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Investigating the carcinogenic and non-carcinogenic health hazards of heavy metal ions in *Spinacia oleracea* grown in agricultural soil treated with biochar and humic acid

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Abstract²

This study addressed the bioaccumulation and human health risk among consumption of *Spinacia oleracea* grown in agricultural soil treated with humic acid and biochars. The biochars came from two local feedstocks of rice husk (RH) and sugar beet pulp (SBP) pyrolyzed at temperatures 300 and 600 °C. The effect of soil treatment with biomass/biochar and humic acid in the accumulation of Heavy metals (HMs) in spinach leaves was considered under the controlled laboratory conditions. Optimum conditions for minimum accumulation of HMs were evaluated according to a central composite design (CCD) under the response surface method (RSM) by Design Expert software. Total concentrations of Cu, Cd, and Ni in soil and biomass/biochar were beyond acceptable standards worldwide. The concentrations of Zn, Cd, Pb, and Ni in spinach exceeded the

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Abbreviations: RH, rice husk; SBP, sugar beet pulp; RH300, rice husk biochar pyrolyzed at 300°C; RH600, rice husk biochar pyrolyzed at 600°C; SBP300, sugar beet pulp biochar pyrolyzed at 300°C; SBP600, sugar beet pulp biochar pyrolyzed at 600°C; HM, heavy metal; Zn, zinc; Fe, iron; Cu, copper; Mn, manganese; Cr, chromium; Cd, cadmium; Pb, lead; Ni, nickel; DTPA, diethylenetriaminepentaacetic acid; RSM, response surface method; CCD, central composite design; ANOVA, analysis of variance; CCI, chlorophyll content index; EDI, estimated daily intake; RfD, oral reference dose; HQ, hazard quotient for noncarcinogenic diseases; HI, hazard index; CR, carcinogenic risk; TCR, cumulative carcinogenic risk; SEPA, environmental quality standards set by the state environmental protection administration.

safety threshold limit, and its consumption is not recommended. Results of the desirability test suggested the minimum values of biomass/biochar application rate due to their metal-contaminated nature. The minimum concentration of humic acid also was suggested due to its role in promoting pollutant bioavailability. Results showed that the treatment of metal-contaminated soil with HMs polluted-biochars fails to stabilize pollution. Also, polluted biochars increased the hazard indices for non-carcinogenic and carcinogenic diseases beyond the permissible levels.

Keywords: Biochar; Spinach; Health risk assessment; Carcinogenic risk; 3D surface plot; Inceptisols.

1. Introduction

Understanding the occurrence and bioavailability of heavy metals (HMs) in soils is critical to human life, environmental health, agroecosystems, food safety, and ecotoxicology (Alloway 2012). Soil as a "universal sink" carries the heavy burden of environmental pollution (Ashraf, Maah et al. 2014). The concentration and chemical form of HMs in soils alter considerably geographically, even in uncontaminated soils. It depends on the geochemistry nature of the rocks, soil properties, and the intensity of soil-forming processes (Cataldo and Wildung 1978, Alloway 2012). Given the persistence in the environment and bio-accumulative nature (Ali, Abdelsalam et al. 2018), contamination from various anthropogenic sources can often lead to elevated

concentrations of HMs, which can impose toxicity in the ecological health of organisms and susceptible plants (Alloway 2012).

The annual growth of the agriculture sector has caused indiscriminately accumulating agricultural solid wastes in Iran. Given the significance of agro-waste and by-products for development purposes, their reuse is essential to bridge the gap in sustainability and waste management. Sugar beet pulp is a by-product after sucrose extraction from sugar beets, containing 22–30% cellulose, 22–30% hemicellulose, 25% pectin, 1.4% fat, 10.3% protein, 3.7% ash, and 5.9% lignin. About 250,000 tons of SBP are produced in Iran annually (Ahmadi, Zamiri et al. 2015). Rice husk is an organic by-product of the rice milling process. In general, paddy has an outer covering called husk that surrounds the edible part of the grain. This coating makes up about 20% of the weight of rice, which contains about 35–40% cellulose, 15–20% hemicellulose, 20–25% lignin (Gao, Guo et al. 2018), 2.9–3.6% protein (Aderolu, Iyayi et al. 2007), and 20% ash (Worasuwannarak, Sonobe et al. 2007). Up to 100 million tons of RH is produced worldwide annually. The estimated production of RH in Iran is more than 0.5 million tons per year (Yaghoubi, Allahyari et al. 2019).

Pyrolysis is an effective solution for dealing with crops and forestry residues. It is a simple and low-cost technology to process a wide variety of agricultural residues, reduces waste destined for landfill, and is considered a potential technique for sustainable energy production (Lee, Choi et al. 2019). Biochar is a biochemically stable carbonaceous polymer acquired from the pyrolysis of organic materials in an oxygen-limited environment. It has attracted the attention of researchers due to its ability to remove

pollutants from water and soil (Liu, Sun et al. 2017, Yang, Ye et al. 2020, Głąb, Gondek et al. 2021, Kumar, Shaikh et al. 2021), use as a soil amendment (Agegnehu, Srivastava et al. 2017, Hagemann, Joseph et al. 2017, Zheng, Zhang et al. 2021), and mitigate climate change (Woolf, Amonette et al. 2010, Lehmann, Cowie et al. 2021), backed by various physicochemical properties. By introducing biochar as an environment-friendly material that can improve the physicochemical quality of soil, its use in agricultural ecosystems is increasingly recommended today (Sultan, Ahmed et al. 2020). Biomass and resultant biochar differ regarding inorganic and organic compositions, surface properties, degree of aromaticity, and environmental stability against biotic and abiotic degradation (Lee, Park et al. 2013, Nakhli, Panta et al. 2019). Pyrolysis caused HMs enrichment in char because of the stable nature of HMs against low temperatures (Chen, Luo et al. 2017, Liu, Wang et al. 2020, Giudicianni, Gargiulo et al. 2021). Char synthesized from hyperaccumulator biomass at 600°C retained more than 98.5% of HMs (Ni, Zn, Cu, Co, and Cr) (Koppolu, Agblevor et al. 2003, Koppolu and Clements 2003, Koppolu, Prasad et al. 2004).

Soil HMs pollution based on biochar application may impose a significant risk to human health through the food chain (Ndirangu, Liu et al. 2019) that, unfortunately, has not been well-documented for to use safely of biomass resources. HMs are toxic, carcinogenic, mutagenic, and teratogenic in high concentrations, especially when they enter the food chain (Zhang, Zou et al. 2021). Food safety has been an important issue in human health in recent decades due to the high demand for food. It is estimated that 13.58% of the world's total cultivated land is contaminated by HMs (0.235 billion hectares of HMs-contaminated arable land divided by 1.73 billion hectares of total cultivated land in 2015)

(Zhang, Li et al. 2019). To avoid inadvertent consequences of extensive applications, a comprehensive understanding of the possible environmental risks of biochars is essential and urgent. A recent study by Wang et al. (Wang, Zou et al. 2021) concluded that the proportions of stable fractions and environmental risk of HMs in chicken manure and water-washed swine manure biochars decreased with increasing pyrolysis temperature. In another report, the content of HMs was determined in three biochars that differed in pyrolysis temperature and biomass feedstock (Zhou 2016). The results showed that the potential ecological risk of Cd in the soil increases significantly after treatment with sewage sludge-derived biochar. On the other hand, several studies have revealed that biochar can immobilize HMs in contaminated soils and reduce their bioavailability and toxicity (Yang, Liu et al. 2016, Lu, Yang et al. 2017, Li, Yu et al. 2021, Gong, Zhao et al. 2022).

However, so far, there has been little discussion about the harmful consequences of amending agricultural soils with biochar concerning its pollutant content. As far as we know, no previous research has quantified the human health risk from consuming crops grown in an agricultural soil treated with HMs-rich biochar. In this research, we investigated whether HMs-rich biochar could play the role of an immobilizing adsorbent in HMs-contaminated soil to prevent the accumulation of these elements in crops beyond acceptable standards worldwide. In the present study, spinach (*Spinacia oleracea*) seeds were planted in soils treated with RH, SBP, and their resultant biochars pyrolyzed at 300 and 600 °C. The aims of this study are, therefore, to (1) analyze the effect of RH, SBP, and their biochars, as well as humic acid treatment on the chlorophyll content and

accumulation of HMs in spinach leaves, and (2) address the human health risk from crop (spinach leaves) ingestion grown on a contaminated soil treated with contaminated biomass/biochar. In humans, a diet rich in vegetables is recommended to ensure a healthy lifestyle. Vegetables are the primary source of human exposure to HMs, accounting for about 90% of total metal intake. In comparison, the remaining 10% occurs through exposure to air pollutants (inhalation or dermal contact) (Khan, Khan et al. 2015). Crops such as spinach may accumulate HMs migrated from biochar and further increase the health risk of the crop (Keeflee, Zain et al. 2020).

Humic substances such as humic and fulvic acids are dark-colored high-molecular-weight organic materials that are dominant in aliphatic components. They are among the most reactive soil components and natural chelating agents that may be applied as a fertilizer, plant growth promotor, and nutrient carrier (Stevenson 1994, Park, Kim et al. 2013, Dubey, Raha et al. 2019). In fertile soils rich in humus, the content of humic acid reaches 5–7% (Bezuglova and Klimenko 2022). Complexation of HMs with humic substances can enhance their phyto-available fraction in soil and stimulate plant growth (Chen and Zhu 2006, Bezuglova and Klimenko 2022).

2. Materials and methods

2.1. Sources and preparation of biochars

In the current study, rice husk (RH) and sugar beet pulp (SBP) were used as raw materials to produce biochar. RH was obtained from rice fields in Lahijan County, Guilan Province, in the north of Iran. Dried SBP was provided from Bisotun sugar factory, Harsin County,

Kermanshah Province, in the west of Iran. The feedstock samples were oven-dried at 70 °C and crushed to pass through a 149 microns sieve (100 mesh). The ground feedstock samples were poured into aluminum cans, each wrapped with double-layer aluminum foil, and carbonized under oxygen-limited conditions in a muffle furnace. Biochars were synthesized at two different low and high pyrolysis temperatures of 300 °C and 600 °C via the traditional slow pyrolysis conversion. The rate of increase of pyrolysis temperature was 10 °C min^{-1} , and each final temperature lasted for two hours. Then, the biochar samples were allowed to cool to room temperature in the main chamber to prevent rapid oxidation and auto-ignition. According to sieve analysis, all biochar samples had a median grain size (D_{50}) of about 50 microns. The biochar samples are labeled RH300, RH600, SBP300, and SBP600 based on the feedstock type and applied pyrolysis temperature. Altogether, the concentrations of Cu, Cd, and Ni in feedstocks and their biochars exceeded the threshold values provided by Adagunodo et al. (Adagunodo, Sunmonu et al. 2018).

2.2. Soil preparation

The soil material used was collected from the topsoil (0- to 20-cm depth) of a sandy-loam Inceptisols located in Kurdistan province, western Iran, with a humid continental climate (Dsa) according to the Koppen climate classification. The soils were first air-dried, homogenized, and then passed through a 2000 microns sieve (10 mesh). The mechanical or sieve analysis and the hydrometer method, respectively, were used to determine the distribution of the larger-sized particles (50 to 2000 microns diameter) and the finer

particles (< 50 microns diameter) according to ASTM D422-63 (Standard 2007). The pH value was measured using a pH meter (HACH HQ40d portable multi pH meter, USA) on a suspension of soil in deionized water (1:1 ratio). Electrical conductivity (EC, dS m^{-1}) was measured using a SWISS 644 conductometer based on saturated soil-paste extract. Phyto-available potassium and phosphorous were measured using 1 N NH_4OAc at pH 7 (Rees, Pettygrove et al. 2013) and 0.5 N NaHCO_3 at pH 8 (Olsen, Cole et al. 1954), respectively. Total nitrogen, cation exchange capacity (CEC, $\text{cmol}_c\text{kg}^{-1}$), and oxidizable organic carbon (OC, %) were determined using the Kjeldahl digestion (Bremner 1996), ammonium-acetate compulsory displacement (1 M NH_4OAc , 1 M KCl, pH = 7) (Sumner and Miller 1996), and potassium dichromate oxidation (Knicker, Müller et al. 2007) methods, respectively. Nitrogen adsorption at 77 K was used to determine the specific surface area (SSA, m^2g^{-1}) using the standard multipoint Brunauer-Emmett-Teller (BET) method. Table S1 in the supporting information file shows some fundamental properties and total concentrations of HMs in the initial (untreated) soil. On the whole, the studied soil had concentrations of Cu, Cd, and Ni beyond the threshold values provided by Adagunodo et al. (Adagunodo, Sunmonu et al. 2018). The acid digestion method was used to determine the total concentration of HMs, as reported in literature (Yuan, Huang et al. 2011).

2.3. Phyto-available fraction of HMs

The DTPA method was applied to assess the plant-available fraction of HMs (Sims and Johnson 1991, Amacher 1996, Xiao, Yuan et al. 2015, Wang, Zou et al. 2021). The

solution extractant was acquired by dissolving 1.96 g DTPA, 14.92 g TEA, and 1.47 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ with 950 ml deionized water, buffered to pH 7.3 using 6 M HCl, and rinsing it well to 1 liter. The final concentration of each reagent is 0.005 M DTPA, 0.1 M TEA, and 0.01 M CaCl_2 . To extract phyto-available fractions of HMs, 10 g of each sample was mechanically shaken for two hours at 25 °C with 50 ml of extractant. In this case, 50 g of soil was mixed with 100 ml extractant. Quantitative filter paper is used to take filtrate.

2.4. Experimental design

A pot experiment was conducted in the Department of Environment, Faculty of Natural Resources, University of Kurdistan, Iran to investigate the role of soil treatment with biomass/biochar and humic acid in the accumulation of HMs in spinach leaves. About 3 kg of the studied air-dried soil was mixed and homogenized thoroughly with various doses of biochar. The mixtures were transferred to plastic containers of about 4 L volumes with a layer of interlining fabric at the bottom to facilitate drainage. Designing an ideal experimental trial, optimizing parameters, and plotting 3D surfaces and contour lines were done in Design Expert software (USA Stat-Ease. 7.0). The optimal design allows the statistical model to be estimated with a minimal number of runs of the experiment (Fathianpour, Taheriyoun et al. 2018, Ghorbani, Kamari et al. 2020). Optimum conditions for minimum accumulation of HMs were searched according to a central composite design (CCD) under the response surface method (RSM). The Box-Wilson central composite design, generally named the central composite design (CCD), is generally used to formulate the response variable by fitting a second-order polynomial

according to the response surface method without using a complete factorial design of experiments. To determine the coefficients of the designed model with quadratic terms, at least three levels must be defined for each factor. In CCD, experimental points fall into three main groups: (I) a group of corner (fractional) points of a cube (design space) where the factor levels are coded to -1 , $+1$; (II) axial points of each face of the cube at a distance of α from the design center and (III) a center point of a cube (Sahoo and Barman 2012, Kumar, Vignesh et al. 2022).

In this study, three independent factors (process variables) of the application rate (A), humic acid concentration (B), and biomass (RH and SBP) or biochar types (RH300, RH600, SBP300, and SBP600) (C) were chosen for optimization, and evaluation spinach leaves in terms of (I) accumulation of HMs, (II) health risks caused by the ingestion, and (III) chlorophyll content as the response items. A total of 78 experiments were designed, including three groups of design points: 24 corner points, 24 axial (star) points, and 30 central points. Table 1 illustrates the organizational experimental design, including three parameters (A, B, and C), each at five levels, coded as -1 , $-\alpha$, 0 , $+\alpha$, and $+1$, respectively. The range of factors based on the levels (± 1) was selected by the user (according to the literature). The range of factors based on the levels ($\pm \alpha$) and (0) was selected by the software. In RSM, the response variable is fitted to a second-order polynomial model as in Eq. (1) (Nikraftar and Ghorbani 2016, Zaib, Jouiad et al. 2019, Ghorbani, Kamari et al. 2020):

$$Y = a_0 + \sum_{i=1}^n a_i X_i + \sum_{i=1}^n a_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=2}^n a_{ij} X_i X_j + \epsilon \quad (1)$$

where Y is the predicted response variable (HMs accumulation, health risk assessment indices, and chlorophyll content), X_i and X_j are coded independent variables (or regressors), and ϵ is the random error. The regression coefficients of a_0 , a_i , and a_{ij} represent model intercept, linear, and quadratic effects, respectively. a_{ij} are cross-product coefficients. The purpose of solving Eq. 1 is to optimize the regression coefficients of the model using the least-squares approach. The least-squares technique is determined by minimizing the sum of squared errors, i.e., the difference between the observed value and the predicted value $(\sum_i (Y_i - f(X_i, \alpha))^2)$. Seventy-eight experiments were conducted according to the scheme mentioned in Table S2. Analysis of variance (ANOVA) was used to evaluate the significance of the model and the input variables. The goodness-of-fit of the statistical model was tested by the coefficient of determination (R^2), and statistical significance was expressed based on the p -value with a 95% confidence level and Fisher's F value. The Pareto analysis (Eq. 2) was applied to rank the effects of process variables on responses (Zaib, Jouiad et al. 2019):

$$P_n = \left(\frac{b_n^2}{\sum b_n^2} \right) \times 100 \quad n \neq 0 \quad (2)$$

where P_n represents the relative contribution of each factor (%), and b_n are coefficients of the polynomial equation (Eq. 1) that are significant at a p -value of 0.05.

Water content at field capacity points of the soil and soil-biochar mixtures was determined before beginning the experiment (Romano and Santini 2002). Twelve baby spinach (*Spinacia oleracea* L. cv. 'Bloomsdale') seeds were sown about 1 cm deep in each pot on 6 December 2021. *Spinacia oleracea* is a member of the Caryophyllales order,

characterized by a relatively fast growth rate, broad green leaves, and a relatively high HM absorption rate, and by being a standard part of the human diet (Alia, Sardar et al. 2015). Two weeks after emergence, seedlings were thinned to seven uniform plants per pot. It was performed on light shelves with yellow-white LED lighting with light intensity more fabulous than 1000 lux and a photoperiod of 15 hours. Day/night temperatures of $25\pm2/15\pm2^{\circ}\text{C}$ were established with a relative air humidity of 45-55%. Soil moisture was maintained at field capacity in all the pots by adding tap water ($EC_w = 0.41\text{ dSm}^{-1}$) to a constant weight (Gavili, Moosavi et al. 2018, Parwada, Chigiya et al. 2020). Technical grade humic acid was purchased from AJ Products Pty. Ltd., Australia, as Potassium Humate (Powder/Flakes) and was applied once in the middle of the growth period with different concentrations in Table 1.

2.5. Chlorophyll content

Relative chlorophyll content non-destructively was measured with the OPTI-SCIENCES CCM-200 chlorophyll content meter (USA) as chlorophyll content index (CCI). Measurements were taken on the fully developed leaf at the end of the growth period before harvest between 11:00 a.m. and 1:00 p.m. for all plants in a pot. The values in each pot were averaged. The measurement area was 71.22 mm^2 . The ratio of radiation transmission at 931 nm to 653 nm, from a light-emitting diode embedded in the device, is termed CCI (CCM-200 user manual). A CCI value of about one indicates an equal transmission of red and NIR radiation and a low amount of chlorophyll in the leaf. CCI

values greater than unity indicate more extraordinary transmission of NIR radiation than red radiation, with larger ratios indicating more chlorophyll.

2.6. Health risk assessment of crop ingestion

2.6.1. Estimated daily intake (EDI) of heavy metals

The EDI values ($\text{mg kg}^{-1} \text{ day}^{-1}$) of each HM were calculated based on their mean concentration in spinach leaves by the following formula according to literature (EPA 2011, Organization 2011, Guo, Hong et al. 2020, Liu, Yang et al. 2021):

$$EDI = \frac{EF \times ED \times IR \times C}{BW \times AT} \times CF \times 0.001 \quad (3)$$

where EF is exposure frequency ($365 \text{ days year}^{-1}$), ED is the exposure duration (77 years) equivalent to the average lifetime of Iranian people (Salehi, Aziz-Mohammdloo et al. 2021), IR is the ingestion rate ($240 \text{ g person}^{-1} \text{ day}^{-1}$) according to the World Health Report on low consumption of fruits and vegetables (Organization 2002), C is the metal content in spinach samples ($\text{mg kg}^{-1} \text{ dry weight}$), BW is the body weight for an adult, which was considered as an average of 73 kg for males and females (Tabrizi, Sadeghi-Bazargani et al. 2018), $AT = EF \times ED$ is the average exposure time, $CF = 0.087$ is a conversion factor to convert the fresh weight of spinach leaves to dry weight (EPA 2011), and 0.001 is a unit conversion factor.

2.6.2. Non-carcinogenic risk

The non-carcinogenic risk of individual HMs, expressed as the hazard quotient (HQ), was calculated using Eq. (17) (Organization 2011, Shaheen, Irfan et al. 2016, Gao, Zhang et al. 2021):

$$HQ = \frac{EDI}{RfD} \quad (4)$$

where RfD is the oral reference dose ($\text{mg kg}^{-1} \text{ day}^{-1}$) that represents the tolerable daily uptake of contaminants through ingestion of foods. The RfD values are 0.7, 0.14, 0.04, 0.3, 0.0035, 0.02, 0.003, and 0.001 $\text{mg kg}^{-1} \text{ day}^{-1}$ for Fe, Mn, Cu, Zn, Pb, Ni, Cr, and Cd, respectively (Waste and RCRA 2003, Can, Ozyigit et al. 2021, Liu, Yang et al. 2021, Giri, Bharti et al. 2022). The hazard index (HI), which indicates the non-carcinogenic risk of multiple HMs, was determined by Eq. (18):

$$HI = HQ \text{ metal } 1 + HQ \text{ metal } 2 + \dots + HQ \text{ metal } n. \quad (5)$$

where values of $HI > 1$ indicate that the exposed population is at increased risk of non-carcinogenic effects of HMs, and vice versa for $HI < 1$.

2.6.3. Carcinogenic risk

The carcinogenic risk (CR) or incremental lifetime cancer risk via oral exposure routes is obtained by multiplying the EDI by the carcinogenic slope factor (SF, kg day mg^{-1}):

$$CR = EDI \times SF \quad (6)$$

SF values for Pb, Ni, Cr, and Cd are 0.0085, 1.7, 0.5, and 0.38, respectively (Zhang, Li et al. 2019, Obasi, Obasi et al. 2020, Can, Ozyigit et al. 2021). The permissible cancer risk

limit is between 10^{-6} and 10^{-4} for a single or multi-element carcinogen. Values surpassing 10^{-4} impose significant health risks (Waste and RCRA 2003, Shaheen, Irfan et al. 2016, Gao, Zhang et al. 2021). The cumulative carcinogenic risk (TCR) can be computed as below:

$$TCR = CR_{metal\ 1} + CR_{metal\ 2} + \dots + CR_{metal\ n}. \quad (7)$$

Since Fe, Mn, Cu, and Zn are essential elements for plant growth and are not pollutants, according to Zhang et al. (Zhang, Li et al. 2019), their cancer risk was not considered.

2.7. Process optimization

After selecting the goal for each factor and response from the menu, the process variables were optimized using the numerical optimization option. The goals were to maximize CCI and minimize bioaccumulation, EDI, HQ, and CR of individual HMs and HI and TCR of multiple HMs by optimizing any combination of the three goals, namely humic acid concentration, application rate, and biomass or biochar types. The desirability function optimizes multiple response processes and yields values between zero and one. The value 1 illustrates the desirable response and the optimal performance for the studied factors (Amdoun, Khelifi et al. 2018). The numerical optimization found a point that minimized the desirability function. The simultaneous objective function is a geometric mean of all responses:

$$D = (d_1 \times d_2 \times \dots \times d_n)^{1/n} = \left(\prod_{i=1}^n d_i \right)^{1/n} \quad (8)$$

where D is the desirability function, d_i is the desirable range for each response, and n is the number of responses.

3. Results and discussion

3.1. Phyto-available fraction

Table S3 shows the phyto-availability of HMs in feedstocks and their biochars pyrolyzed at 300 and 600 °C and also the grading standard values of the phyto-available amounts of soil Zn, Fe, Cu, and Mn based on the classification given by the Chinese agricultural division data (Yang, Yu et al. 2014). As shown in Table S3, the phyto-available fractions of Zn, Fe, Cu, Mn, Pb, and Ni extracted by DTPA follow a distinct decreasing pattern with increasing pyrolytic temperature. For example, in evaluating the phyto-available content of Cu, RH600, compared to raw RH, showed a decrease of 19.08%. By contrast, the phyto-available fraction of Cr and Cd in both RH and SBP biochars increased with increasing pyrolytic temperature. For example, phyto-available contents of Cr in RH600 increased by 49.50% compared to RH. The solubility of Cr is affected by the oxidation-reduction potential of the environment, which needs further investigation.

Table S3 also illustrates that Zn, Fe, Cu, and Mn extracted by DTPA in RH were 4.4, 1.3, 2.8, and 4.4 times greater than those introduced as abundance thresholds for soil nutrients based on Chinese agricultural division data, respectively. Concerning RH600, phyto-available portions of these elements extracted by DTPA were 2.03, 1.26, 2.37, and 2.57 times higher than the abundance threshold for soil nutrients, respectively.

3.2. HMs-bioaccumulation

Total heavy metal concentrations in spinach leaves ($mg\ kg^{-1}$ dw) are shown in Table 2, and decreased, respectively, in the order of $Fe > Zn > Mn > Ni \geq Cu > Pb > Cr > Cd$. Fe showed the highest concentration in spinach leaves. Cd was the least bioaccumulated element by leaves. Additionally, Table 2 contains Indian standards (Awashthi 1999) and the Environmental Quality Standards set by the State Environmental Protection Administration (SEPA) (SEPA 2005) for HM concentrations in food. Human exposure through digestion (i.e., oral route) provides one of the main routes for HMs to enter the human body. Generally, the concentrations of Zn, Cd, Pb, and Ni in spinach leaves exceeded the safety threshold limit, and its consumption is not recommended, especially for a long time.

Cd is a highly toxic non-essential element its high concentrations in the soil naturally are associated with parent material (black shales). During the 20th century, human activities like mining, the burning of fossil fuel, industrial dust, disposing of Cd-containing waste, and using chemical fertilizers (especially phosphatic fertilizers) and sewage sludge have increased Cd levels in the soil. It is not easily absorbed by plant roots because it can be adsorbed by metal oxides and lime at low and high pH, respectively. Cd has a half-life of 15-1100 years in the soil (Alloway 1995, Oliver 1997). Plants have different abilities to absorb and accumulate Cd. Lettuce, celery, spinach, and cabbage can accumulate high levels of Cd (Davis and Carlton-Smith 1980). Various agencies (e.g., Environmental Protection Agency (EPA), Joint FAO/WHO Expert Committee on Food Additives (JECFA), and Agency for Toxic Substances and Disease Registry (ATSDR)) have

reported different daily maximum safe exposure levels for Cd from 0.1 to 1 $\mu g Cd kg^{-1} day^{-1}$ (Wong, Roberts et al. 2022). Cadmium is a non-biodegradable heavy metal with a long biological half-life. Therefore, long-term exposure to low levels of Cd can also have a significant impact on public health. The significant health risk of Cd is its chronic accumulation in the renal cortex, which causes renal tubule dysfunction if its concentration exceeds 200 $\mu g Cd kg^{-1}$ of fresh weight (Oliver 1997). The contamination of rice with Cd has been widely reported in the literature (Yan, Sun et al. 2021). In waterlogged (anaerobic reducing) conditions where soil redox potential (Eh) is small, Cd is held as CdS which is insoluble and has low bioavailability. Higher bioavailability of Cd occurs when the soil is aerated (aerobic oxidizing conditions), in which case Eh increases, and CdS is oxidized to free soluble Cd^{+2} (Meng, Li et al. 2019). Cd concentration in agricultural soils is reported in 0.01-0.7 $mg kg^{-1}$ (Khan, Khan et al. 2015).

Pb biologically has no proven function and has toxic effects on humans and other living organisms, even at low concentrations. Pb is a widespread soil contaminant with high immobility that tends to accumulate in the roots. The transfer of lead from the roots to the aboveground organs depends on the type of plant and is generally not done quickly (Oliver 1997, Bonanno and Giudice 2010, Egendorf, Groffman et al. 2020). For hyperaccumulator species, plant uptake in the acidic soils ranged from 934 to 4500 $mg Pb kg^{-1}$ and ranged from 1496 to 4253 $mg Pb kg^{-1}$ in alkaline soils (Egendorf, Groffman et al. 2020). Pb occurs naturally in the environment from the parent materials and anthropogenically through environmental pollution from industrial wastes and atmospheric depositions. It enters the food chain by being taken up or settles on by plants.

Pb that deposits on plants cannot be entirely removed by washing or other food processing steps, especially those sealed with wax (Van der Ent, Baker et al. 2013, Gavelek, Spungen et al. 2020). Pb concentration in agricultural soils is reported in 2-100 mg kg⁻¹ (Khan, Khan et al. 2015). Pb concentration in spinach leaves found in this study was 13.43 mg kg⁻¹, five times higher than the threshold standard for food according to the Indian standards for HM concentrations in food (Awashthi 1999). It has been shown that airborne Pb i.e., atmospheric suspensions (dust, exhaust fumes, and industrial fallout), can settle on plants and even enter residences (Caravanos, Weiss et al. 2006, Hu, Wang et al. 2014, Egendorf, Groffman et al. 2020). Foliar uptake of airborne Pb via internalization through the cuticle or penetration through stomata openings has been reported by numerous authors (Schreck, Foucault et al. 2012). These findings suggest that airborne Pb cannot be ignored, and in urban communities, it should be considered an essential source of Pb contamination of many plants. Nowadays, one of those rare plausible sources for reference values for Pb is the Interim Reference Levels (IRLs) set by the FDA in 2018, which derived the IRL of 0.26 $\mu\text{g kg}^{-1}\text{day}^{-1}$ in young children and 0.16 $\mu\text{g kg}^{-1}\text{day}^{-1}$ in older children and adults (Gavelek, Spungen et al. 2020, Wong, Roberts et al. 2022). In 2021, the U.S. Food and Drug Administration (FDA) updated its interim reference levels (IRLs) for Pb from food to 2.2 $\mu\text{g day}^{-1}$ for children and 8.8 $\mu\text{g day}^{-1}$ for females of childbearing age (Flannery and Middleton 2022). In general, the more Pb there is in the soil, the higher its concentration is in plants and, in food (Oliver 1997, Bower, Lister et al. 2017). Concerning the human-available portion of Pb in many

soils, a 30% phyto-available fraction (of total Pb) has been recommended as a conservative estimate (Egendorf, Groffman et al. 2020).

Zn is an essential element for both man and plant health growth. It plays a crucial role in plant growth, development, signaling, and metabolism. The concentration of Zn in natural soil depends on the parent materials and the extent of weathering processes and typically varies between 10-300 mg kg⁻¹ (overall mean of around 50 mg kg⁻¹). However, its toxicity usually results from anthropogenic activities, especially from sewage sludge, wastewater irrigation, agrochemicals, pesticides, mining, and atmospheric fallout (Oliver 1997, Long, Yang et al. 2003, Balafrej, Bogusz et al. 2020). Zn-toxicity in plants increases with its bioavailable fraction in soil. The latter depends on soil properties, root exudates, and microbial communities (Balafrej, Bogusz et al. 2020). Long et al. (Long, Yang et al. 2003) showed that the shoot growth of Chinese cabbage, pakchoi, and celery is significantly reduced at Zn-DTPA concentrations above 170 mg kg⁻¹ in soil. Most plants contain 30-100 mg Zn kg⁻¹ dry matter. It is generally assumed that leaf Zn levels above 300 mg kg⁻¹ dry weight are considered toxic, which can be harmful to plants, animals, and humans (Marschner 1995, Long, Yang et al. 2003, Noulas, Tziouvalekas et al. 2018). However, while humans can tolerate high Zn concentrations, the risk of Zn toxicity increases by overconsumption of Zn, which can cause anemia, stomach cramps, vomiting, nausea, and skin irritations. Excessive Zn exposure can damage the pancreas, disrupt protein metabolism, and cause atherosclerosis (Ihedioha, Okoye et al. 2014). A provisional maximum tolerable daily intake of 1 mg kg⁻¹ body weight was established by JECFA for this element (Ihedioha, Okoye et al. 2014).

Ni is an essential micronutrient for average plant growth in small quantities. The role of this metal in activating the urease enzyme required for nitrogen metabolism has been confirmed (Klucas, Hanus et al. 1983, Correia, Marrocos et al. 2018). Ni in the soil can be formed by anthropogenic and lithogenic/pedogenic processes (El-Naggar, Ahmed et al. 2021). High levels of Ni in the soil are mainly the result of human endeavors, such as mining, smelting, agricultural activities, and the burning of Ni-containing diesel (Wuana and Okieimen 2011, Correia, Marrocos et al. 2018). Generally, the level of Ni in agricultural soils is 3-1000 mg kg⁻¹ (El-Naggar, Ahmed et al. 2021). In the environment of contaminated soil, all living organisms are affected by the toxicity caused by excessive levels of Ni over the permissible limit (100 mg kg⁻¹) (Adagunodo, Sunmonu et al. 2018). High doses of dietary Ni intake induce DNA damage and have harmful effects on the immune system, endocrine glands, and neurological and cardiovascular activities (Das, Reddy et al. 2019). High levels of nickel have been found in legumes, spinach, lettuce, and nuts (Das, Reddy et al. 2019). Correia et al. (Correia, Marrocos et al. 2018) confirmed that soil Ni concentrations close to 70 mg kg⁻¹ could be an optimal concentration for the cultivation of tomatoes as a sensitive plant to HM pollution. In contrast, they showed that at a concentration of 105 mg kg⁻¹, Ni in the soil reaches a toxic level that affects the intake of macronutrients and the growth of tomato plants. World Health Organization has set a maximum tolerable daily intake of 5 µg kg⁻¹ body weight for Ni (Organization 1996, Ihedioha, Okoye et al. 2014).

3.3. Effects of biomass/biochar application rate

440 The levels of HMs-bioaccumulation found in the analyzed samples (*Spinacia oleracea* L.
441 cv. 'Bloomsdale') affected by biomass or biochar application rate are listed in Fig. 1. All
442 metal concentrations were expressed in dry weight basis. The graph considers the average
443 bioaccumulation for each application rate (regardless of whether it is biomass or biochar).
444 A similar pattern was found for Zn, Fe, Cu, Mn, Cr, and Pb in the way that by increasing
445 the application rate up to 4.35%, the bioaccumulation of these HMs is gradually
446 increased. However, the applied rate of 5.10% causes it sharply to decrease. On the other
447 hand, the bioaccumulation of Cd and Ni showed a slight decrease with increasing
448 application rates up to 4.35% and 2.55%, respectively. A higher applied rate resulted in a
449 sharp increase in the Cd- and Ni-bioaccumulation. The Zn, Cu, and Cd-bioaccumulation
450 have taken the most minor contribution from the biochar application rate. The EDI values
451 of eight considered HMs were calculated according to the mean concentration of each
452 metal in spinach leaves and the respective consumption rates. The above order was the
453 same also for EDI (Fig. 2), HQ (Fig. 3), and CR (Fig. 5). EDI and HQ values for Zn, Fe,
454 Cu, Mn, Cr, and Pb increased with biomass/biochar treatments being maximum in 4.35%
455 application rate compared to the control (0.00% application). The minimum values of the
456 EDI and HQ for these elements were obtained at the application rate of 0.00% or 5.10%.
457 CR values for Cr and Cd also followed a similar pattern. However, treatment of soils with
458 biomass/biochar up to 4.35% and 2.55% slowly reduced the EDI-Cd and EDI-Ni
459 compared to the control, respectively. However, the treatment of 5.10% resulted in a rapid
460 increase of 8% and 41%, respectively. Adding 5.10% biomass/biochar into the soil gave
461 the maximum amount of EDI for Cd and Ni. Application of 4.35% and 2.55% of

462 biomass/biochar caused the minimum value for the EDI-Cd and EDI-Ni, respectively.
463 Such a pattern can also be seen for HQ and CR. The application of 4.35%
464 biomass/biochar decreased the EDI, HQ, and CR values of Cd by approximately 8% as
465 compared to the control. Compared to the control, EDI, HQ, and CR values of Ni
466 decreased by 5% after the application of 2.55% biomass/biochar amendment. Soil
467 incorporation of 4.35% biomass/biochar increased EDI, HQ, and CR values of Cr by 25%
468 and Pb by 50% compared to the control.

469 The combined non-carcinogenic effects of multiple HMs from consuming contaminated
470 spinach leaves by adult populations were assessed based on the hazard index (HI).
471 Consumption of edible substances with $HI > 1$ will likely impose harmful effects on the
472 health of the exposed population (Shaheen, Irfan et al. 2016). HIs of the multiple HMs are
473 figured in Fig. 4. It is evident from Fig. 4 that the HIs of all the biomass/biochar
474 treatments were >1 , indicating the detrimental consequences of its consumption on health.
475 The highest (3.69) and lowest (3.15) values of HI were recorded by 4.35% and 0.00%
476 application rates. The target CRs derived from the intake of Cr, Cd, Pb, and Ni were
477 calculated because these elements are classified as carcinogens and may increase the risk
478 of carcinogenesis and cancer-related diseases (Kim, Kim et al. 2015) depending on the
479 period and dose of exposure. The carcinogenic effects of consuming spinach leaves
480 contaminated with Cr, Cd, Pb, and Ni by adult populations were evaluated based on the
481 cumulative carcinogenic risk (TCR). Accordingly, the TCR (Fig. 5) showed a slight
482 decrease with the increase in the biomass/biochar application rate up to 2.55% compared
483 to the control. However, the application of 5.10% biomass/biochar increased the TCR by

36% compared to the control. TCR values ranged from 0.0085 (2.55% biomass/biochar application rate) to 0.0119 (5.10% biomass/biochar application rate), all of which were greater than 10^{-4} and considered unacceptable.

Farmlands polluted with HMs in Iran are still in production and produce crop products rich in HMs. Some of these HMs-rich crop residues may be used as precursors for biochar production. After using contaminated biochar in agricultural soils, their toxic elements are returned to the soil and accumulate in plants. Plants can easily take up an unstable (phyto-available) fraction of HMs, to a certain extent, within a given period. Adding more biochar leads to a more phyto-available fraction of HMs entering the soil environment. (Shen, Huang et al. 2016) reported that soil treatment with HMs-rich biochar made from rice straw increased the soil Cd accumulation risk and total Cd content of tobacco. A similar increase in DTPA extractable soil Cd after the addition of Cd-contaminated rice straw has also been reported by Wang et al. (Wang, Huang et al. 2015). They also reported increased Cd content from 13.9 to 84.1% in Chinese cabbage. The soil treatment with biochar releases large amounts of dissolved organic matter (DOM), resulting in the formation of DOM-HM complexes, which increases the phyto-availability of HMs in soils (Meng, Tao et al. 2018, Wang, Liu et al. 2020, Sun, Xiong et al. 2021). High DOC content released from biochar could facilitate the formation of soluble Cu complexes (Oustriere, Marchand et al. 2016). Higher transfer of HMs from soil to plant means strong bioaccumulation of these metals in plant cells (Khan, Khan et al. 2015). For example, Beesley et al. (Beesley, Moreno-Jiménez et al. 2010) indicated that the incorporation of hardwood biochar increased Cu and As more than thirty-fold in soil pore water, which

stems from a significant increase in DOM. Abbas et al. (Abbas, Rizwan et al. 2017) indicated that soil treatment with rice straw-derived biochar increased the bio-intake of Zn and Mn approximately by 56%, 36%, and 124% and 50%, 54%, and 70% in shoots, roots, and grains of wheat compared to the control, respectively. They also showed an increase in AB-DTPA extractable fractions of Zn and Mn in the soil when biochar levels increased from 1.5 to 5%. Namgay et al. (Namgay, Singh et al. 2010) stated that activated wood biochar pyrolyzed at 550 °C increased the DTPA-extractable Zn in soil. In contrast, Cu did not show any significant change, Pb declined, and Cd displayed an inconsistent trend. They also reported an increase in Zn, Pb, and Cu in maize shoots following the addition of 5 g kg⁻¹ biochar + 50 mg kg⁻¹ ZnCl₂, 15 g kg⁻¹ biochar + 50 mg kg⁻¹ Pb(NO₃)₂, and 15 g kg⁻¹ biochar + 10 mg kg⁻¹ CuSO₄·5H₂O, respectively, compared with the control. The influence of biochar on activity and phyto-availability of HMs in soil and the rate of HMs-bioaccumulation by plants are sometimes inconsistent. For example, biochar derived from Orchard prune residues synthesized by Beesley et al. (Beesley, Marmiroli et al. 2013) increased the arsenic content in soil pore water. However, no increase in arsenic accumulation was observed in tomato roots. Soil-incorporated biochar can increase the mobility of anionic toxic elements [e.g., CrO_4^{-2} , AsO_3^- , and $Sb(OH)_6^-$] and their bioavailability (Guo, Song et al. 2020).

The HMs in the soil are not always easily bioavailable. Only a tiny portion of the total HM content in the soil exists as soluble components and is phyto-available. Cu, Zn, Cd, and Ni are more mobile and bioavailable for plants than Cr and Pb. The mobility and bioavailability of Fe and Mn are between these two categories (Yan, Wang et al. 2020).

528 The stabilization or mobility of HMs in soil intensely depends on their interplays with
529 inorganic and organic solid soil components and, thus, the formation of different HMs,
530 solid adsorption, complexes, or chelates. HMs phyto-uptake is also largely dependent on
531 HMs overall abundance, soil properties, and plant species (Li, Wang et al. 2022). Some
532 studies have reported an increase in the stabilization and a decrease in the mobility and
533 phytotoxicity of HMs after adding biochar to the soil. Therefore, stabilization efficiency
534 mainly ascribed to the different individual characteristics of the HM, biochar, and soil, as
535 well as their complex interactions (Houben, Evrard et al. 2013, Bian, Joseph et al. 2014,
536 Al-Wabel, Usman et al. 2015, Jia, Wang et al. 2017, Guo, Song et al. 2020, Wang, Liu et
537 al. 2020, Li, Wang et al. 2022). In the present study, stabilization of HMs following
538 biochar addition at low application rates was not observed except for Cd and Ni. Humic
539 acid may be responsible for part of this complex behavior. Houben et al. (Houben, Evrard
540 et al. 2013, Houben, Evrard et al. 2013) and Qian et al. (Qian, Chen et al. 2015)
541 considered the primary mechanism of HMs stabilization by biochar in slightly acidic soils
542 to be the liming effect of biochar, during which the pH value of the soil and subsequently
543 metal precipitation in pore water increases. Burgos et al. (Burgos, Madejón et al. 2006)
544 amended Spanish soils with 30 $mg\ ha^{-1}$ biochar for one year, representing an increase in
545 total organic carbon content by about 32%, during which $CaCl_2$ -extractable Pb decreased
546 by about 24%, suggesting an essential role of organic components in reducing available
547 Pb in soils. Under alkaline status, metal ions can replace hydrogen ions in an ion
548 exchange process and precipitate as insoluble metal phosphate species or form other
549 metals displacing species such as hydroxo-metal complexes. In some cases, hydroxo-

550 metal complexes like those formed with Zn, Cd, and Ni are more soluble than those
551 formed with Cr and Fe (Olaniran, Balgobind et al. 2013). However, Kabata-Pendias and
552 Pendias (Kabata-Pendias 2000) have pointed out that an increase in soil pH does not
553 necessarily result in a decrease in HM availability. For instance, a high Cd accumulation
554 and toxicity in a high pH soil have been reported (Emurotu and Onianwa 2017). The
555 formation of $\text{Pb}(\text{OH})_2$ is also reported as a probable mechanism for Pb stabilization
556 (Abdelhafez, Li et al. 2014). According to earlier investigations, the nutrient elements
557 such as P, Ca, and Si provided by biochar may act as immobilizing agents for Pb through
558 the formation of insoluble precipitation or co-precipitation (Yang, Liu et al. 2016, Lu,
559 Yang et al. 2017). Nie et al. (Nie, Yang et al. 2018) were able to significantly reduce the
560 accumulation of Cu (14.9%–37.6%), Cd (61.6%–75.5%), and Pb (17.3%–49.1%) in the
561 shoots of Brassica by applying biochar derived from bagasse. The above researchers
562 raised the possibility that biochars rich in phosphorus may increase the residual fraction
563 of Pb in the soil matrix by forming pyromorphite. Biochar is known to have high CEC,
564 adsorption capability, and surface area, which helps in holding positively charged ions.
565 Therefore, in soil and water environments, HMs with high electronegativity can replace
566 basic cations on the surfaces of biochar particles (Mansoor, Kour et al. 2021). Such
567 cations are more slowly available to plants than soluble cations and have low leachability.
568 Oxygen-containing functional groups also increase specific adsorption of HMs by
569 enabling binding sites for HMs to form complexes (Mansoor, Kour et al. 2021).
570 It is often stated that biochar amendment negatively affected the concentration of labile
571 forms of HMs in soil and consequently decreased the HMs bioaccumulation in plant

organs. However, our research shows that the above may not be accurate, especially when applying HMs-contaminated biochar in HMs-polluted soil, as well as in the early years of cultivation and for all HMs. According to the HMs concentration in spinach leaves in the present study, it is clear that soil treatment with biochar failed to reduce the bioavailability of HMs below the risky level except for Cu. In addition, the bioaccumulation of Zn and Pb, which did not exist at the critical level in the soil, exceeded the safety threshold limit in spinach after loading biochar.

3.4. Effects of biomass/biochar type

The bioaccumulation of Zn, Fe, Cu, Mn, Cr, Cd, Pb, and Ni in the leaves of spinach (*Spinacia oleracea* L. cv. 'Bloomsdale') affected by biomass/biochar type are shown in Fig. 6. The HMs-bioaccumulation greatly varied among the selected biomass/biochar type. The concentration of Zn, Cu, Fe, Mn, Cd, and Ni in the leaves of spinach of biochar-treated soils decreased compared to soil treated with biomass.

For example, the Zn, Fe, Cu, Mn, Cd, and Ni concentrations in spinach leaves grown in RH600-treated soil were decreased by 10%, 25%, 40%, 11%, 27%, and 26%, respectively, compared to the soil treated with RH. Similarly, in comparison with SBP-amended soils, the bioaccumulation of Zn, Fe, Cu, Mn, and Ni in spinach leaves grown in

594 SBP600-treated soils were reduced by 28%, 16%, 37%, 26%, and 14%, respectively, as
595 shown in Fig. 6. In this case, no difference was observed in Cd concentration. By contrast,
596 the bioaccumulation of Cr and Pb in spinaches grown in soils treated with RH and SBP-
597 derived biochars increased compared to soils treated with biomass. For example, soil
598 treatment with RH600 increased Cr- and Pb-bioaccumulation by 32 and 38% compared to
599 soil treatment with RH. Similar patterns were also observed for EDI, HQ, and CR, shown
600 in Figs. 7, 8, and 10, respectively. The highest and lowest EDI and HQ values of Zn, Fe,
601 Cu, Mn, Cd, and Ni were obtained when the soil was treated with raw residues (RH and
602 SBP) and high-temperature biochars (RH600 and SBP600), respectively. These results
603 indicate the better efficacy of (i) high-temperature biochars than those derived at lower
604 temperatures in reducing phyto-availability and HMs-bioaccumulation and (ii) biochar as
605 a soil amendment than feedstock in alleviating the bioaccumulation of HMs and the
606 potential health risk. On the other hand, the EDI, HQ, and CR values for Cr and Pb
607 increased in the case of biochar application, especially biochars produced at high
608 temperatures, compared to biomass. The EDI, HQ, and CR values increased by 32% for
609 Cr and 38% for Pb after the application of RH600 compared to RH. Similarly, the
610 SBP600 application increased EDI, HQ, and CR values for Cr and Pb contents of the
611 spinach leaves by 22% and 48%, respectively, compared to SBP. The highest contribution
612 to carcinogenic risk (CR) belonged to Ni ranging from 0.0065 (SBP600-treated soils) to
613 0.0090 (RH-derived soils). Also, Pb and Cd showed the highest contribution to non-
614 carcinogenic risk. Contrary to expectation, the HI did not follow a clear pattern
615 considering HMs concentration in spinach grown in soil treated with RH and RH-derived

616 biochars (Fig. 9). In addition, spinach grown in soils treated with SBP-derived biochars
617 showed higher HI than those treated with biomass. These results can be explained by the
618 different patterns that HMs follow; on the one hand concentration of Zn, Fe, Cu, Mn, Cd,
619 and Ni in leaves of spinach decreased when the soil was treated with biochars than
620 biomass. However, on the other hand, spinach leaves accumulated more Cr and Pb in soil
621 treated with biochar than biomass. According to RfD values (Eq. 5), Cr, Pb, and Cd have
622 lower tolerable daily uptake than other considered HMs. Therefore, Cr, Cd, and Pb play
623 the most important role in the calculation of HI ($Cd > Cr > Pb > Ni$). According to the
624 agency for toxic substances and disease registry, four HMs of As, Pb, Cd, and Hg are
625 considered the most serious toxicants because of their carcinogenic/ toxicological
626 mechanisms (Wynne 2008, Mansoor, Kour et al. 2021). The application of biochar also
627 decreased the TCR values from 0.0101 in RH to 0.0079 in RH600 and from 0.0087 in
628 SBP to 0.0078 in SBP600. These values are significant for carcinogenic risk since they
629 are above the upper limit of the acceptable carcinogenic risk of 10^{-4} . On the other hand,
630 the probability of developing cancer over time due to the consumption of spinach grown
631 in the studied soil under RH and RH300 treatments was higher than SBP and SBP300.
632 Consequently, although many researchers found biochar as an efficacious solution in
633 alleviating the mobility of HMs in soils (Houben, Evrard et al. 2013, Bian, Joseph et al.
634 2014, Al-Wabel, Usman et al. 2015, Jia, Wang et al. 2017, Wang, Liu et al. 2020, Li,
635 Wang et al. 2022), the results may differ from expectations when applying multi-element
636 contaminated biochars. Contradictory effects during biochar application to multi-element
637 polluted soil can result in the bioavailability and toxicity of specific elements (Beesley,

638 Moreno-Jiménez et al. 2010). Kutrowska et al. (Kutrowska, Małecka et al. 2017)
639 evaluated antagonistic and synergistic interactions between metal ions as important for an
640 efficient phyto-uptake or reduction of non-essential metal uptake in crops. For example,
641 the high bioavailability of Cu or Zn in the soil can give rise to high Pb bioaccumulation.
642 Increasing Zn concentration in soil solution stimulates the ions transport system through
643 the apoplastic route or/and activates non-selective ion channels (Kutrowska, Małecka et
644 al. 2017). Cd sits right below Zn, an essential micronutrient for plants and animals, on the
645 periodic table and therefore shares many of its same chemical properties. This may
646 partially account for Cd toxicity (Wuana and Okieimen 2011).

647 As mentioned earlier, the abundance of surface functional groups, and thus the CEC, is an
648 essential attribute in determining the capacity of biochar to immobilize HMs through
649 adsorption-based interactions. Therefore, biochar pyrolyzed at 300 °C is expected to have
650 a greater ability to reduce the bioaccumulation of HMs than that produced at 600 °C.
651 However, this pattern was valid only for the bioaccumulation of Cr and Pb. The
652 maximum bioaccumulation of Zn, Fe, Cu, Mn, Cd, and Ni was found in biomass-
653 amended soil. Pots treated with biochars pyrolyzed at 600°C demonstrated the lowest
654 bioaccumulation of those HMs. It seems that the dominant factor explaining the
655 bioaccumulation beyond the established standard threshold is the abundance of phyto-
656 available portions of HMs. As previously described, biochars pyrolyzed at low
657 temperatures generally contain greater amounts of the phyto-available fraction of HMs
658 than at high temperatures. As long as the phyto-availability of HMs in the soil solution is
659 high, the uptake by plants will also be high (Khan, Khan et al. 2015).

3.5. Effects of applied humic acid

The effect of the humic acid application on the HMs-bioaccumulation in spinach leaves is also picturized in Figure 12. In general, soil application of humic acid increased the bioaccumulation of HMs in spinach leaves, although a slight decrease was sometimes observed at high concentrations (2310 mg L^{-1}). The pattern was the same for EDI (Fig.13), HQ (Fig. 14), and CR (Fig. 16). Increasing the concentration of applied humic acid in the pots caused an apparent increase (upward trend) in the TCR, although it decreased in the concentration of 2310 mg L^{-1} . According to the study conducted by Vaughan (Vaughan 1974), low concentrations (up to 50 mg L^{-1}) of humic acid enhances root cell elongation, but at higher concentrations, it is inhibitory. The effectiveness of the humic acid application rate also depends on the soil condition and crop type being grown (Ampong, Thilakaranthna et al. 2022). As shown in Figs. 15 and 16, humic acid is a pivotal factor in increasing HI and TCR. The HMs bonded with dissolved OM show high bioavailability to soil flora (Krishnamurti, Megharaj et al. 2004). Therefore, the high application of humic substances in agricultural soils, especially in soils with high content of HMs or in combination with HMs-contaminated biochars, can lead to excessive bioaccumulation in crops beyond the permitted limits and is not suitable. Further, it may induce a reduction in the biomass of the sensitive plants.

The interaction between humic substances and metal ions, thereby decreasing or increasing their speciation in soils, is highly pH-dependent (Li, Wang et al. 2022). Humic substances have been found to enhance the phyto-extraction of HMs in alkaline soils.

682 These substances are insoluble in acidic conditions, but their solubility increases with
683 increasing pH, and as a result, they can increase the solubility, mobility, translocation, and
684 bioavailability of HMs (Fox and Comerford 1990, Evangelou and Marsi 2001, Halim,
685 Conte et al. 2003, Marschner and Kalbitz 2003, Chen and Zhu 2006, Park, Kim et al.
686 2013, Mani, Kumar et al. 2015, Noroozisharaf and Kaviani 2018, Kleber and Lehmann
687 2019, Abourayya, Kaseem et al. 2020, Li, Wang et al. 2022). Probably, the acidic nature
688 of humic substances plays an essential role in their reactivity and performance in alkaline
689 soils. The abundance of different functional groups in humic acid, especially acid
690 functional groups like carboxyl, determines their adsorption capacities for HMs (Yang,
691 Miao et al. 2015, Bezuglova and Klimenko 2022). In expressing the effect of humic
692 substances in the growth process of plants, the following have been reported: I)
693 improvement in root formation and development of the assimilation apparatus (Canellas,
694 Olivares et al. 2002, Zandonadi, Santos et al. 2010, Mora, Baigorri et al. 2012), changes
695 of phosphate metabolism and energy transfer (Rosa, Silva et al. 2018, de Castro, Berbara
696 et al. 2021), acceleration in protein metabolism (Vaughan 1974, Vaccaro, Ertani et al.
697 2015), increase in the intensity of respiration processes, photosynthesis, and water
698 exchange and increasing the concentration of chlorophyll and ascorbic acid (Liu, Cooper
699 et al. 1998, Lotfi, Kalaji et al. 2018, Shamsullah 2020).

700 It has been shown that many factors affect the mobility and phyto-availability of HMs in
701 soil and, as a result, their accumulation in edible parts of plants, among which pH is of
702 particular importance. Alkalinity encourages the adsorption of HMs to metal binding sites
703 and declines the release of HMs into the soil solution. As long as the availability of HMs

in soil solution is low, the uptake by plants will also be low (Khan, Khan et al. 2015). At lower pH, HMs inclined to be more mobile and toxic because they are more soluble (Kuo, Huang et al. 2004, Khan, Khan et al. 2015, Li, Wang et al. 2022). Analysis of the Cd uptake from sewage sludge-amended soils by Swiss chard (*Beta vulgaris* L.), lettuce (*Lactuca sativa* L.), and corn (*Zea mays* L.) leaves revealed that a combination of total Cd concentration and pH of the soil is a reasonable predictor of plant tissue Cd concentration (McBride 2002). Adams et al. (Adams, Zhao et al. 2004) used a multiple regression model to predict the concentration of Cd in wheat grain (*Triticum aestivum* L.). They showed that the model, including total soil Cd and pH, explained 53% of the variability of wheat grain Cd. In the current study, the produced biochars all had high pH in the range of 8.56 (SBP300) to 10.66 (SBP600). Therefore, their incorporation with soil prepared in the current study with pH = 7.05 should reduce the bioavailability and bioaccumulation of HMs. Nevertheless, it is important to note that the HMs in the prepared biochars mostly had values beyond the acceptable limits. High HMs concentrations may be the most critical contributor to the observed high plant uptake (Egendorf, Groffman et al. 2020). In addition, the use of humic acid has increased the bioavailability and bioaccumulation of HMs in spinach leaves. Salinity can also affect the accumulation of some HMs in plants. Chloride enhances Cd mobility and bioavailability in soil solution by forming relatively stable and phyto-available complexes of CdCl_n^{2-n} (Filipović, Romić et al. 2018, Raiesi and Sadeghi 2019, Guo, Chen et al. 2022). The EC values of biochars produced in this study ranged from 0.81 (RH300) to 6.38 (SBP600).

3.6. Fitting of process models

To optimization of the response variables as well as explore the relationships between controlled experimental factors (process variables) and response variables, the CCD under RSM was applied, and 78 runs of experiments were designed. The final equations in terms of coded factors were obtained by fitting the experimental data to Eq. 1. The mathematical models were formulated taking into consideration linear (A, B, C), quadratic (A^2 , B^2 , C^2), and interactive (AB, AC, BC,) effects of all variables as follow:

$$CCI = 5.89 - 1.84(C1) - 0.025(C2) + 0.33(C3) - 0.16(C4) + 0.39(C5) \quad (9)$$

Zn – bioaccumulation

$$= 115.56 + 13.39(B) + 7.52(C1) + 14.90(C2) - 6.94(C3) - 27.33(C4) + 8.18(C5) \quad (10)$$

Fe – bioaccumulation

$$\begin{aligned} &= 750.83 + 83.00(B) + 89.41(C1) + 121.70(C2) \\ &- 58.04(C3) - 58.43(C4) - 95.48(C5) + 220.05(AC1) \\ &- 202.18(AC2) + 58.56(AC3) - 73.26(AC4) \\ &+ 38.02(AC5) - 39.58(BC1) + 183.40(BC2) \\ &+ 19.87(BC3) + 43.07(BC4) - 62.63(BC5) + 64.97B^2 \end{aligned} \quad (11)$$

Cu – bioaccumulation

$$\begin{aligned} &= 15.63 + 1.58(B) - 1.14(C1) + 2.88(C2) + 0.64(C3) \\ &- 4.68(C4) - 1.20(C5) \end{aligned} \quad (12)$$

Mn – bioaccumulation

$$\begin{aligned} &= 70.09 + 7.07(C1) - 1.59(C2) + 0.27(C3) - 14.10(C4) \\ &+ 3.43(C5) - 9.41(AB) + 10.88(BC1) + 17.39(BC2) \\ &- 0.87(BC3) + 5.82(BC4) - 22.32(BC5) \end{aligned} \quad (13)$$

Cr – bioaccumulation

$$\begin{aligned} &= 6.32 + 0.51(B) - 0.43(C1) - 0.78(C2) + 0.38(C3) \\ &+ 0.49(C4) - 0.38(C5) - 0.55A^2 \end{aligned} \quad (14)$$

Cd – bioaccumulation

$$\begin{aligned} &= 2.88 + 0.62(B) - 0.11(C1) + 0.47(C2) - 0.25(C3) \\ &- 0.19(C4) + 0.65(C5) + 0.42B^2 \end{aligned} \quad (15)$$

Pb – bioaccumulation

$$\begin{aligned} &= 15.48 - 3.31(C1) - 0.44(C2) - 1.20(C3) + 0.82(C4) \\ &- 1.02(C5) - 1.67(BC1) + 4.26(BC2) - 1.89(BC3) \\ &- 3.18(BC4) + 0.37(BC5) - 1.72A^2 - 1.63B^2 \end{aligned} \quad (16)$$

Ni – bioaccumulation

$$\begin{aligned} &= 14.79 + 1.10(A) + 1.25(B) - 0.095(C1) + 0.74(C2) \\ &- 0.95(C3) + 0.54(C4) + 2.34(C5) + 1.90A^2 \end{aligned} \quad (17)$$

733 The negative (–) and positive (+) signs of regression terms represents antagonistic and
734 synergistic effects, respectively. Insignificant terms with $p > 0.05$ were removed from the
735 models. All bioaccumulations possessed the same linear significant term C. Linear term A
736 only had a significant effect on Ni-bioaccumulation, and term B exhibits significant

effects on all response variables except Pb and Mn. All bioaccumulations possessed the quadratic term(s) except Zn, Cu, and Mn. The interaction terms showed significant effects only on Pb, Mn, and Fe. Readers can find other extracted significant regression models in terms of coded factors in the Supporting Information file Eqs. S1-S10.

3.7. Analysis and diagnostics of the model

In this step, the fitted regression models were analyzed to identify the best type of regression model for estimating responses. The diagnosis of the best fitting regression model i.e., linear, 2FI, quadratic and cubic models of all responses, is made by comparing the p-values, lack of fit, and R^2 statistics between the models (Tables S4-S34 available in the Supporting Information file). ANOVA estimates a significant portion of the variance in the model to identify important terms in the model (Kumar, Vignesh et al. 2022). For example, the CCI response regression (Table S4) was statistically significant at F -value of 3.25 and p -value of 0.0048. As can be seen from Table S4, ANOVA confirmed that biomass/biochar type significantly affects the CCI response. At the same time, the application rate and humic acid treatments had no significant effects, so they were not included in the final model. The "Lack of Fit F -value" of 0.77 implies the Lack of Fit is not significant relative to the pure error. There is a 78.38 % chance that "Lack of Fit F -value" occurs due to the noise. The non-significance of the "Lack of Fit F -value" in fitting a statistical model is desirable. A p -value between 0.05 and 0.1 may be considered significant, which can be judged based on the subject matter knowledge. The value of $R^2 = 0.24$ between the predicted and experimental data indicated that the developed

759 model has failed to explain 0.76 % of the total variation. There was a difference of less
 760 than 0.2 between the predicted and adjusted R^2 , showing their reasonable agreement.
 761 Adeq precision measures the signal-to-noise ratio. The more this ratio exceeds 4, the more
 762 reasonable it is. By comparing the p-value, lack of fit, and R^2 statistics between the fitted
 763 models, a linear model was suggested for CCI response. Concerning low R^2 , the ANOVA
 764 analysis shows the inapplicability of the model to predict CCI.

765 Take Table S10 for another example; the F -value of 2.45 and small p -value (0.0043)
 766 illustrated that most variations in the Cd-bioaccumulation could be explained by a
 767 quadratic regression equation. The p -values ≤ 0.05 showed that the model terms were
 768 statistically significant with a 95% confidence interval. The 0.11% lack-of-fit value shows
 769 the probability of the model getting affected by the noise, which is significant. The
 770 significant lack-of-fit indicates that the model fails to fit the data accurately. The value of
 771 $R^2 = 0.46$ between the predicted and experimental data indicated that the developed
 772 model has failed to explain 0.55 % of the total variation. Since the ratio is slightly greater
 773 than 4, it can be concluded that the constructed model shows a somewhat good signal-to-
 774 noise ratio. The ANOVA results suggested that the humic acid concentration (F -value =
 775 17.83, p -value < 0.0001, SS = 18.58) had the most significant effect on Cd-
 776 bioaccumulation followed by biomass/biochar type (F -value = 2.67, p -value = 0.0309,
 777 SS = 13.91).

778 By and large, our results showed that the quadratic (second-order polynomial) model is
 779 more significant than other models for the response variables. In general, the obtained
 780 ANOVA results (Supporting Information Tables S4-S34) revealed that the developed

response regression models for EDI-Mn, EDI-Cd, EDI-Pb, HQ-Zn, HQ-Mn, HQ-Cd, HQ-Pb, HI, CR-Cr, and CR-Pb were statistically significant at 95% of probability level. Although, the final diagnosis of the regression models' efficiency should be made by evaluating the described statistics.

To evaluate the models, the actual data were plotted against the corresponding predicted values and compared with the 1:1 line (Fig. 17). The plots between actual and predicted values from models in Equations 9-17 yielded R^2 values range from 0.24 to 0.54, indicating not a good correlation of experimental data with predicted values (Tables S47-S12). Other experimental versus predicted plots are given in Supporting Information Figures S1-S3.

Pareto analysis was performed to quantify the contribution of individual and interacting factors (Figs. 18 and 19). As an example, in the case of CCI in Eq. 9, Pareto analysis revealed that C1 contributes 91% to the changes in the relative chlorophyll content (Fig. 18). Figure 19 also shows that B, C5, and BC3 were each responsible for about 18% of Cd-bioaccumulation (Eq. 15). Readers can find more information about the relative contribution of process variables on other modeled responses in Supporting Information Figures S4-S6.

3.8. Model analysis via graphical 3D response surfaces and 2D contour plots

Diagnosing and quantifying interactions between different components involved in a process is of vital importance, especially when multivariate optimization methods are used in solving technical problems. Graphical 3D response surfaces and 2D contour plots

allow the identification and measurement of these interactions, based on calculations of developed regression models. For a process involving two factors, one contour plot and one 3D surface plot are generated. According to the bioaccumulation of 8 HMs examined in this study following soil application of 6 biomass/biochar types, 48 response surface, and 48 contour plots were drawn, the complete list of which is given in the supplementary information file (Figures S7-S101). Saddle and ridge-shaped 3D graphs [(inverse) hyperbolic contour plots] suggest the existence of a significant mutual interaction between the experimental variables (Rehman, Arslan et al. 2022), while linear contour lines represent a non-significant interaction (Ali, Abdelsalam et al. 2018).

For example, the response surfaces and corresponding contour plots due to the effects of SBP600 and various doses of humic acid on the response HMs-bioaccumulation are illustrated in Figs. 20-23. The elliptical nature of the contour lines of Fe-bioaccumulation in Fig. 20(d), Mn-bioaccumulation in Fig. 21(d), Cr- and Cd-bioaccumulation in Fig. 22(b, d), and Pb- and Ni-bioaccumulation in Fig. 23 (b and d) implies that there is a significant interaction between the application rate and the humic acid. High interactions between the process variables reveal the presence of inflection points in the response surface, which help to estimate the optimization process. From the contour plot in Fig. 20 (b), the nearly linear contour lines imply that the interaction between the applied doses of SBP600 and humic acid is weak. Fig. 20(c) shows that the Fe-bioaccumulation increases significantly with the increase in the applied dose of humic acid. Applied dose of SBP600 also decreases Fe-bioaccumulation. According to the contour plot in Fig. 20(d), the maximum Fe-bioaccumulation was achieved approximately after applying 1800 ppm at a

825 lower value of applied SBP600. The 3D surface and contour plots for the response of Cu-
826 bioaccumulation were mainly dependent on the soil application of humic acid (Fig. 21a,
827 b). As shown in Fig. 21(c, d), the increase in Mn-bioaccumulation is affected by the
828 increase in humic acid concentration and biochar applied dose. However, the mutual
829 effect of these two independent variables on Mn-bioaccumulation is decreasing. To obtain
830 the minimum value of Mn-bioaccumulation, the values of humic acid and applied dose of
831 SBP600 should be kept to a minimum, as illustrated in (Fig. 21c, d). According to Fig.
832 22b, the correlation of humic acid and SBP600 has a significant influence on the Cr-
833 bioaccumulation. In contrast to previously, the maximization of humic acid concentration
834 decreased the bioaccumulation of Cr. Here, to obtain the minimum accumulation, we
835 need to maximize the humic acid concentration and minimize the SBP600 application rate
836 (Fig. 22a, b). As shown in Fig. 22(c, d), the Cd-bioaccumulation has a minimum value at
837 the central point of humic acid concentration (1250 ppm), and SBP600 applied dose (2.55
838 %w/w), and the response decrease in both directions away from it. The response Pb-
839 bioaccumulation decreases by increasing the humic acid concentration to reach a
840 minimum value of 9.7 (Fig. 23a, b). The bioaccumulation of Pb decreased with
841 decreasing dose of SBP600 and increasing humic acid concentration, as observed in Fig.
842 23(a, b). A slight decrease in bioaccumulation of Pb was detected at high levels of
843 SBP600. For the last part (Fig. 23c, d), the minimum Ni-bioaccumulation can be achieved
844 by minimizing the humic acid concentration at the central point of the SBP600 applied
845 dose (~ 3 %w/w).

The interaction of SBP600 dosage with humic acid concentration displays a remarkable relation with Fe-, Mn-, Cr-, Pb-, and Ni-EDI (Fig. S41c, d; Fig. S42c, d; Fig. S43a, b; Fig. S44a, b; Fig. S44c, d, respectively). For the contour plots in Fig. S41 (a, b), Fig. S42 (a, b), and Fig. S43 (c, d), the nearly linear contour lines imply the non-significant interaction between the applied doses of SBP600 and humic acid on Zn-, Cu- and Cd-EDI, respectively. It can be seen from the contour lines in Fig. S41 (d) that by increasing the humic acid concentration, Fe-EDI increased. A lower Fe-EDI was observed in the high SBP600 application rate. Minimum Fe-EDI occurs at high doses of SBP600 and low concentrations of humic acid. The results show that the Mn-EDI increases sharply with increasing humic acid concentration and SBP600 dosage, but the amount of Mn-EDI under the mutual effect of these two independent variables decreases when the application rate of SBP600 exceeds ~ 3.5% (Fig. S42c, d). Results display that by increasing the amount of SBP600 up to about 3.3%, the Cr-EDI increases, and further addition does not consider its impact on it (Fig. S43a, b). The minimum Cr-EDI occurs in high concentrations of humic acid and low doses of SBP600. The Pb-EDI increases with the enhancement of SBP600 dosage and reaches a maximum value (~ 0.0045 mg kg⁻¹ day⁻¹) after about 3.5% SBP600 incorporation (Fig. S44a, b). Pb-EDI will be minimal at low SBP600 application rates (< 0.75) and high humic acid concentrations. As shown in Fig. S44(c, d), the minimum Ni-EDI can be achieved by minimizing the humic acid concentration and SBP600 applied dose.

The 3D surfaces and contours in Figs. S65-S68 represents the interaction of two independent variables on the HMs-HQ amounts. According to contour lines in Fig. S65b,

868 Zn-HQ is mainly affected by changes in humic acid concentration, and increasing the
869 applied dose of SBP600 does not affect it. Similar results also were observed in Cu-HQ
870 (Fig. S66a, b) and Cd-HQ (Fig. S67c, d). With the increase of the humic acid
871 concentration and decrease of the applied dose of SBP600, the Fe-HQ increases (Fig
872 S65c, d). When the humic acid concentration reaches ~ 1800 ppm at the low to medium
873 SBP600 ($< \sim 3$ %w/w), the Fe-HQ reaches its maximum. As we can see from Fig. S66 (c,
874 d), when the concentration of humic acid and applied dose of SBP600 be at minimum
875 amounts, the Mn-HQ reaches its minimum. From contour lines in Figs. S67 (b) and S68
876 (b), to obtain the minimum Cr- and Pb-HQ, we need to maximize the humic acid
877 concentration and minimize the SBP600 application rate. As shown in Fig. S68 (d),
878 increasing the concentration of humic acid in the presence of low levels of SBP600
879 maximized the Ni-HQ. The minimum Ni-bioaccumulation can be achieved by minimizing
880 the humic acid concentration at the central point of the SBP600 applied dose (~ 3 %w/w).
881 Fig. S85 (a, b) presents the interactive effects of the humic acid concentration and
882 SBP600 application rate on the HI as an index for evaluating the risk of non-carcinogenic
883 risk of multiple HMs. As can be seen, HI increases sharply with increasing humic acid
884 concentration. Also, HI is much more affected by the applied concentration of humic acid
885 than the application rate of SBP600. However, an SBP600 application dose of more than
886 ~ 3.4 %w/w is not recommended. The minimum HI can be achieved by minimizing the
887 humic acid concentration, and SBP600 applied dose.

888 The 3D surfaces and contours in Figs. S91 and S92 represent the interaction of two
889 independent variables on the CR-HMs values. The minimum CR-Cr can be obtained at

high concentrations of humic acid and low doses of SBP600 (Fig. S91a, b). From the contour plot in Fig. S91 (d), the nearly linear contour lines imply that the interaction between the applied doses of SBP600 and humic acid on CR-Cd is non-significant. CR-Cd is mainly affected by changes in humic acid concentration, and increasing the applied dose of SBP600 does not affect it. In order to obtain the minimum value of CR-Pb we need to maximize the humic acid concentration and minimize the SBP600 application rate (Fig. S92a, b). The minimum CR-Ni conditions are a moderate application of SBP600 (~3.4 %w/w) and 500 ppm humic acid (Fig. S92c, d).

Fig. S100 (a, b) presents the interactive effects of the humic acid concentration and SBP600 application rate on the TCR as an index for evaluating the risk of carcinogenic risk of multiple HMs via oral exposure pathways. As can be seen, TCR increases with increasing humic acid concentration and decreases with increasing SBP600 dosage. The minimum TCR value can be achieved by minimizing the humic acid concentration and maintaining SBP600 applied dose at around 3.4 %w/w.

The chlorophyll content in spinach leaves, which are presented in the form of CCI, affected by the SBB600 application rate and humic acid concentration, are shown in Figure S31. It is indicated that results for different treatments are not significantly affected, and chlorophyll level was steady in all biomass/biochar or humic acid application rates. Although, these results differ from some published studies (Huang and Wang 2010), which found a decline in photosynthetic pigments and chlorophyll amount in the leaves of two mangrove plants seedlings (*Kandelia candel* and *Bruguiera gymnorhiza*) species under HM stress. Haider et al. (Haider, Koyro et al. 2015) found

that loading biochar or humic acid can stimulate photosynthesis by increasing the electron transport rate of photosystem II and the ratio between effective photochemical quantum yield and non-photochemical quenching.

3.9. Optimal multiresponse by the desirability function approach

From the desirability results (Tables 3 and 4) as an example for SBP600 application, the obtained responses optimal (minimum) values of Zn-, Fe-, Cu-, Mn-, Cr-, Cd-, Pb-, and Ni-bioaccumulation are 74.97, 639.40, 9.82, 38.23, 6.22, 3.19, 14.10, and 16.51 $mg\ kg^{-1}$ at optimum process parameters of application rate of 0.75 %w/w and 500 ppm humic acid concentration. Numerical optimization by RSM also resulted in HI and TCR of 2.98 and 0.0093, respectively. Tables 3 and 4 illustrate that none of the investigated biomass or resultant biochars can be used in soil due to their metal-contaminated nature. Treating soil with biomass or biochar containing potentially toxic elements increases the risk of producing unhealthy crops by enhancing crop uptake of HMs. The desirability results also recommend using the minimum concentration of humic acid, which indicates the role of humic acid in promoting pollutant bioavailability and mobility.

4. Conclusion

The findings of this research prove that all the tested materials fail to meet the requirement of an adsorbent for ameliorating contaminated soil conditions due to their HMs-polluted nature. It also shows that the treatment of metal-contaminated soil with HMs polluted-biochars fails to stabilize (immobilize) and reduce pollution in any way.

The HI and TCR values of all treatments were greater than their threshold limits of the acceptable carcinogenic and non-carcinogenic risks. Therefore, there is a strong possibility that consuming crops grown in soil treated with HMs polluted-biochars in the long term increases the risk of developing cancerous and non-cancerous diseases. The most striking result to emerge from the data is that treating HMs polluted-soils with humic acid can increase the risk of excessive bioaccumulation of HMs in crops. In addition, humic acid can increase the bioavailability of potentially toxic HMs in polluted applied biochars. Applying pyrolyzed biochars at 600 °C to contaminated soil was more effective in reducing the cumulative carcinogenic risk than biomass and low pyrolyzed biochars.

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Appendix A. Supplementary data

Additional information on the studied (initial) soil properties, experimental design, the bioavailable fraction of HMs in soil, feedstocks, and biochars, multiple regression models, tables of regression analysis, predicted vs. observed regression scatter plots, Pareto charts, and 3D surface and 2D contour plots are presented here.

Conflict of Interest statement

The authors declare that they have no conflict of interest.

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Table 1

Independent variables and their coded levels and experimental ranges for the central composite design (CCD).

Numeric factors								
Independent variables	Units	Labels	Range (coded)					
			$-\alpha$	-1	0	+1	$+\alpha$	
Application rate	%	(A)	0.00	0.75	2.55	4.35	5.10	
Humic acid	$mg\ L^{-1}$	(B)	189	500	1250	2000	2310	
Categorical factors								
			Levels coded					
			1	2	3	4	5	6
Biomass/Biochar type	-	(C)	SBP	RH	SBP300	SBP600	RH300	RH600

Table 2

Total concentration of HMs in spinach leaves and Indian and SEPA standards for HMs in food.

Source	Zn	Fe	Cu	Mn	Cr	Cd	Pb	Ni
	$mg\ kg^{-1}$							
Leaves	116.00	765.27	15.70	70.00	5.80	3.31	13.43	15.91
Threshold standard ^a	50	- ^c	30	- ^c	20	1.5	2.5	1.5
Threshold standard ^b	100	- ^c	20	- ^c	0.5	0.1-0.2	9	10

^a Awashthi, (2000)

^b SEPA (2005)

^c Not enlisted.

Table 3

Desirability results for numerical optimization of HMs-bioaccumulation, EDI, HQ, HI, CR, and TCR with three goals of biomass/biochar type, humic acid concentration, and application rate. Obtained desirability in the range of independent variables applied in designing experiments and the responses set as minimize.

Optimum value						
Factors	Goal	Upper limit	Lower limit	SBP	SBP300	SBP600
Biomass/biochar types	SBP-SBP600	-	-			
Application rate (%w/w)	is in range	0.75	4.35	0.75	0.75	0.75
Humic acid (ppm)	is in range	500	2000	500	500	500
Responses						
Zn-bioaccumulation (mg/kg)	minimize	48.22	217.80	109.81	95.35	74.97
Fe-bioaccumulation (mg/kg)	minimize	270.45	1463.40	576.58	531.17	639.40
Cu-bioaccumulation (mg/kg)	minimize	5.92	34.05	13.36	15.14	9.82
Mn-bioaccumulation (mg/kg)	minimize	23.02	139.42	38.86	59.53	38.23
Cr-bioaccumulation (mg/kg)	minimize	1.42	9.75	3.46	5.76	6.22
Cd-bioaccumulation (mg/kg)	minimize	1.42	6.15	3.20	2.11	3.19

Pb-bioaccumulation (mg/kg)	minimize	1.72	33.15	11.00	9.72	14.10
Ni-bioaccumulation (mg/kg)	minimize	8.62	31.72	14.51	13.55	16.51
EDI-Zn (mg/kg/day)	minimize	0.0138	0.0623	0.0194	0.027	0.0231
EDI-Fe (mg/kg/day)	minimize	0.0774	0.4867	0.1642	0.154	0.1817
EDI-Cu (mg/kg/day)	minimize	0.0016	0.0142	0.0038	0.004	0.0029
EDI-Mn (mg/kg/day)	minimize	0.0066	0.041	0.0111	0.017	0.0109
EDI-Cr (mg/kg/day)	minimize	0.0004	0.0035	0.0010	0.0017	0.0018
EDI-Cd (mg/kg/day)	minimize	0.0004	0.0018	0.0007	0.0007	0.0007
EDI-Pb (mg/kg/day)	minimize	0.0005	0.0095	0.0031	0.0028	0.0040
EDI-Ni (mg/kg/day)	minimize	0.0006	0.0091	0.0038	0.0035	0.0040
HQ-Zn (-)	minimize	0.046	0.208	0.105	0.092	0.072
HQ-Fe (-)	minimize	0.110	0.695	0.235	0.220	0.260
HQ-Cu (-)	minimize	0.041	0.355	0.096	0.109	0.071
HQ-Mn (-)	minimize	0.047	0.288	0.079	0.124	0.078
HQ-Cr (-)	minimize	0.136	1.158	0.323	0.558	0.613
HQ-Cd (-)	minimize	0.365	1.759	0.741	0.706	0.739
HQ-Pb (-)	minimize	0.141	2.709	0.898	0.801	1.147
HQ-Ni (-)	minimize	0.030	0.454	0.206	0.189	0.235
HI (-)	minimize	1.95	5.79	2.69	2.85	2.98
CR-Cr (-)	minimize	0.00020	0.00174	0.00048	0.00084	0.00092
CR-Cd (-)	minimize	0.00014	0.00067	0.00028	0.00027	0.00028
CR-Pb (-)	minimize	4.2×10^{-6}	8.0×10^{-5}	2.7×10^{-5}	2.4×10^{-5}	3.4×10^{-5}
CR-Ni (-)	minimize	0.00102	0.01543	0.00700	0.00641	0.00799
TCR (-)	minimize	0.0022	0.0169	0.0079	0.00750	0.0093
Desirability				0.737	0.705	0.678

Table 4

Desirability results for numerical optimization of HMs-bioaccumulation, EDI, HQ, HI, CR, and TCR with three goals of biomass/biochar type, humic acid concentration, and application rate. Obtained desirability in the range of independent variables applied in designing experiments and the responses set as minimize.

Factors	Goal	Optimum value				
		Upper limit	Lower limit	RH	RH300	RH600
Biomass/biochar types	RH-RH600	-	-			-
Application rate (%w/w)	is in range	0.75	4.35	0.75	0.75	0.75
Humic acid (ppm)	is in range	500	2000	500	500	500
Responses						
Zn-bioaccumulation (mg/kg)	minimize	48.22	217.80	116.96	110.45	105.96
Fe-bioaccumulation (mg/kg)	minimize	270.45	1463.40	442.70	596.77	853.80
Cu-bioaccumulation (mg/kg)	minimize	5.92	34.05	16.49	13.30	18.01
Mn-bioaccumulation (mg/kg)	minimize	23.02	139.42	52.99	93.62	69.07
Cr-bioaccumulation (mg/kg)	minimize	1.42	9.75	3.32	3.98	6.16
Cd-bioaccumulation (mg/kg)	minimize	1.42	6.15	3.74	3.14	3.19
Pb-bioaccumulation (mg/kg)	minimize	1.72	33.15	5.54	7.86	14.69
Ni-bioaccumulation (mg/kg)	minimize	8.62	31.72	18.89	15.01	11.93
EDI-Zn (mg/kg/day)	minimize	0.0138	0.0623	0.0330	0.0304	0.0289
EDI-Fe (mg/kg/day)	minimize	0.0774	0.4867	0.1276	0.1703	0.2434
EDI-Cu (mg/kg/day)	minimize	0.0016	0.0142	0.0047	0.0038	0.0051

EDI-Mn (mg/kg/day)	minimize	0.0066	0.041	0.0154	0.0268	0.0198
EDI-Cr (mg/kg/day)	minimize	0.0004	0.0035	0.0009	0.0011	0.0017
EDI-Cd (mg/kg/day)	minimize	0.0004	0.0018	0.0009	0.0009	0.0006
EDI-Pb (mg/kg/day)	minimize	0.0005	0.0095	0.0017	0.0022	0.0041
EDI-Ni (mg/kg/day)	minimize	0.0006	0.0091	0.0047	0.0046	0.0032
HQ-Zn (-)	minimize	0.046	0.208	0.1130	0.105	0.101
HQ-Fe (-)	minimize	0.110	0.695	0.1823	0.243	0.348
HQ-Cu (-)	minimize	0.041	0.355	0.1183	0.096	0.129
HQ-Mn (-)	minimize	0.047	0.288	0.1103	0.191	0.141
HQ-Cr (-)	minimize	0.136	1.158	0.3056	0.373	0.583
HQ-Cd (-)	minimize	0.365	1.759	0.8919	0.952	0.617
HQ-Pb (-)	minimize	0.141	2.709	0.4784	0.633	1.184
HQ-Ni (-)	minimize	0.030	0.454	0.2720	0.217	0.174
HI (-)	minimize	1.95	5.79	3.37	3.04	3.31
CR-Cr (-)	minimize	0.00020	0.00174	0.00046	0.00056	0.00087
CR-Cd (-)	minimize	0.00014	0.00067	0.00034	0.00036	0.00023
CR-Pb (-)	minimize	4.2×10^{-6}	8.0×10^{-5}	1.4×10^{-5}	1.8×10^{-5}	3.5×10^{-5}
CR-Ni (-)	minimize	0.00102	0.01543	0.00925	0.00738	0.00593
TCR (-)	minimize	0.0022	0.0169	0.0101	0.0083	0.0072

Desirability			0.680	0.655	0.639
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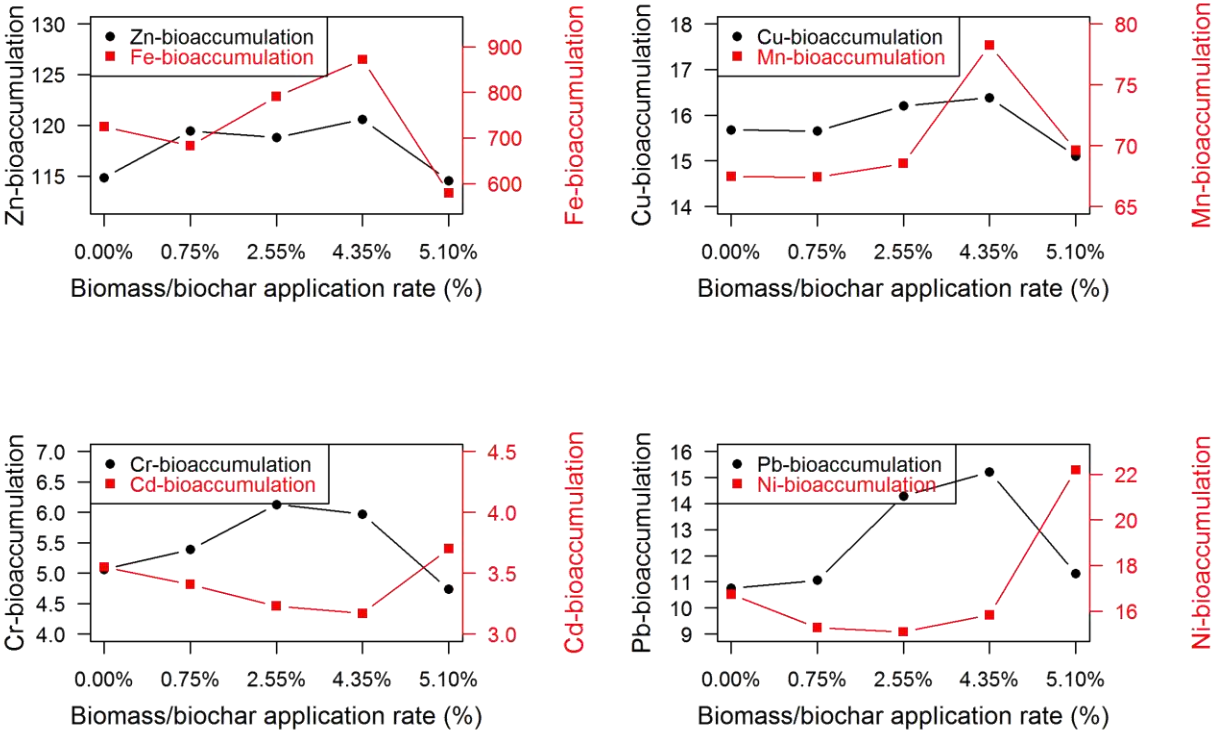


Fig. 1. Changes in HMs-bioaccumulation ($mg\ kg^{-1}$) in spinach leaves as affected by biomass or biochar application rate.

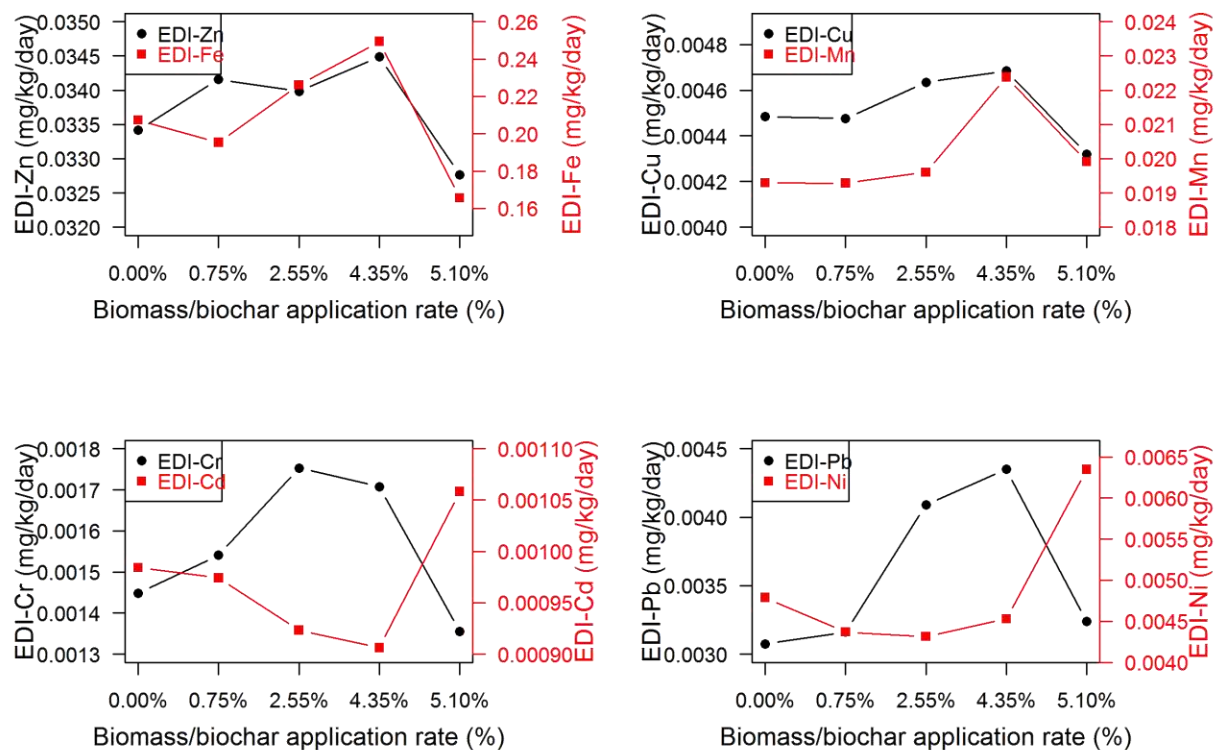


Fig. 2. Changes in estimated daily intake (EDI) of HMs from consumption of spinach leaves as affected by biomass or biochar application rate.

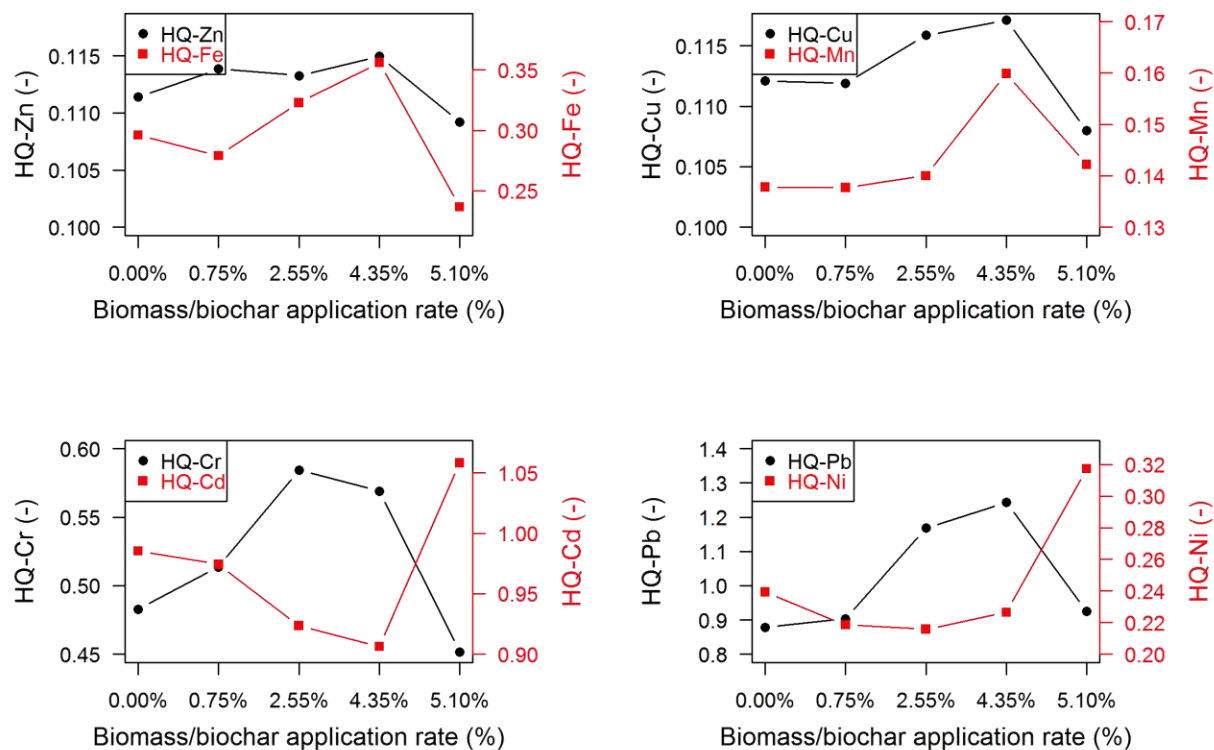


Fig. 3. Changes in hazard quotient (noncarcinogenic risk) (HQ) of individual HMs from consumption of spinach leaves as affected by biomass or biochar application rate.

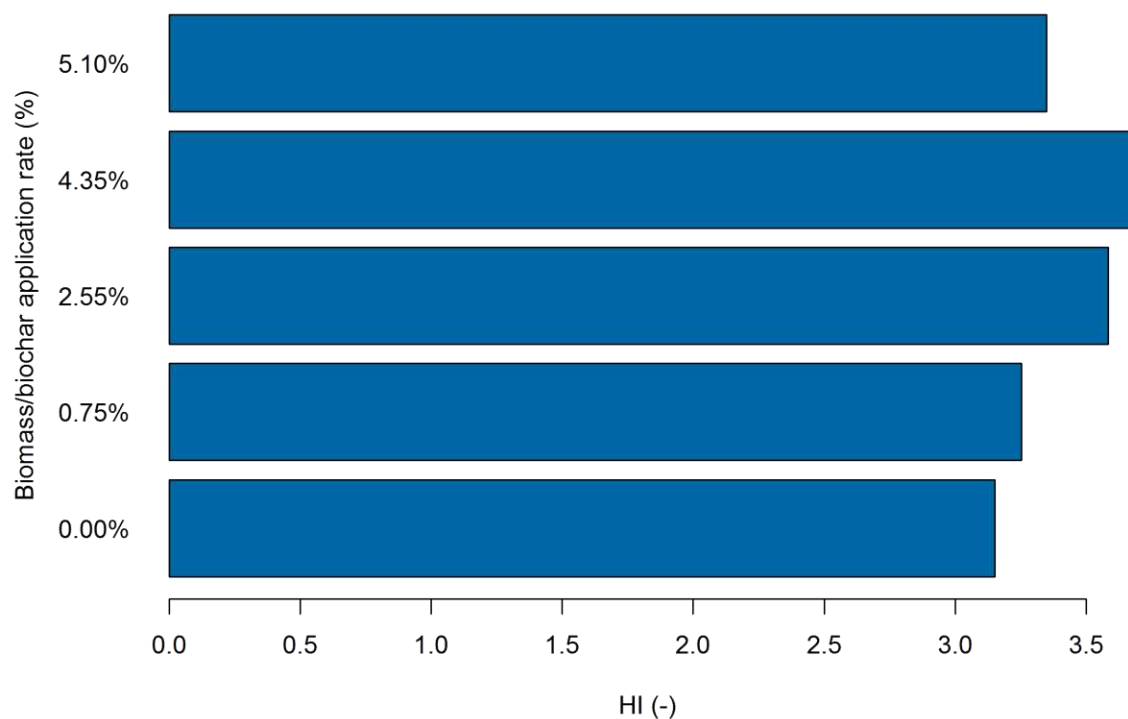


Fig. 4. Changes in the hazard index (HI) of multiple HMs from consumption of spinach leaves as affected by biomass or biochar application rate.

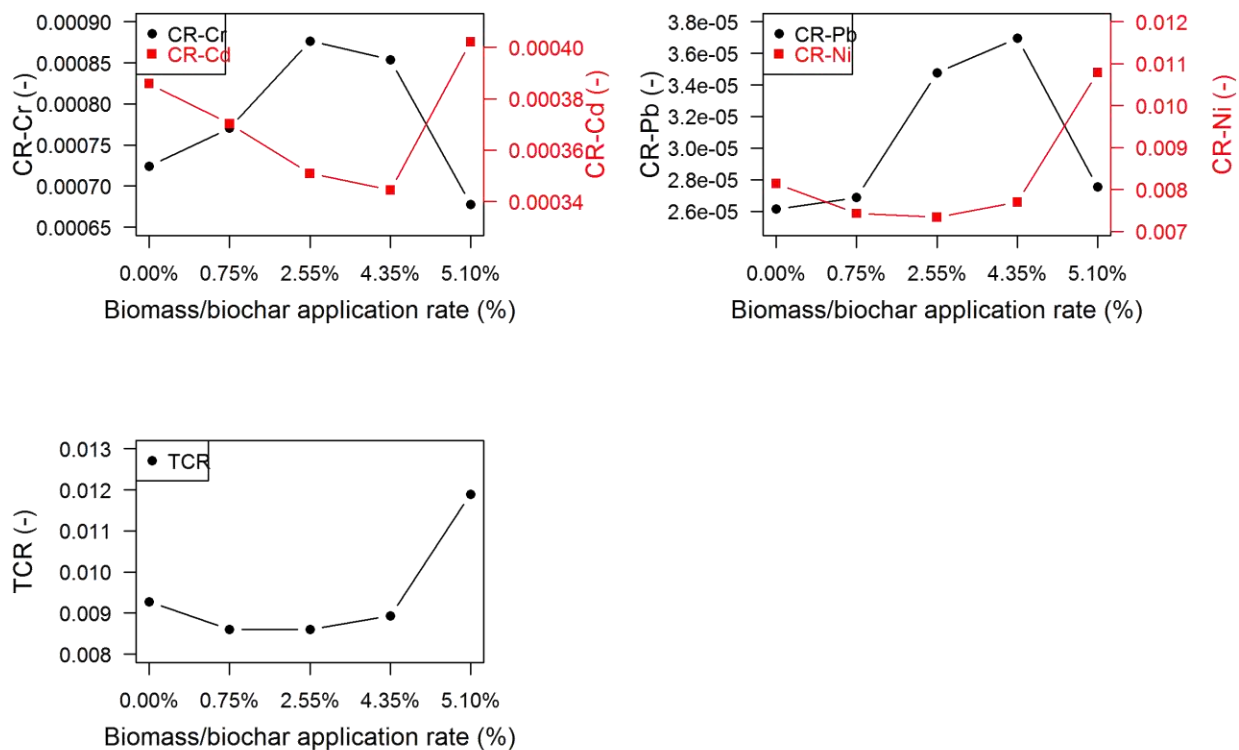


Fig. 5. Changes in the carcinogenic risk (CR) and cumulative carcinogenic risk (TCR) of HMs from consumption of spinach leaves as affected by biomass or biochar application rate.

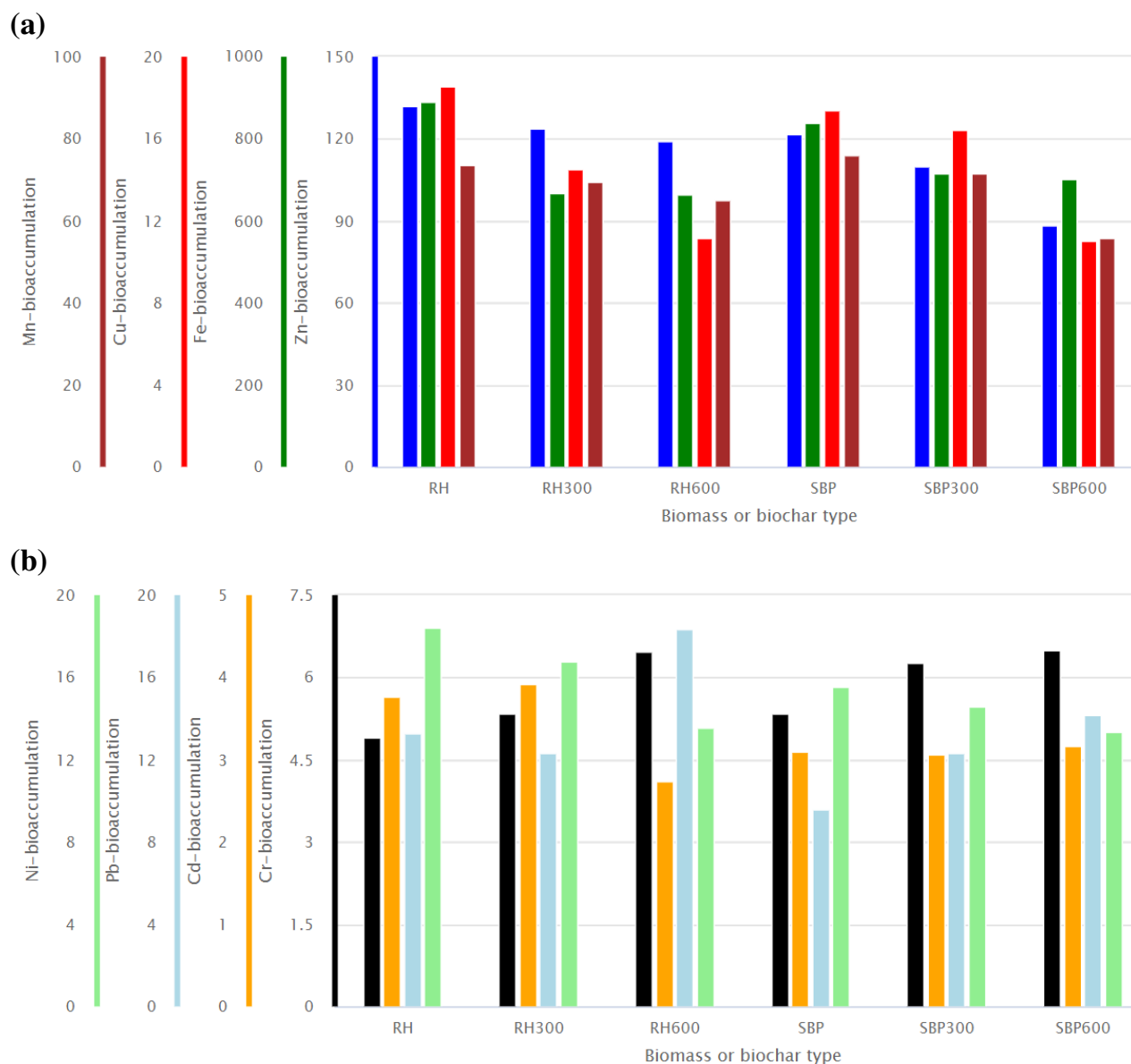


Fig. 6. Changes in bioaccumulation ($mg\ kg^{-1}$) of (a) Zn, Fe, Cu, and Mn and (b) Cr, Cd, Pb, and Ni in spinach leaves as affected by biomass or biochar type.

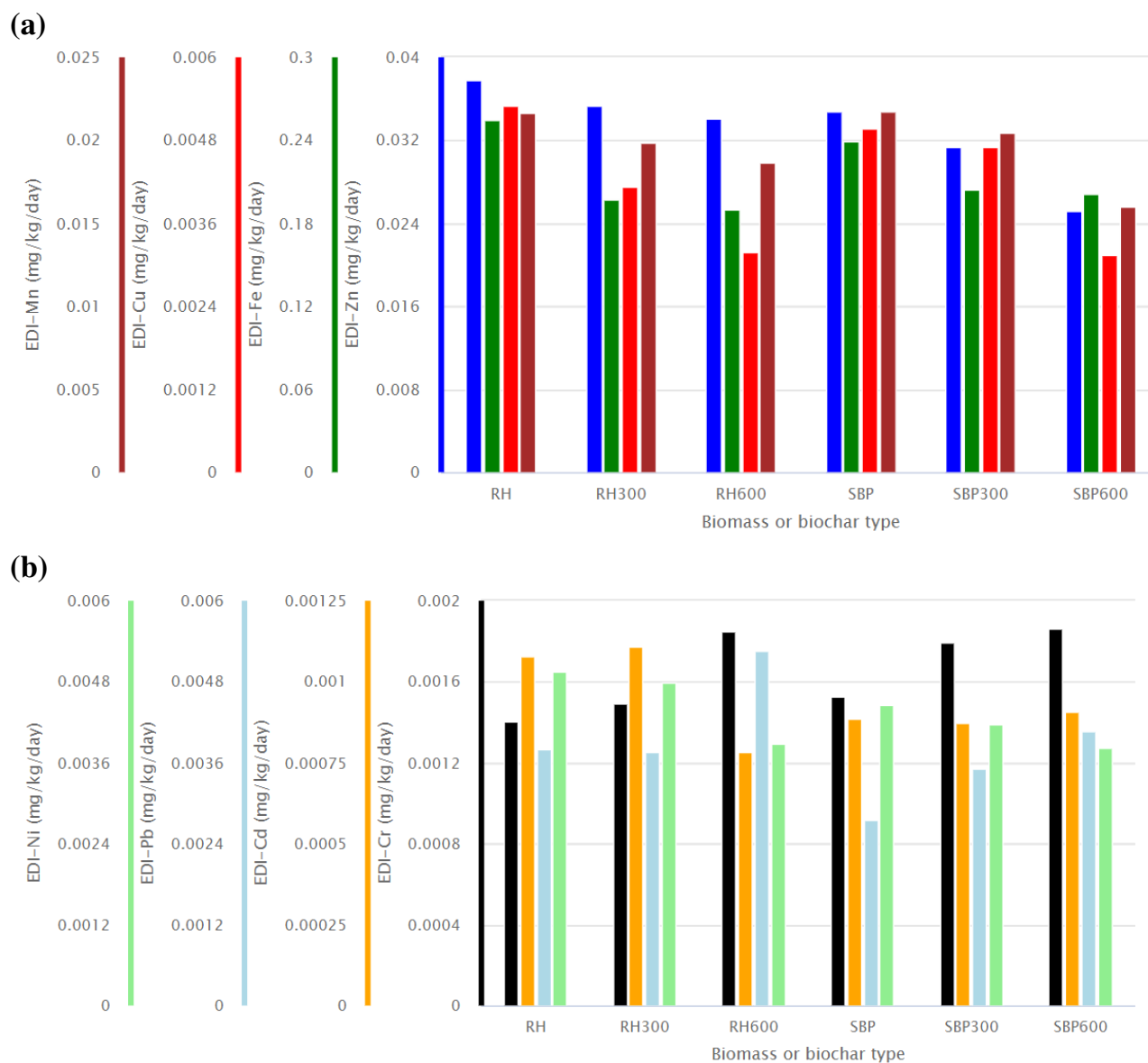


Fig. 7. Changes in estimated daily intake (EDI) of (a) Zn, Fe, Cu, and Mn and (b) Cr, Cd, Pb, and Ni from consumption of spinach leaves as affected by biomass or biochar type.

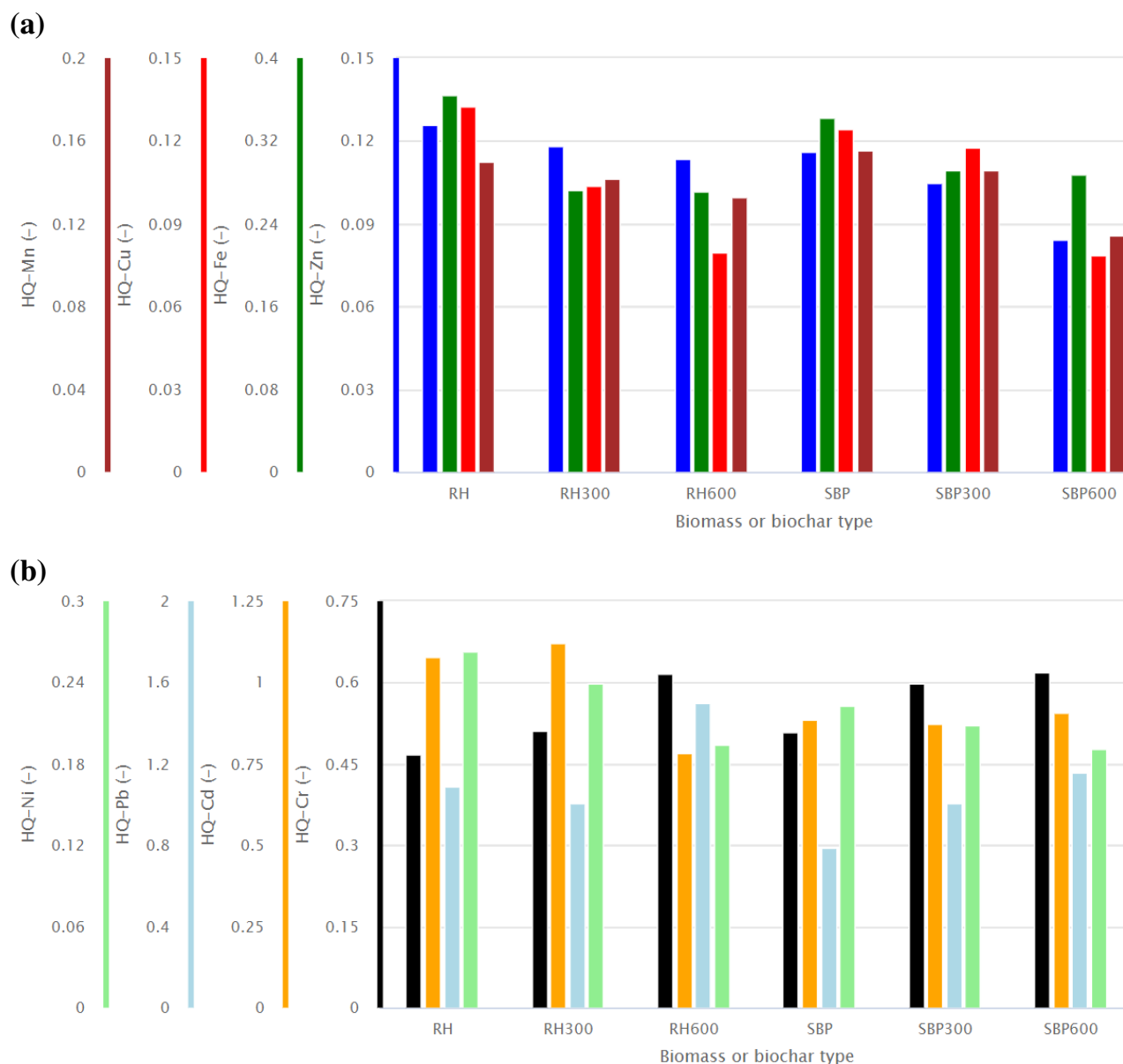


Fig. 8. Changes in hazard quotient (noncarcinogenic risk) (HQ) of (a) Zn, Fe, Cu, and Mn and (b) Cr, Cd, Pb, and Ni from consumption of spinach leaves as affected by biomass or biochar type.

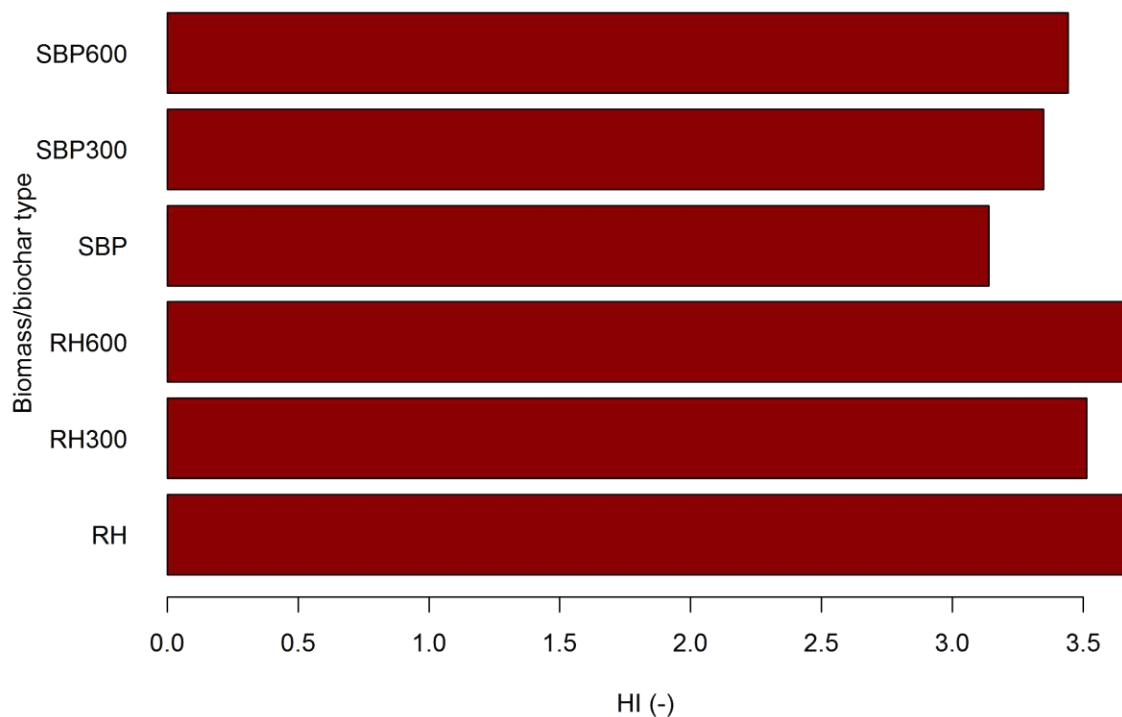


Fig. 9. Changes in the hazard index (HI) of multiple HMs from consumption of spinach leaves as affected by biomass or biochar type.

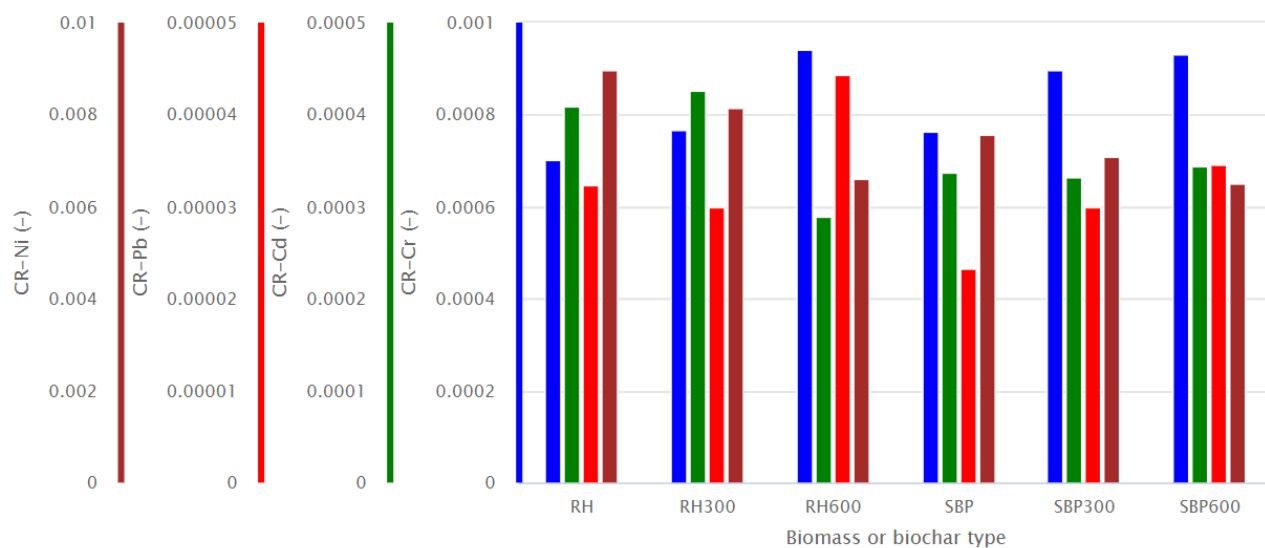


Fig. 10. Changes in the carcinogenic risk (CR) of HMs from consumption of spinach leaves as affected by biomass or biochar type.

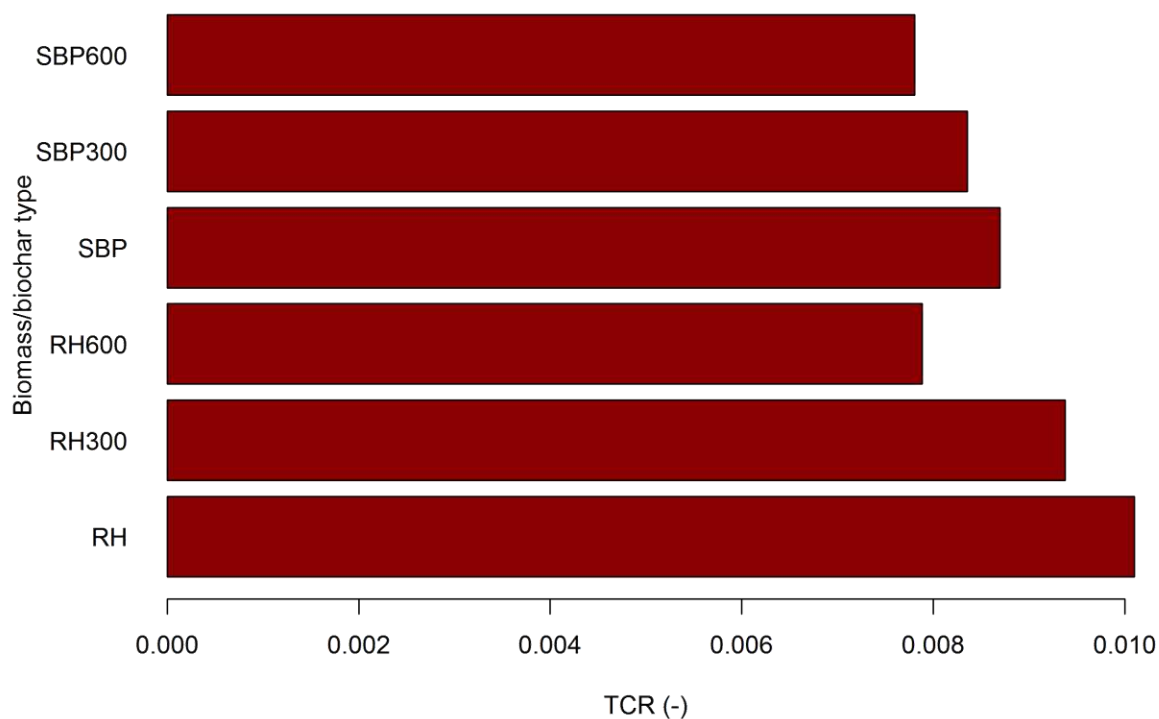


Fig. 11. Changes in the cumulative carcinogenic risk (TCR) of HMs from consumption of spinach leaves as affected by biomass or biochar type.

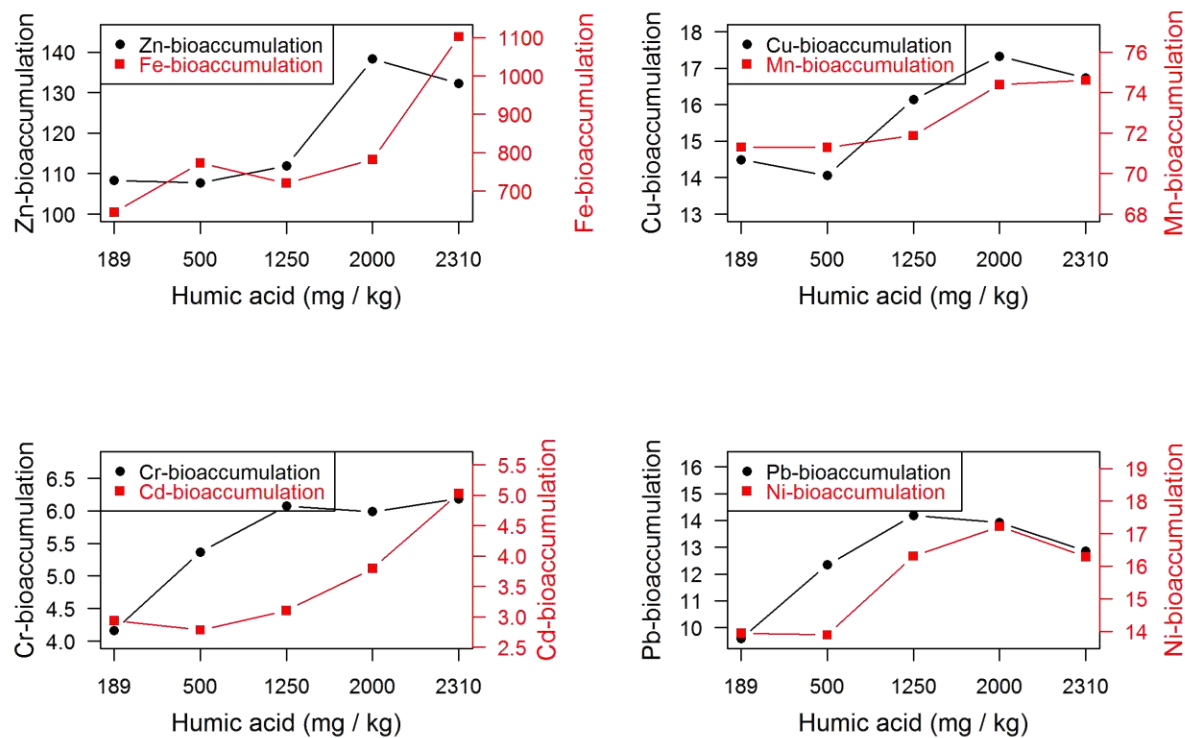


Fig. 12. Changes in HMs-bioaccumulation in spinach leaves as affected by different concentrations of humic acid.

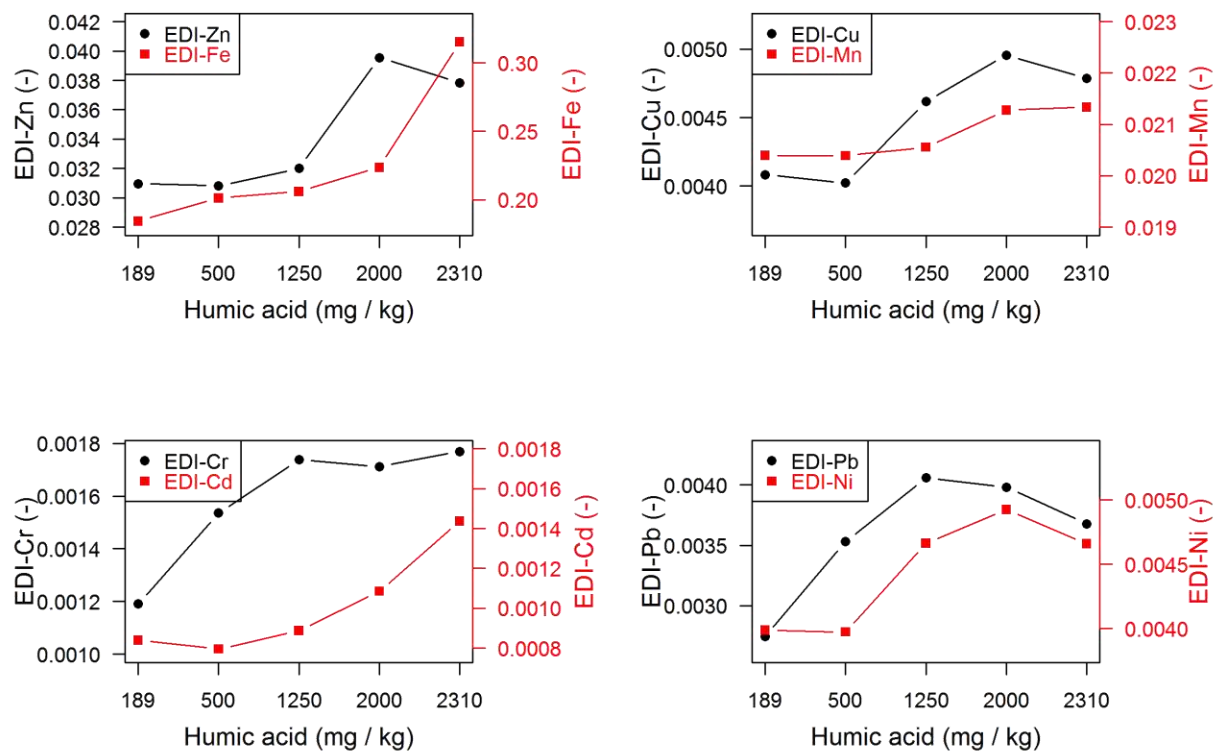


Fig. 13. Changes in estimated daily intake (EDI) of HMs from consumption of spinach leaves as affected by different concentrations of humic acid.

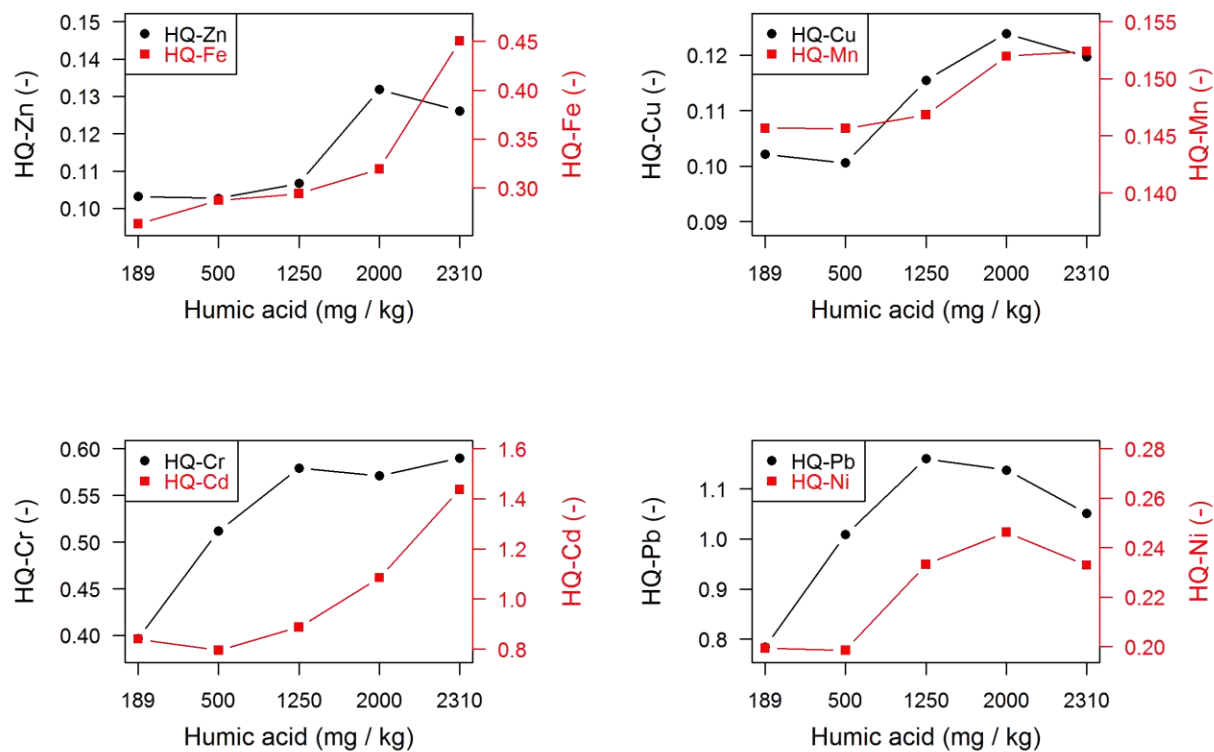


Fig. 14. Changes in hazard quotient (noncarcinogenic risk) (HQ) of individual HMs from consumption of spinach leaves as affected by different concentrations of humic acid.

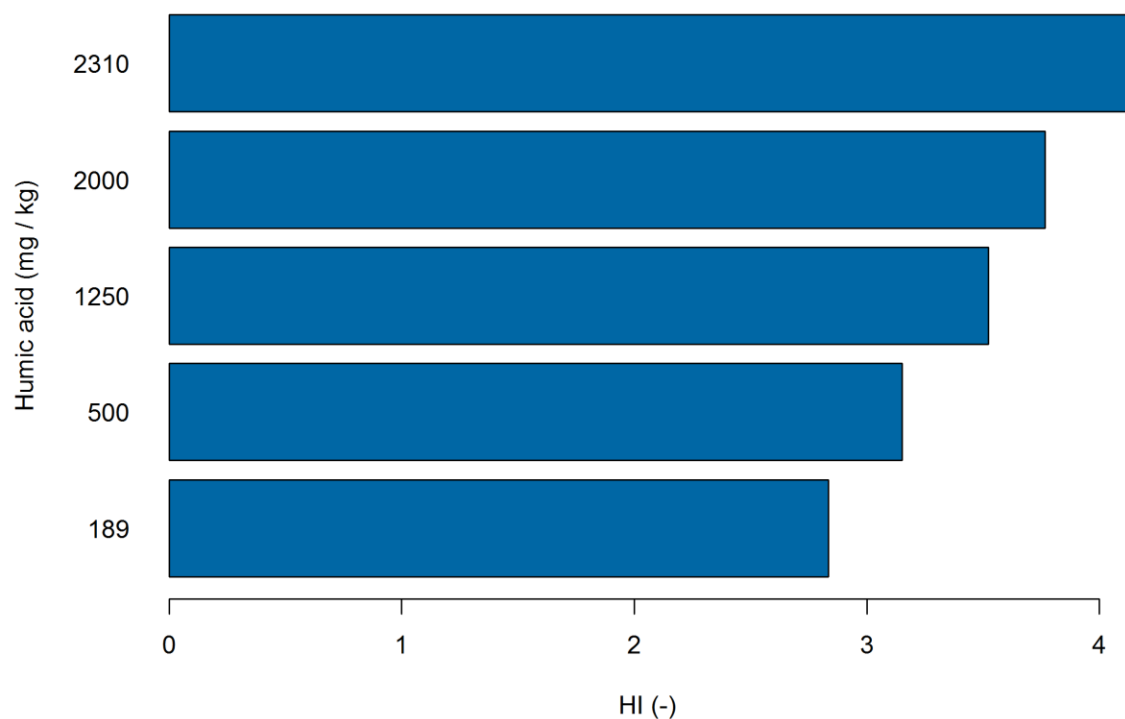


Fig. 15. Changes in the hazard index (HI) of multiple HMs from consumption of spinach leaves as affected by different concentrations of humic acid.

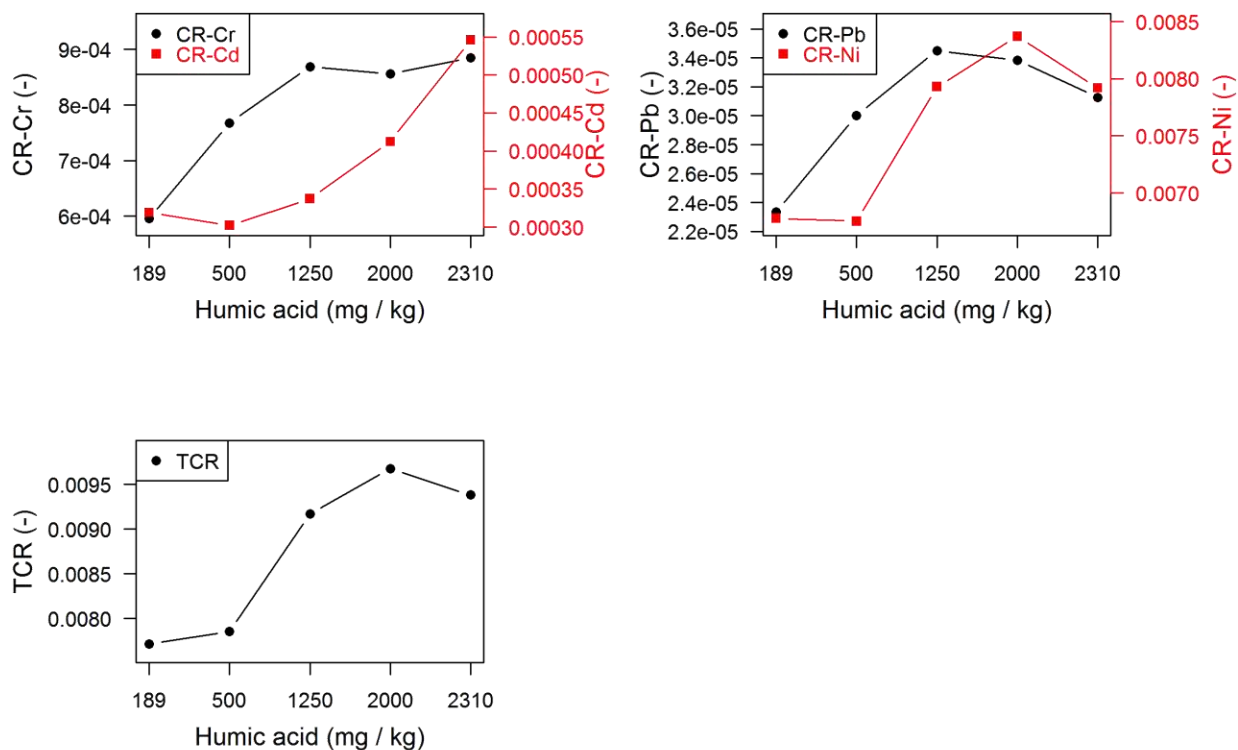


Fig. 16. Changes in the carcinogenic risk (CR) and cumulative carcinogenic risk (TCR) of HMs from consumption of spinach leaves as affected by different concentrations of humic acid.

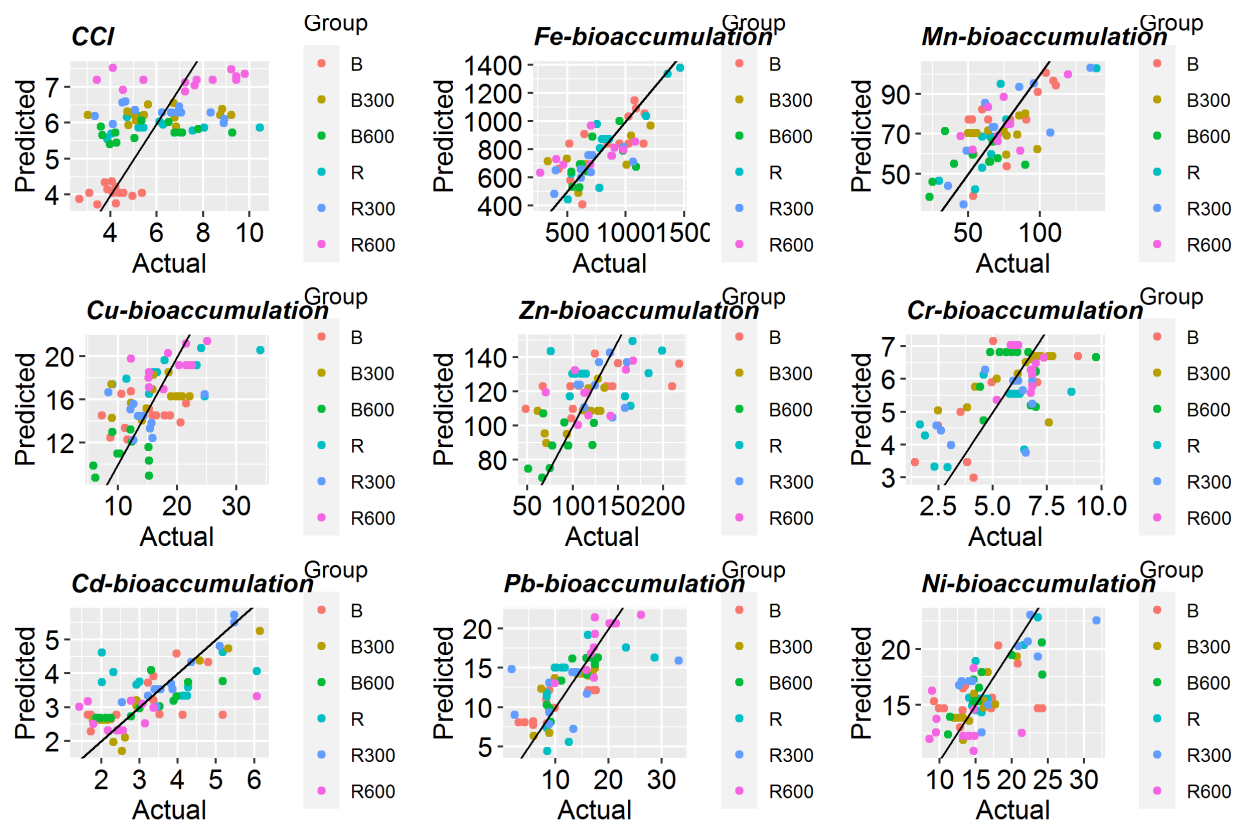


Fig. 17. Predicted vs. observed regression scatter plots derived from the models presented in Equations 9-17 along with 1:1 corresponding lines for CCI and HMs-bioaccumulation.

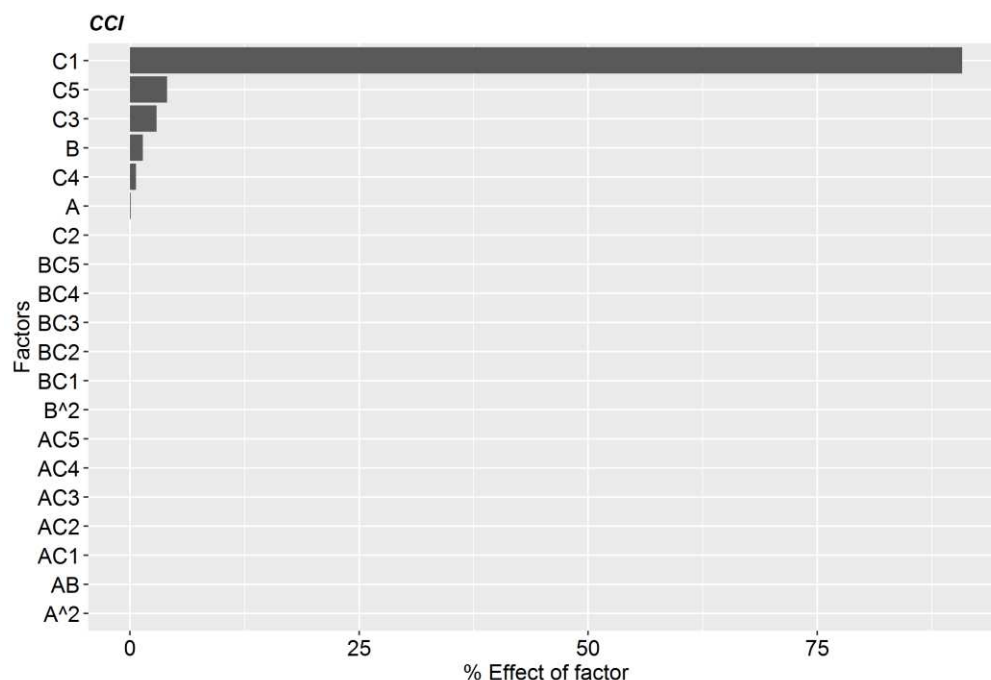


Fig. 18. Pareto chart presenting percent contribution of application rate (A), humic acid concentration (B), and biomass/biochar type (C) to percent CCI.

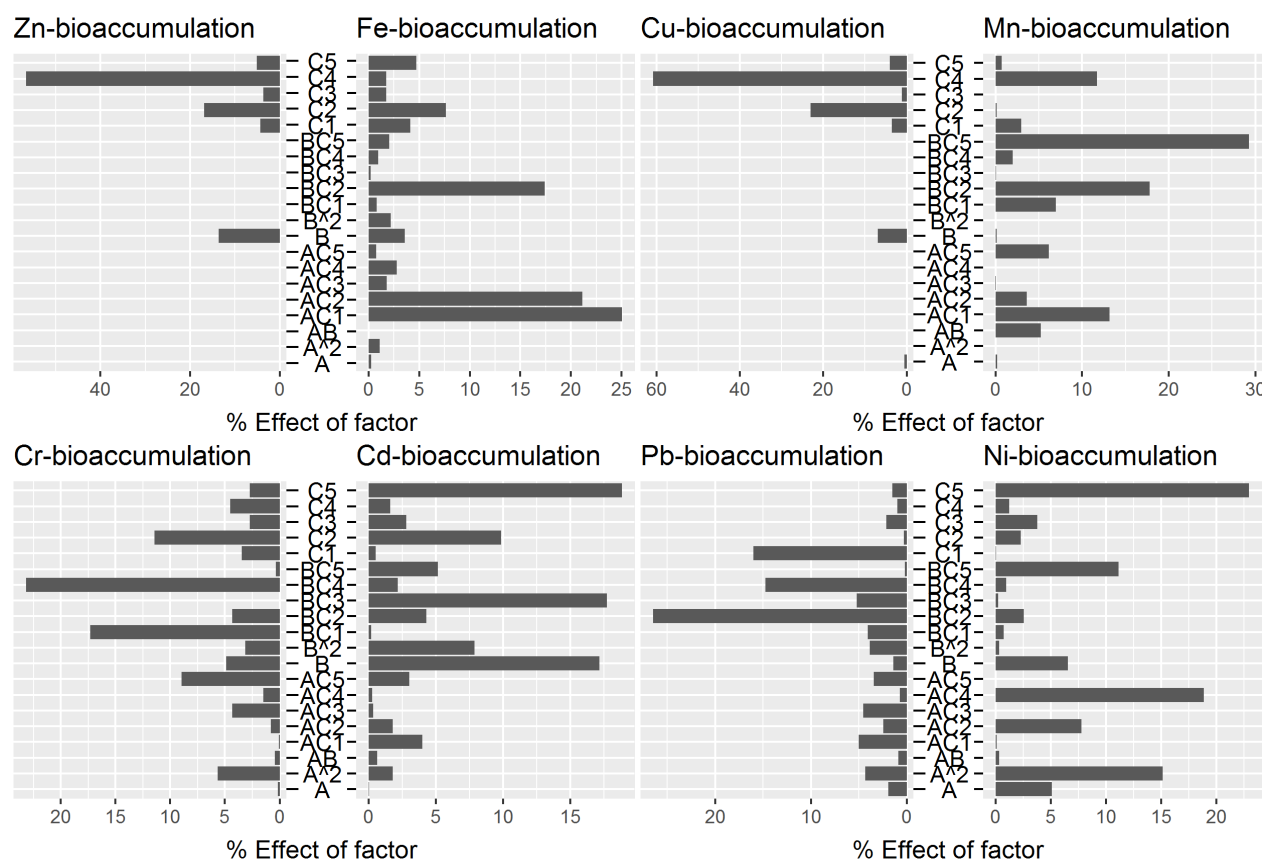
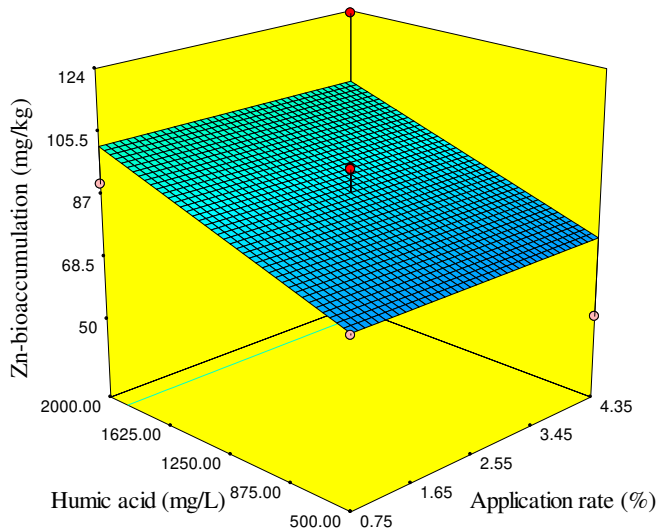
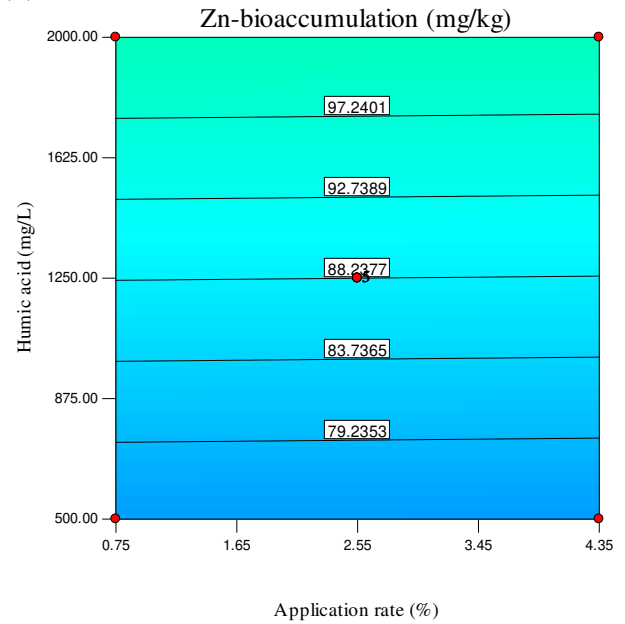


Fig. 19. As Fig. 18, but to percent HMs-bioaccumulation.

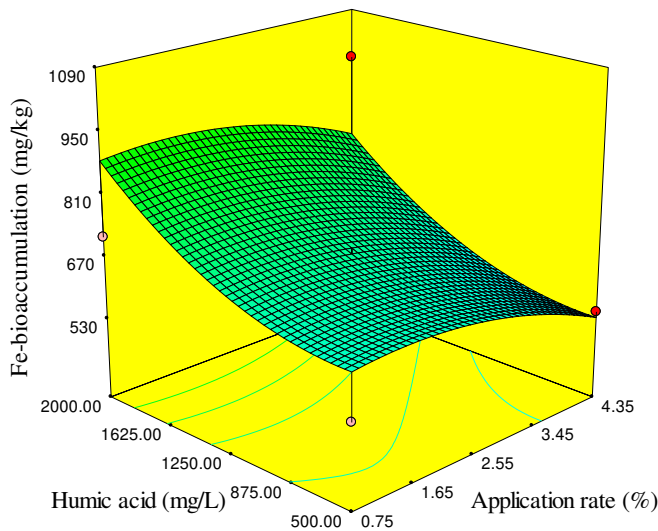
(a)



(b)



(c)



(d)

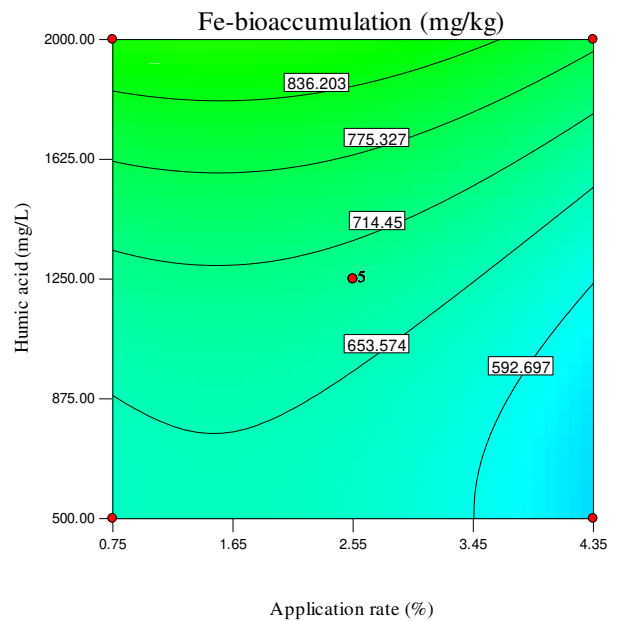
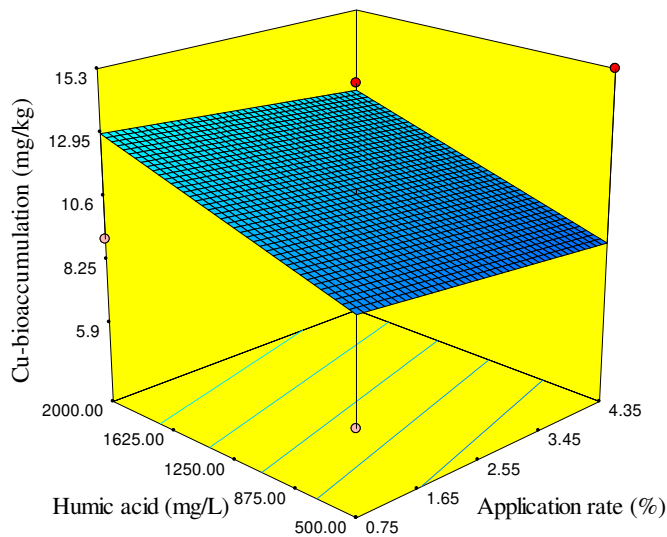


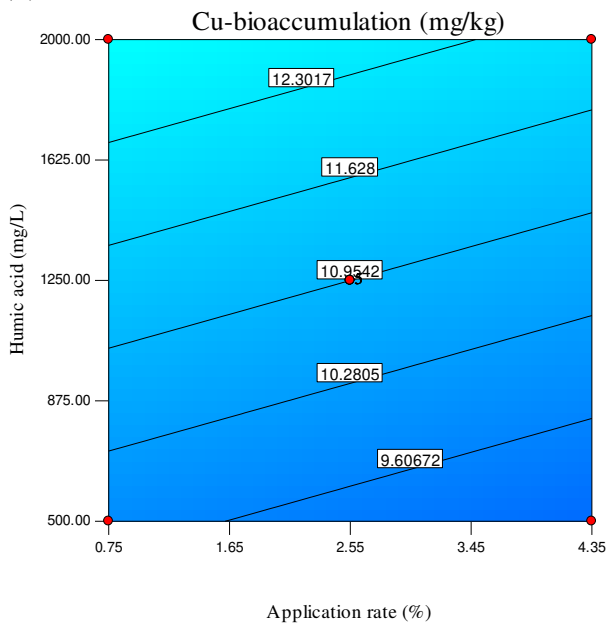
Fig. 20. 3D surface (a and c) and contour plots (b and d) of response Zn- and Fe-bioaccumulations in spinach leaves affected by humic acid concentration and SBB600 application rate.

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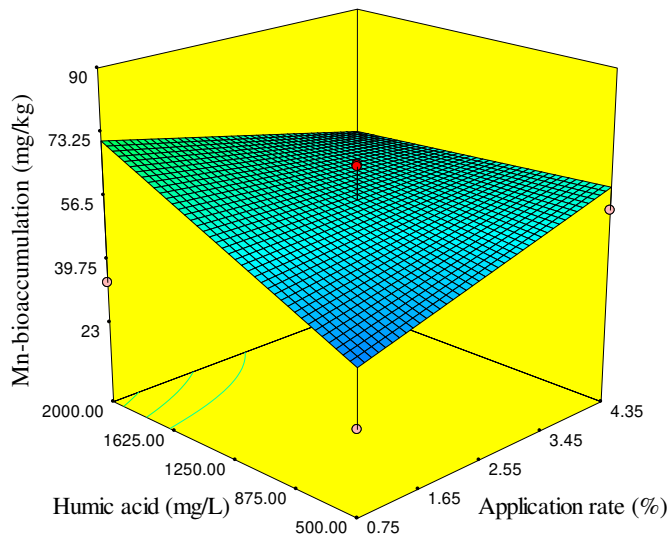
(a)



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(d)

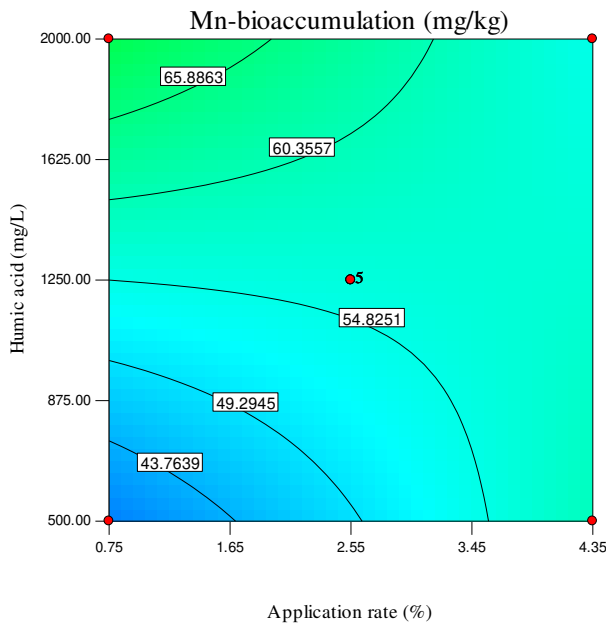
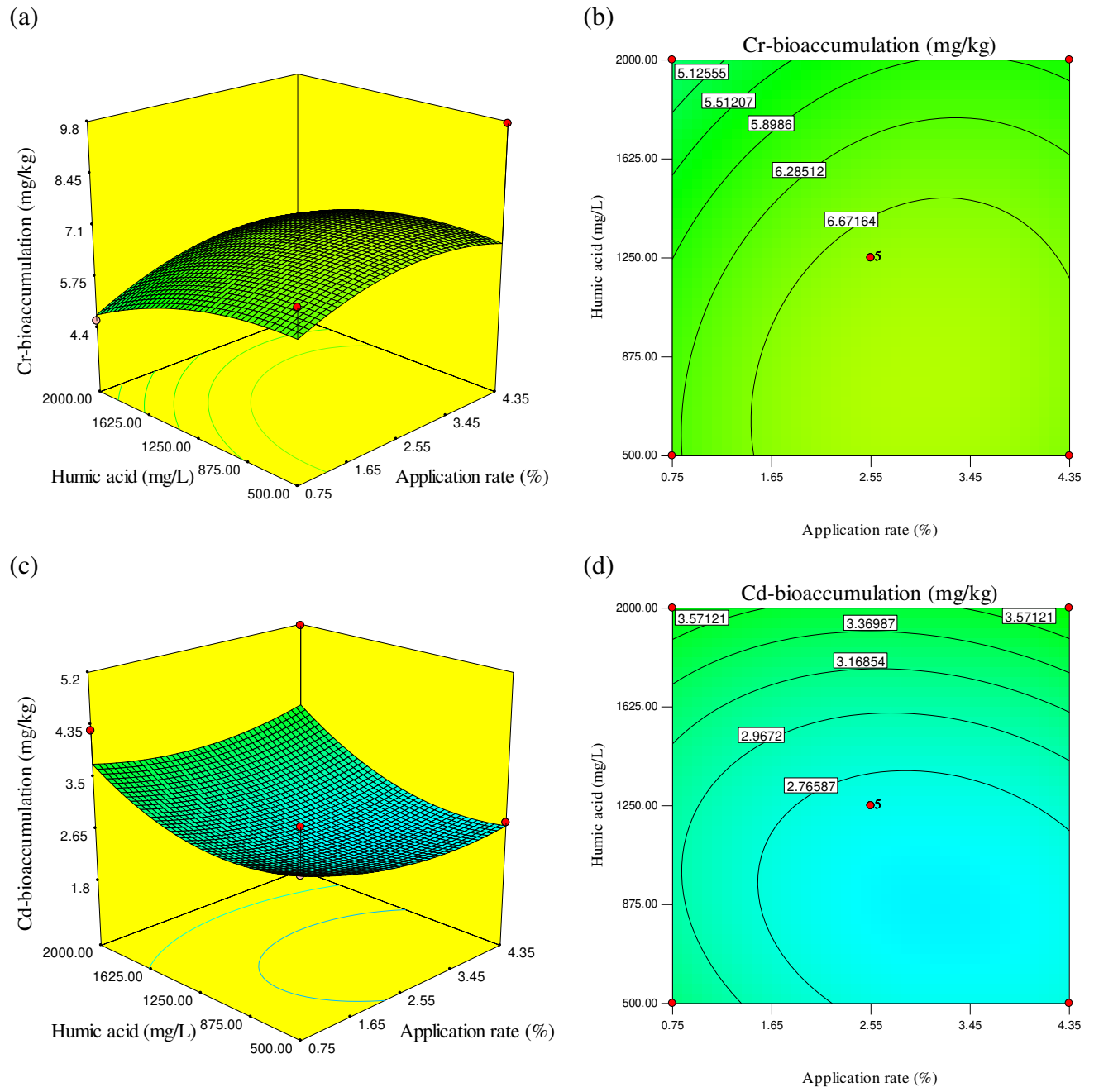


Fig. 21. 3D surface (a and c) and contour plots (b and d) of response Cu- and Mn-bioaccumulations in spinach leaves affected by humic acid concentration and SBB600 application rate.

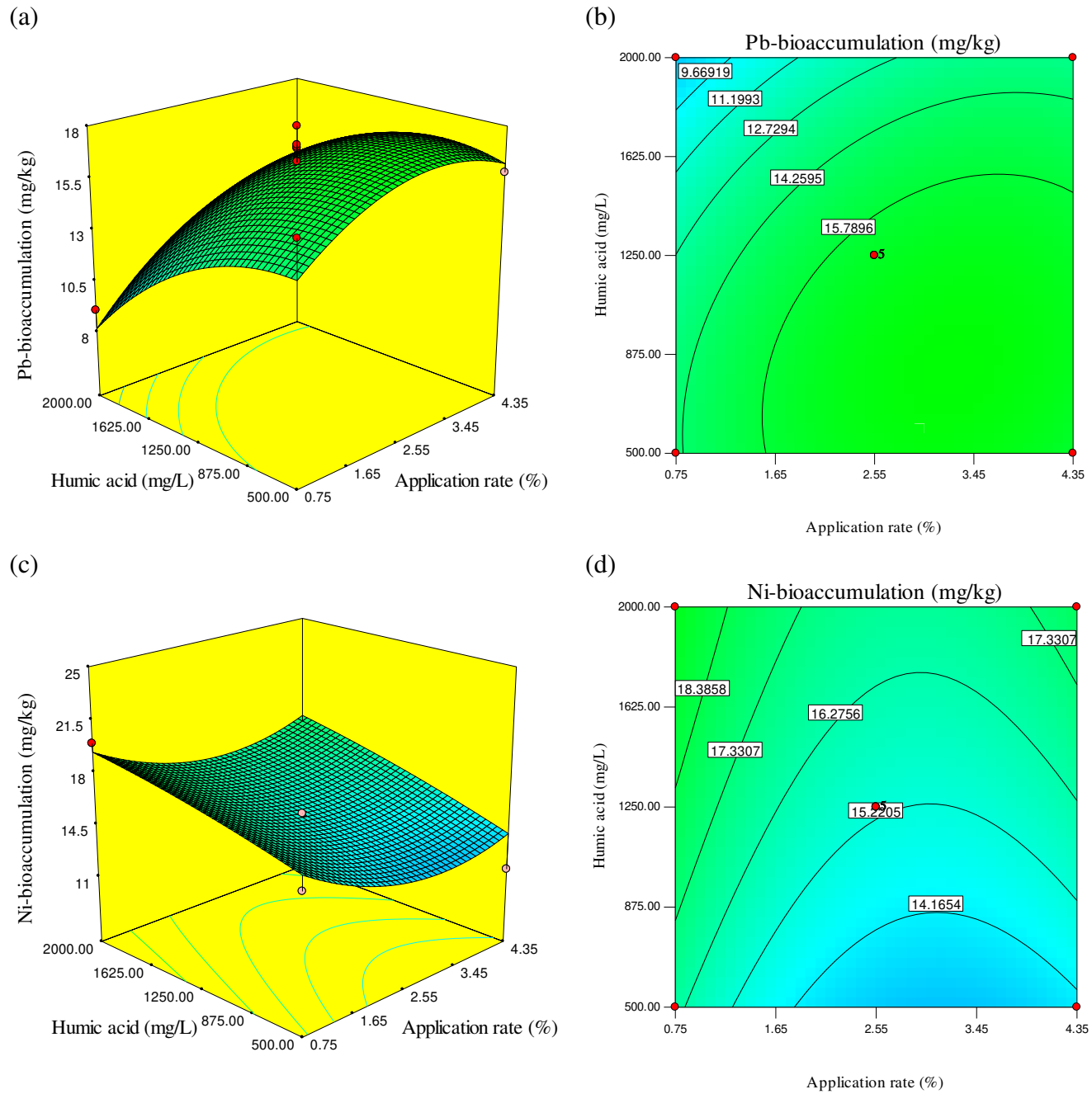
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1549 **Fig. 22.** As Fig. 20 but for Cr- and Cd-bioaccumulations.
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Fig. 23. 3D surface (a and c) and contour plots (b and d) of response Pb- and Ni-bioaccumulations in spinach leaves affected by humic acid concentration and SBB600 application rate.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementary.docx](#)