Modeling Approach with Box-Behnken Design for Optimization of Pb Bioaccumulation Parameters in *Gammarus pulex* (L., 1758)

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INTRODUCTION

Pollution of the aquatic systems is a severe environmental problem and due mostly to anthropogenic activities (1, 2). Industrial contaminants, especially heavy metals, are toxic and pose a serial risk to human health and the aquatic flora and fauna (3). Heavy metals such as cadmium, copper, and lead do not decompose in the environment, so their concentrations must be monitored to admissible levels (4). Lead is hazardous even at trace levels, is used in industrial processes including paints and leaded gasoline, glass and batteries (5, 3, 6), and can lead to disorders of the liver, kidneys, heart, in addition to severe damage to erythrocytes and leucocytes resulting in susceptibility to infections (7, 8).

To evaluate the potential human influence on the ecosystems, heavy metals bioaccumulation strategies as well as concentrations of chemicals in organisms of the aquatic systems and their levels have to be well established (9). This depends on some important parameters including biological species and the element type, measure of exposure time to accumulation, and the development of an adequate experimental design on different spatial and temporal scales (10-13). Aquatic organisms exposed to chronic contamination accumulate metals directly via water or via their diet. In biological tissues, metals analysis

ABSTRACT

The aim of this research was to evaluate the suitability of *Gammarus pulex* (L., 1758), an amphipod crustacean, as a bio-monitor for lead (Pb). A Box-Behnken experimental approach combined with response surface modeling (RSM) and quadratic programming was employed to increase the knowledge about Pb²⁺ bioaccumulation in *Gammarus pulex* (L., 1758). Independent variables, such as temperature ranging from 18 to 22 °C, contact time from 8 to 48 hours, and Pb²⁺ ion solution concentrations from 50 to 100 µg L⁻¹, were studied and tested using analysis of variance (ANOVA). The standardized effects of the independent variables and their interactions on the dependent variable were also investigated according to a Pareto chart. The selected variables' optimum values were obtained by solving the quadratic regression model along with analyzing the response surface contour plots. Optimum experimental conditions were obtained at 22.0 °C, contact time of 8 hours, and Pb concentration at 69.5 µg L⁻¹. Based on the Box-Behnken design, the applied model is significant because the model F-value and R² were obtained at 77.80 and 0.9901, respectively. Bioaccumulation of Pb was determined using electrothermal atomic absorption spectrometry (ETAAS). The obtained data suggest that *G. pulex* can be used as a Pb biomonitor.

is thus relevant to keep the bioavailable fraction of metals potentially toxic for the aquatic animals under observation. In fact, the bioaccumu-

lation process completes the ability of organisms to regulate metals and the effects of water chemistry on metal bioavailability (14, 15). Among potential biomonitors, many researchers have recently focused on the genus *Gammarus pulex* (L., 1758), an amphipod crustacean, for the study of inorganic and organic pollutants in establishing the quality of the environment (16, 17) as well as its important ecological role and usage for ecotoxicological research. These organisms are commonly found and are important components of marine, freshwater and brackish water, and play a vital role in the aquatic food webs, and can be easily used for experimental studies. Furthermore, gammarids, due to their ability to accumulate metals, are interesting organisms for the development of bio-indicator systems and for biological observation (18, 19). They are also suitable candidates for the development of exposure biomarkers because metal accumulation can induce their sublethal alterations in terms of physiological, biochemical, and behavioral levels (20, 21). Recently, several models, including biologically-based, have been proposed to evaluate metal bioaccumulation (22, 23). These predictive models are generally constructed in the laboratory by establishing kinetic parameters describing physiological mechanisms involved in the uptake of bio-available metals and elimination of bio-accumulated metals. Some biodynamic approaches were successfully carried out in various aquatic species, especially the gammarids (24-29). Several researchers have reported that the amount of metal measured

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in some aquatic organisms depends on body size for the determination and evaluation of bio-concentration factors (30). Generally, there are three possible mechanisms for metal intake such as passive transport, active uptake, and specific cellular uptake (31).

In several studies, invertebrates from freshwater have been used for aquatic toxicology studies on heavy metals toxic effects by assaying enzyme activities and histologic changes. For example, Pb, Cd, and Cu in the gills of *Mytilus galloprovincialis* were used as a biomarker in investigating the single and combined effects of heavy metal pollution in terms of biochemistry (32). In another study, physiological and behavioral effects were investigated for the single and combined administration of Cd and AsO_4^{3-} in *G. pulex* (33). Also, the effect of Cd, Ni, and Pb on the intracellular effect of *G. fossarum* was studied by comparative methods (34).

In the present study, *G. pulex* was selected as a bioindicator organism to determine aquatic pollution in terms of Pb sensitivity in freshwater systems. This study focuses on the experimental parameters with Pb bioaccumulation in gammarids using the Box-Behnken design methodology as a tool to assess and verify bioaccumulation. To our knowledge, no studies have been published using the Box-Behnken experimental design and RSM (Response Surface Modeling) for modeling of Pb^{2+} ion bioaccumulation in *G. pulex*.

Classical and conventional methods cannot depict all combinations that affect an experiment. At the same time, these methods require much time to obtain the optimum levels. Using a statistical experimental design, which optimizes all of the effecting parameters collectively, these limitations can be eliminated. For modeling of process parameters, RSM contains a small number

of experiments and is, therefore, widely used in various processes. The primary target of RSM is to detect the optimum operational conditions for the system or to detect a region that compensates the operating specifications (35, 36). In this study, the influence of some operating variables, including solution temperature, contact time, and solution lead ion concentration, was investigated.

EXPERIMENTAL

Reagents and Standard Solutions

The chemicals used were of analytical grade and obtained from Merck (Darmstadt, Germany). A stock solution of 1000 mg L^{-1} Pb^{2+} was prepared by dissolving an accurate quantity in ultrapure water acidified with 0.5 M HNO_3 . All other concentrations were prepared using the stock solutions by dilution.

Collecting and Storing of Samples

Individuals of the *G. pulex* organisms were collected with dip nets from the Munzur River (39.156820 N , 39.499640 E) in Tunceli, Turkey. The samples were kept in plastic bottles with screw tops with holes so oxygen can flow into them, then filled with river water, and immediately brought to the laboratory. These organisms were stored in a stock aquarium for at least 15 days in a climate-controlled room at $18 \pm 0.5 \text{ }^\circ\text{C}$ and a 12:12 light:dark cycle. They were fed on refuted willow leaves and checked every 24 hours. For this purpose, experimental groups were constructed in which different concentrations of Pb (ranging from $50 \text{ } \mu\text{g L}^{-1}$ to $100 \text{ } \mu\text{g L}^{-1}$) were applied to *G. pulex* under various temperatures (ranging from $18 \text{ }^\circ\text{C}$ to $22 \text{ }^\circ\text{C}$) for periods of time ranging from 8 to 48 hours.

Application of Lead Solution to *Gammarus pulex* (L., 1758)

First, the acute toxicity (LC_{50}) values (about 150 ppm) of Pb were determined according to static non-renewal tests (37) in the organisms. Three different sublethal concentrations (50 , 75 , and 100 ppm) lower than the LC_{50} value were determined. Experiments were carried out in one-liter glass bottles. For each experimental group, 30 organisms were added to each bottle and the trials carried out in three replicates.

Box-Behnken Experimental Design and Optimization by RSM

The optimum conditions for Pb^{2+} bioaccumulation by *G. pulex* were determined by means of a three-factor, three-level Box-Behnken experimental design, combined with RSM and quadratic programming. According to single-factor experiments, a Box-Behnken design was used to determine three independent variables: temperature (X_1), Pb concentration (X_2), and contact time (X_3) on bioaccumulation of Pb^{2+} by *G. pulex* (Y). In order to reduce unexplained variability effects on the observed response, randomized experimental order was carried out. Seventeen experimental points including replications of the central points experimental design were used. Table I lists the different parameters such as variable conditions, run order, experimental values, and predicted values. A final equation was obtained from RSM in terms of actual factors by using the following equation:

Eq. 1

$$Y = +76192.34 - 9810.03X_1 + 261.16X_2 + 824.16X_3 - 3.71X_1X_2 - 49.86X_1X_3 + 0.21X_2X_3 + 289.58X_1^2 - 0.31X_2^2 + 2.50X_3^2$$

After data analysis by ANOVA in order to determine the lack of fit, other parameters were evaluated including the effects of quadratic,

linear, and interaction variables for bioaccumulation of Pb²⁺ by *G. pulex*. Response surface modeling and data analyses were carried out with Design Expert software program (Design Expert Version 10, Stat-Ease, USA). All bioaccumulation experiments were performed in flasks containing Pb²⁺ ion synthetic solutions under parameters affecting accumulation, including temperature (18-22 °C), Pb concentration (50-100 µg L⁻¹) and contact time (8 to 48 hours). The *G. pulex* were separated from the aquatic medium by filtering and digesting in a microwave digestion system (Coolpex, P.R. China). The microwave digestion program is listed in Table II. Clear solutions were analyzed using the AAnalystTM 800 ETAAS for Pb determination (PerkinElmer, Inc., Shelton, CT, USA). The operating conditions are listed in Table III. In this study, an EDL was not used because it generates much more light intensity than the HCL, which has short warm-up times and better stability.

RESULTS AND DISCUSSION

Second-order Polynomial Model

Response surface modeling with a Box-Behnken design and ANOVA was used for Pb²⁺ bioaccumulation by *G. pulex* and the parameters are given in Table I and Table IV, respectively. The Model F-value of 77.80 shows that the model is significant. The probability that an F-value of this magnitude can occur due to noise is 0.01%. Values less than 0.05 of "Probe > F" indicate statistical significance of model terms. Because the model coefficient (R²) was 0.9901, it can be said that 99.01% of the model-predicted values matched the experimental bioaccumulated Pb values in *G. pulex*. The "Pred R-Squared" of 0.8670 is in reasonable agreement with the "Adj R-Squared" of 0.9774; i.e., the difference is less than 0.2. "Adeq Precision" measures

TABLE I
Box-Behnken Design and Observed Responses of Pb²⁺ Bioaccumulation (µg g⁻¹)

Run	Temp.	Pb Concentration	Contact Time	Pb (qe, µg g ⁻¹)
	X ₁ (°C)	X ₂ (µg L ⁻¹)	X ₃ (hour)	Observed
1	20	100	8	314
2	18	100	28	395
3	20	100	48	143
4	20	75	28	140
5	18	75	48	3456
6	18	75	8	450
7	20	75	28	0
8	20	75	28	0
9	22	75	48	35
10	18	50	28	221
11	22	100	28	252
12	20	75	28	0
13	22	75	8	5006
14	20	75	28	254
15	20	50	8	596
16	20	50	48	0
17	22	50	28	819

TABLE II
***G. pulex* Sample Digestion Method**

Biological Sample	Weight (g)	Solvent	Volume (mL)	Temp. (°C)	Pressure (atm)	Time (min)
<i>G. pulex</i>	0.2	HNO ₃	4	150	15	4
		H ₂ O ₂	1	180	25	5

TABLE III
Instrumental Operating Conditions of AAnalyst 800 ETAAS

Step	Temp. (°C)	Ramp Time (s)	Hold Time (s)
Dry 1	110	1	30
Dry 2	140	15	30
Pyrolysis	850	10	20
Atomization	1600	0	5
Clean-out	2450	1	3
Wavelength	283.3 nm		
Hollow cathode lamp current	440 mA		
Background correction	Zeeman-effect		
Injection volume	20 µL		
Slit width	0.7 nm		

the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratio of 30.890 obtained in this study indicates an adequate signal. This model can be used to navigate the design space. On the other hand, Ince and Kaplan Ince (38) reported that when $R^2 > 0.75$, the model is adequate. This study revealed that the lack of fit that measures the fitness of the model was not significant ($P > 0.05$); the "Lack of Fit F-value" of 6.17 implies that the Lack of Fit is not significant relative to pure error. There is a 5.56% chance that a "Lack of Fit F-value" this large could occur due to noise. A non-significant lack of fit is good. In addition, the number of experiments was sufficient to determine the effects of the independent variables for Pb bioaccumulated values in *G. pulex*. The results expressed by using the statistical model were adequate in order to predict the Pb levels and fitted to a second-order polynomial equation. In this case, while X_3 , X_1^2 , X_2^2 , X_3^2 , and X_1X_3 are highly significant ($P < 0.01$), X_1 , X_2 , X_1X_2 , X_2X_3 , they are significant model terms in terms of Pb bioaccumulation values in *G. pulex*. The quadratic and linear coefficients affecting the factors on the response of Pb bioaccumulation in *G. pulex* follow the order of contact time > temperature > Pb concentration.

RSM Analysis

Contour plots (2D) and response surface plots (3D) were presented because they are useful in determining maximum, middle, and minimum response points. With contour plots, the level of the variables can be determined and also contribute to a desired response; in addition, the variables' levels are plotted in a curve with equal response. Because of these reasons, contour plots are easier to interpret. Figure 1 shows the effect of Pb concentration and temperature on Pb bioaccumulation in *G. pulex*.

TABLE IV
Analysis of Variance (ANOVA) for the Quadratic Polynomial Mode

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2.982E+007	9	3.314E+006	77.80	< 0.0001	Significant
X_1 -Temp.	3.160E+005	1	3.160E+005	7.42	0.0296	
X_2 -Lead conc.	35431.22	1	35431.22	0.83	0.3920	
X_3 -Contact time	9.333E+005	1	9.333E+005	21.91	0.0023	
$X_1 X_2$	1.373E+005	1	1.373E+005	3.22	0.1157	
$X_1 X_3$	1.591E+007	1	1.591E+007	373.52	< 0.0001	
$X_2 X_3$	45071.29	1	45071.29	1.06	0.3378	
X_1^2	5.649E+006	1	5.649E+006	132.65	< 0.0001	
X_2^2	2.799E+006	1	2.799E+006	65.71	< 0.0001	
X_3^2	4.208E+006	1	4.208E+006	98.81	< 0.0001	
Residual	2.981E+005	7	42589.66			
Lack of Fit	2.451E+005	3	81706.61	6.17	0.0556	Not significant
Pure Error	53007.80	4	13251.95			
R-Squared	0.9901					
Adj R-Squared	0.9774					
Pred R-Squared	0.8670					

* $P < 0.01$ highly significant; $0.01 < P < 0.05$ significant; $P > 0.05$ not significant.

Fig 1. Effects of Pb concentration and temperature on Pb bioaccumulation.

The bioaccumulated Pb amount increased with an increase in temperature until it reaches 22 °C, indicating that there are further improvements for bioaccumulation of the Pb levels. At the same time, contact time has important effects on Pb bioaccumulation in *G. pulex*. The effects of contact time and temperature on Pb bioaccumula-

tion in *G. pulex* are shown in Figure 2. The interaction of these two variables had a significant effect on the adsorption process, including from the quadratic variables (contact time, $P < 0.01$; temperature $P < 0.05$). As a result, the impact of contact time on the bioaccumulation level was more significant than the temperature. The effects of

Fig. 2. Effects of contact time and temperature on Pb bioaccumulation.

Fig. 3. Effects of contact time and Pb concentration on Pb bioaccumulation.

contact time and Pb concentration on Pb bioaccumulation in *G. pulex* are shown in Figure 3. The bioaccumulated Pb amount increased with a decrease in contact time until it reached a plateau at 8 hours, indicating that there was further improvement in the bioaccumulated Pb level.

Confirmation of Experiments

Under the optimized conditions (temperature 22 °C, Pb concentration 75 µg L⁻¹, and contact time 28 hours) and to support the optimized data given by numerical modeling, experiments were performed with the parameters as suggested by the model.

There are many studies and different results were reported about heavy metal bioaccumulation in various aquatic organisms. Clason et al. (17) used *Gammarus oceanicus* (Segerstrale, 1947) as a biomonitor for heavy metals such as Pb, Cd, Ni, and Cr. The environmental quality was determined by using a two-compartment model. They reported that their kinetic BCFs at theoretical equilibrium were for Cd, Cr, Cu, Pb, and Ni at 80–202, 80–112, 306, 251–520, and 23, respectively. According to these results, *G. oceanicus* organisms have sensitivity for soluble metals in the order of Cd (0.6 µg L⁻¹), Pb (0.8 µg L⁻¹), Cr (2.5–2.9 µg L⁻¹), Cu (8.5 µg L⁻¹), and Ni (11 µg L⁻¹).

Lead bioaccumulation was modeled in *G. pulex* samples and taken from aqueous (concentrations of Pb), dietary (poplar leaves added Pb), and exposed samples (29). At the end of the experimental studies it was found that biodynamic parameters contributing to contamination, such as the natural/available living/feeding environment for determining Pb bioaccumulation, are important factors.

In another study, electron microscopy techniques were used to determine Pb acetate effects on *G. pulex*. It was reported that Pb acetate may induce some ultrastructural changes on hepatopancreatic ceca of the digestive system (39). Lebrun et al. (40) also researched waterborne Cu influence on the digestive metabolism of *G. pulex*. Wang and Zauke (31) discussed bioaccumulation of metals in the amphipod *Gammarus zaddachi* and its relationship to the permeable body surface area.

CONCLUSION

The Box-Behnken experimental design combined with RSM was successfully fitted for Pb²⁺ determination and the results revealed that the *G. pulex* is suitable for biomonitoring studies for Pb²⁺ bioaccumulation. This aspect is additionally supported by a net accumulation strategy found for Pb²⁺ bioaccumulation. Furthermore, this model and the amphipod can serve as a predictive tool to evaluate the quality of the environment in terms of Pb²⁺ contamination in aquatic systems.

According to research by the authors, no report has been published on the relationship between the Box-Behnken model parameters and heavy metals bioaccumulation in *G. pulex*. This study supports the idea that Pb²⁺ bioaccumulation in *G. pulex* is mainly dependent on three factors: temperature, metal concentration, and contact time. The results present a successful

model verification for Pb bioaccumulation and provides information for *G. pulex* as a biomonitor. When this approach is compared to other models and conventional studies, it ensures a convenient operation. In conclusion and based on the results, the amphipod *G. pulex* is suitable for biomonitoring studies and the Box-Behnken approach applied for metal exposure concentrations can serve as a powerful tool to predict and assess environmental quality. This study can make a breakthrough in identifying biomonitoring necessary to evaluate the bioaccumulation potential in aquatic systems.

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