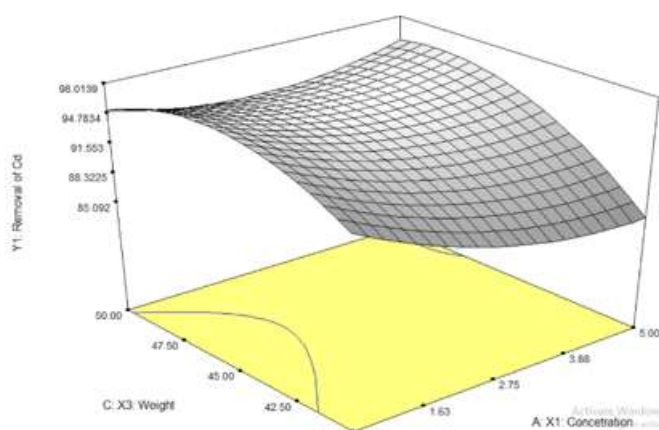
Positive sign in front of the terms indicates synergistic effect, whereas negative sign indicates antagonistic effect. The quality of the model developed was evaluated based on the correlation coefficient value. The closer R^2 value to unity, the better the model will be as this will give predicted values which are closer to the actual values for the response, were considered high, indicating that there was agreement between the experimental and the predicted in the removal of cadmium, nickel and lead from this model. The adequacy of the models was further justified through analysis of variance (ANOVA).

The ANOVA for response surface quadratic model for Cd removal (Y_1) onto *Azolla* showed that (F-value) of 86.85 and (Prob > F) of less than 0.0001 implied that this model was also significant. In this case, x_2 and x_{22} were significant model terms whereas x_1 , x_3 , x_{12} , x_{32} , x_{1x2} , x_{1x3} and x_{2x3} were insignificant to the response. The ANOVA for response surface quadratic model for Ni removal (Y_2) onto *Azolla P.* is shoed (F-value) of 85.71 and (Prob > F) of less than 0.0001 implied that this model was also significant. In this case, x_2 and x_{22} were significant model terms whereas x_1 , x_3 , x_{12} , x_{32} , x_{1x2} , x_{1x3} and x_{2x3} were insignificant to the response. The ANOVA for response surface quadratic model for Pb removal (Y_3) onto *Azolla P.* showed (F-value) of 37.44 and (Prob > F) of less than 0.0001 implied that this model was also significant. In this case, x_1 , x_2 and x_{22} were significant model terms whereas x_3 , x_{22} , x_{32} , x_{1x2} , x_{1x3} and x_{2x3} were insignificant to the response. The experimental observation for both the exposurer time and initial concertation revealed that they have significant effects on the removal of Cd, Ni, and Pb onto *Azolla P.* whereas plant weight showed the least effect on this response. The quadratic effect of exposurer time was also higher compared to the quadratic effects of concertation and *Azolla* weight on the same response. The interaction effect between x_1 , x_2 and x_3 on the removal of Cd, Ni, and Pb were found to moderate. Figures 3, 4 and 5 shows the three-dimensional response and the interaction effects between the variable's initial concertation, exposurer time and *Azolla* weight on the Cd, Ni, and Pb removals.

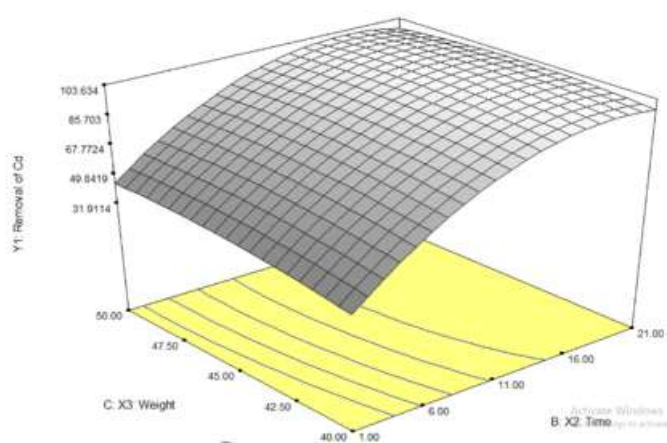
Figures 3, 4 and 5 shows the three dimensions plots for the interaction between exposure time, initial concertation and the weight of *Azolla P.* (optimum conditions) on the response surface (removal of Cd, Ni, and Pb).



(a)

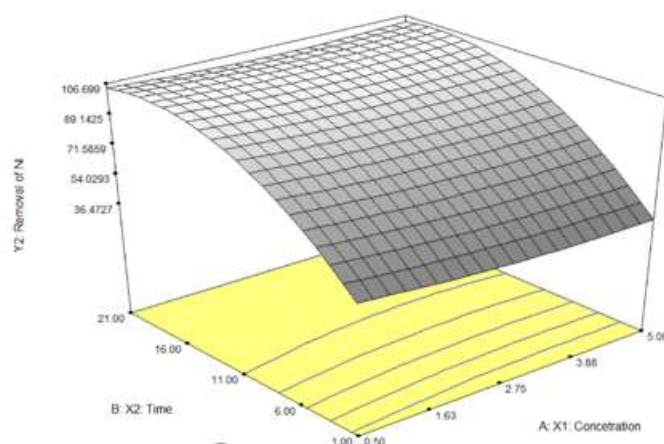


(b)

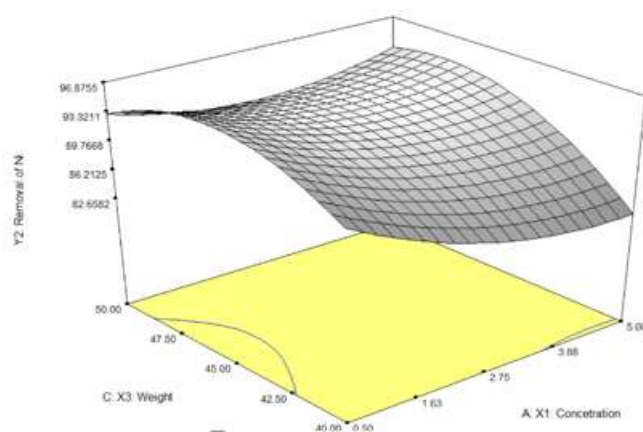


(c)

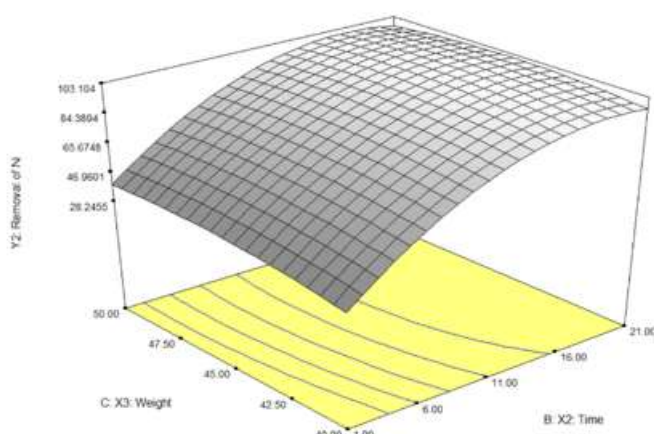
Figure 3: Combined effect of (a) initial concentration and exposure time, (b) initial concentration and Azolla P. weight, (c) Azolla P. weight and exposure time on phytoremediation removal of Cd



(a)

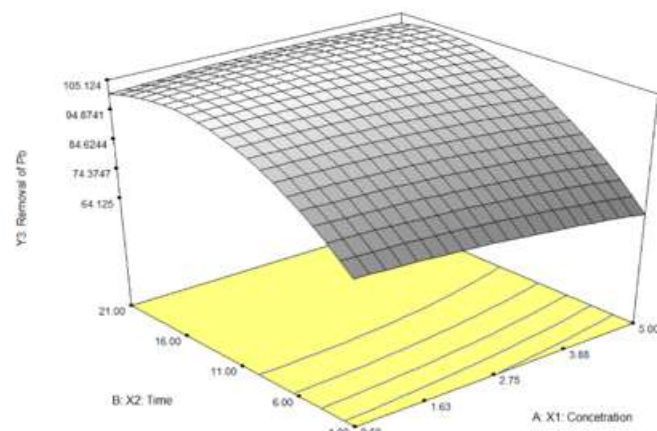


(b)

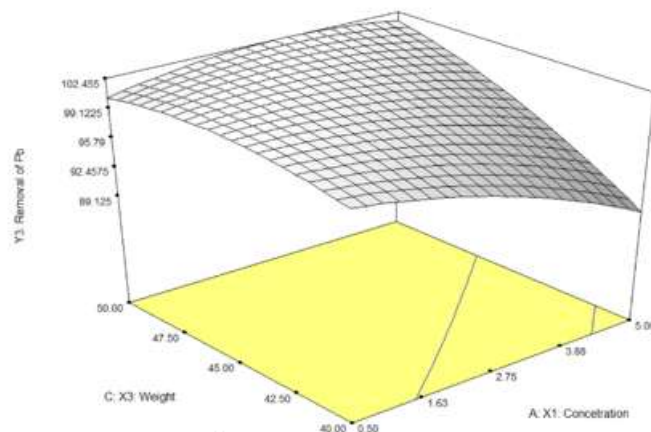


(c)

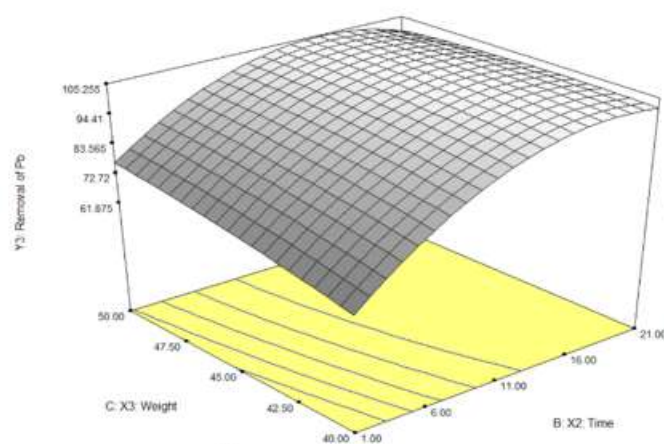
Figure 2: Combined effect of (a) initial concentration and exposure time, (b) initial concentration and Azolla P. weight, (c) Azolla P. weight and exposure time on phytoremediation removal of Ni



(a)



(b)



(c)

Figure 3: Combined effect of (a) initial concentration and exposure time, (b) initial concentration and Azolla P. weight, (c) Azolla P. weight and exposure time on phytoremediation removal of Pb

3.4 Discussion

It was showed from the above three dimensions plots the effect of exposure time, initial concentration and the weight of Azolla P. on the response surface (removal of Cd, Ni, and Pb) with weight of Azolla P. being fixed at zero level ($W=45.0$ g), the effect of Azolla P. weight and initial concentration on the response surface (removal of Cd, Ni, and Pb) with exposure time being fixed at zero level of (11 day), and effect of exposure time and Azolla P. weight on the response surface (removal of Cd, Ni, and Pb) with initial concentration of elements being fixed at zero level (Conc.= 2.75 mg/L). It was observed from these figures that the removal of Cd, Ni, and Pb by phytoremediation using Azolla P. generally increased with increasing exposure time. More of Cd, Ni, and Pb were removed as a result of longer exposure times and lower starting Cd, Ni and Pb concentrations. The results demonstrated that the exposure duration and starting Cd, Ni and Pb concentration had a significant impact on the reaction and percentage elimination of metals. The media and plant surface adsorption active sites were still depleted at an early exposure time. The quantity of metal ions rose along with the metal's concentration, which had an impact on Cd, Ni, and Pb elimination.

It was found from ANOVA results the optimum conditions for completely removal of Cd, Ni, and Pb by phytoremediation technique using Azolla pinnata for the range of initial concentrations selected in this study were treatment time of 18.4 day and Azolla pinnata weight of 49.4 g.

4. CONCLUSION

It was found from RSM results that the optimized conditions which resulted in the predicted maximum simultaneous removal of Cd, Ni, and Pb for the initial metals concentrations selected (0.5-5 mg/L). were approximately 18.4 days of treatment time and 49.4 g of Azolla pinnata biomass.

The application of RSM improved the practical potential of the removal process by identifying operating conditions that maximize treatment performance. Therefore, Azolla pinnata-based phytoremediation could be considered a sustainable alternative or complementary treatment to conventional methods for the remediation of heavy metals contaminated wastewater.

Overall, the findings indicated that Azolla pinnata can serve as a promising remediate material for heavy-metal removal from wastewater, particularly where low cost and environmentally friendly treatment technologies are required

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AUTHOR CONTRIBUTIONS

Alaa Jassem Mohammed: Investigation, Laboratory Analysis.

Basim A. Hussain: Methodology

Ferdaus Mohamat Yusuff: Supervision, Data Curation, Formal Analysis.

Faradiella Mohd Kusin: Conceptualization

Mohd Yusoff Ishak: Data Curation

Jasim Mohammed Salman: Writing—Review & Editing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS STATEMENT

Ethical approval was not required for this study.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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