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International Masters of Science in Environmental Technology and Engineering

Master’s dissertation submitted in partial fulfilment of the requirements for the joint degree of

EU Erasmus+ Master course organized by

University of Chemistry and Technology, Prague, the Czech Republic

IHE Delft Institute for Water Education, Delft, the Netherlands

Ghent University, Ghent, Belgium

Academic year 2022

Biochar as a soil amendment to decrease the uptake of cadmium in spinach

Ghent University, Ghent, Belgium

Ifeoluwapo Ayomikun Babalola

Promotor: Prof. dr. ir. Filip Tack

Tutors: Antoine Delemazure and Maria Laura De Donno

[September 2022]

Co-funded by the Erasmus+ Programme of the European Union

UNIVERSITY OF CHEMISTRY AND TECHNOLOGY PRAGUE

IHE DELFT Institute for Water Education under the auspices of UNESCO

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Declaration

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Ifeoluwapo Ayomikun Babalola

...to my late father

Abstract

Leafy vegetables such as spinach have the tendency to accumulate cadmium (Cd) beyond the permissible limits provided by the European Commission in 2015, i.e., 0.2 mg Cd/kg (fresh weight). Despite its cultivation on agricultural lands with baseline concentrations of Cd i.e., 0.01 – 1 mg Cd/kg, spinach can still contain Cd above the limit. This research aims to use biochar as a soil amendment to control the uptake of Cd in spinach. Among various factors influencing the uptake of Cd by spinach, the impact of watering regime was studied. Cultivation of spinach was carried out in a greenhouse, on agricultural soil (sandy loam) with baseline concentrations of Cd i.e., 0.42 mg/kg. Three treatments included, control soil (soil without biochar), soil with 2% biochar (2% BC), and soil with 4% biochar (4% BC). Moreover, for each treatment, the watering regime implemented was three-times watering a week (3XW), one-time watering a week (1XW), dry-wet watering (DWW) i.e., one-time watering for the first 3 weeks then three-times watering for the last three weeks, and wet-dry watering (WDW) i.e., three-times watering for the first 3 weeks then one-time watering for the last three weeks. After 2 weeks of equilibration, easily extractable Cd (using 0.01 M $CaCl_2$) in substrates with 2% BC and 4% BC had significantly reduced ($P < 0.05$) by 36% and 52% respectively compared to the control soil, while there was no significant difference ($P > 0.05$) in Cd extracted by ammonium acetate-EDTA (pH 4.65). With the aid of a diffusive gradient in thin film (DGT), the ratio (R) which depicts the resupply of Cd from the solid phase to the soil solution was high ($R = 0.8$) for the control soil, but low ($R = 0.3$ and 0.2) for the treatment with 2% BC and 4% BC respectively. In addition, after 6 weeks of treatment, biochar application significantly reduced ($P < 0.05$) Cd concentration in the soil pore-water by 11% - 38% and 53% - 74% for treatments with 2% BC and 4% BC respectively. After harvest i.e., after 10 weeks of cultivation, above-ground biomass (stem, leaves, flowers, and seeds) of spinach was analysed. Highest Cd concentration (0.71 mg Cd/kg) and least Cd concentration (0.29 mg Cd/kg) in spinach were measured under treatments with 2% BC (WDW) and 4% BC (3XW) respectively. In conclusion, Cd most times exceed permissible limits in spinach when cultivated on this agricultural soil because of the continuous increase in bioavailable Cd in the soil pore water, attributable to the exchangeable and reducible fraction of Cd in the soil, coupled with a high resupply of Cd from the solid phase. Both biochar amendments were not able to significantly reduce ($P > 0.05$) Cd uptake by spinach, despite significant effects on soil solution. Therefore, application of this particular biochar may be insufficient in attenuating the risk of exceeding permissible limits of Cd in spinach when cultivated on this soil.

Acknowledgment

Firstly, I will love to express my profound gratitude to the Almighty God for giving me the grace and enablement to successfully complete my thesis. I will also like to render my appreciation to my wonderful supervisor, in person of Prof. Filip Tack, for his undeniable support and efforts throughout my experiment. His kind words, advise, and corrections helped me a lot through this journey and I am more than grateful for having him as my promoter.

Appreciation also goes to my tutors, Antoine Delemazure and Maria Laura De Donno for their support, encouragement, corrections, and guidance during the course of my thesis. It has been a privilege being under their tutelage and mentorship and I cannot take their contributions for granted. I also acknowledge their outstanding assistance and efforts during the cultivation period of my plants.

I cannot neglect the contributions provided to me by the laboratory technicians namely, Joachim Neri, Rosaline Blanckaert, and Amelia Parao. I duly recognize their assistance and advice during my research, most especially during the operation of the laboratory equipment.

Lastly, I love to appreciate the support and encouragement provided to me by my family and friends back in Nigeria. They were more than a driving force toward the successful completion of my thesis.

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Abbreviations

3XW	Three-times watering per week
1XW	One-time watering per week
DWW	Dry-Wet watering i.e. one-time watering for the first three weeks then three-times watering for the last three weeks
WDW	Wet-Dry watering i.e. three-times watering for the first three weeks then one-time watering for the last three weeks
2% BC	Soil with 2% biochar
4 % BC	Soil with 4% biochar
CEC	Cation exchange capacity
TOC	Total organic carbon
EC	Electrical conductivity
DGT	Diffusive gradient in thin films
FAO	Food and Agriculture Organization
USDA	United States Department of Agriculture
BCR	Bureau Communautaire de Reference
DBL	Diffusive boundary layer
EFSA	European Food Safety Authority
WHO	World Health Organization
LOD	Limit of detection
LOQ	Limit of quantification
QC	Quality control
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
DW	Dry weight
BAF	Bioaccumulation factor

1 INTRODUCTION

Cadmium (Cd) is classified as a non-essential, toxic, and relatively mobile heavy metal which is naturally present in the soil (Alloway, 2013, Zahedifar, 2020). Cd concentrations in the soil typically range from 0.01 to 1 mg/kg (Kubier et al., 2019), while the average Cd concentration in European Union (EU) soils is 0.3 mg/kg (Alloway, 2012). Cd concentrations in cultivated crops have been a major concern since the outbreak of the Itai-Itai disease in Jinzu Valley of Japan in the 20th century, which resulted in chronic health disorders, such as bone disease and renal disorders, due to the ingestion of food and water with high levels of Cd leading to the mortality of hundreds of people (Liu et al., 2017, Hayat et al., 2019). Elevated Cd in the soil is majorly attributed to anthropogenic activities (Luo et al., 2009) and geological weathering (Liu et al., 2013).

Some crops such as leafy vegetables have a natural tendency to accumulate high levels of Cd from the soil (Baldantoni et al., 2016, Nawrot et al., 2010). Permissible limit of Cd in cultivated crops such as spinach (*Spinacia oleracea L.*), a widely consumed vegetable in Belgium has been set to 0.2 mg/kg (European Commission, 2015). However, there is a large variability in Cd accumulation by spinach and it sometimes exceed the permissible limits even when cultivated on soils with baseline concentrations of Cd (Smolders, 2001, Khan et al., 2020). This phenomenon has therefore led to various experiments seeking ways on reducing the uptake of Cd by spinach.

The uptake of Cd from the soil is influenced by several factors such as soil pH (Cheng et al., 2020, Qi et al., 2018), organic matter content (Kubier et al., 2019, Muehe et al., 2013), cation exchange capacity, redox potential (Petruzzelli et al., 2020), plant physiology (Yin et al., 2016) and seasonal conditions e.g. rainfall pattern, sunshine, wind, and temperature (Tack, 2017). Cd can exist in different forms, such as soluble Cd^{2+} which can be easily taken up by plants, or insoluble Cd in the form of CdS, $CdCO_3$, and $Cd_3(PO_4)_2$, which prevents absorption of Cd through the roots of plants (Cheng et al., 2020). Therefore, the speciation of Cd in the soil influences the uptake and accumulation of Cd in cultivated plants.

Biochar has been used as a soil amendment over time to improve soil fertility and also mitigate metal accumulation by plants (Park et al., 2011, Sui et al., 2018). Diverse properties such as large surface area, elevated pH, highly porous structure, high cation exchange capacity, and presence of active functional groups make biochar a suitable option for reducing environmental health risks of contaminants (Egene et al., 2018). Some studies have proven biochar to be successful in reducing the uptake of Cd in spinach (Nobaharan et al., 2022, Qayyum et al., 2019, Younis et al., 2016). In addition, the application rate of biochar has an important influence on its role in decreasing the uptake of metals by plants (Egene et al., 2018) and this should be taken into account when using biochar as a soil amendment.

Apart from impeding plant growth and nutrient absorption, water deficiency also has a major impact on the uptake of Cd by leafy vegetables. There seems to be an increase in Cd concentration in spinach when cultivated in water-stressed conditions (Tack, 2017). Therefore, variation in precipitation plays a role in the levels of Cd obtainable in harvested crops.

The main objective of this study was to determine the influence of biochar as a soil amendment on a Belgian soil with baseline concentrations of Cd and evaluate its efficiency in reducing the uptake of Cd by cultivated spinach (*Spinacia oleracea L.*). This involves assessing the plants' response as a function of biochar application rate, time since application, growing conditions, the intensity and frequency of watering, while important uptake of essential elements such as zinc (Zn) or iron (Fe) are to be assessed.

The specific objectives include:

a) evaluate the effectiveness of biochar in decreasing uptake of Cd by spinach cultivated on soil with baseline concentrations
b) assess Cd fractionation across various pools in the soil, as influenced by the addition of biochar
c) determine the influence of varying watering regimes on the uptake of Cd by spinach

2 LITERATURE REVIEW

2.1 Heavy metals

Heavy metal contamination raises more concern especially when present in agricultural soils, wherein uptake by plants results in negative impacts on food security (CEC, 2006). Factors such as concentration level and non-biodegradability of some heavy metals make them highly detrimental to the environment. As a result of soil contamination which may eventually lead to the degradation of underground water and the environment in general (Kubier et al., 2019), the food chain can become potentially affected. Despite the well-known information as regards the negative impact posed by heavy metals in contaminated areas, so far there has been relatively little documentation and data to show the real extent of the issue in the European Union (Tóth et al., 2016).

The toxicity of heavy metals is dependent on some factors such as plant species, soil composition, metal concentration, pH, chemical form, and specific metal (Jaishankar et al., 2014). Heavy metals such as Cd, mercury (Hg), and arsenic (As) have a strong negative impact on plant growth and important enzymes which are metal sensitive, thereby possibly resulting to plant death (Nagajyoti et al., 2010). Most heavy metals also get accumulated in the trophic food chain through gradual uptake by the primary producers i.e. plants and the final consumers i.e. humans.

2.2 Cadmium

Naturally, Cd occurs in low quantities in the soil but due to anthropogenic activities, its level tends to be on the increase over the years (Luo et al., 2009, Kubier et al., 2019). This increase in Cd could vary from location to location based on various factors such as variation in soil composition/formation, closeness to the pollution source, and soil management practices such as application of Cd contaminated fertilizers, manure, or sludge from sewage (Grant et al., 1998).

There is an increase in Cd concentrations within the soil when phosphate fertilizers are been applied. This is because Cd is naturally present in them (Yi et al., 2020). This leads to an overall increase in Cd levels above the background concentrations. This statement aligns with that of Toth et al., (2016), wherein high levels of Cd in Western Europe soils were attributed to the use of fertilizers produced from phosphate rock gotten from Morocco, while Eastern Europe soils have relatively low or no Cd due to the use of fertilizers produced from Kola phosphate rock from Russia. It is noteworthy that increased levels of Cd (> 1 mg/kg) detected in most industrialized areas are attributed to past centuries when smelters carried out their operations under less strict regulations and also when Cd was not recovered during the production of Zn (WHO, 2000).

Irrespective of the fact that Cd is a non-essential element required by plants , the bioaccumulation factor of Cd in plants is relatively higher in comparison to other trace elements, due to its low coefficient of adsorption and high soil-to-plant mobility (Shahid et al., 2016). Cd has been recognized for having the potential to alter the morpho-physiological and biochemical status of plants (Farooq et al., 2016, Ehsan et al., 2014), thereby affecting the overall plant condition and expected growth pattern.

Long-term exposure or occupational hazards relating to Cd contributes to the development of health issues such as acute pneumonitis and lung cancer (WHO, 2000). However, this perception has changed over the years as several studies have documented the health hazards posed by cadmium-contaminated food (Yang et al., 2016). Excessive consumption of food high in Cd can lead to pulmonary edema, chest pain, cough, and inflammation of certain vital organs in the body (Roy et al., 2013, Hayat et al., 2019). Vegetables that are cultivated on Cd contaminated soils were proven to have altered mineral contents of Zn, copper (Cu), manganese (Mn), and magnesium (Mg). However, the negative effects of altered minerals concentrations in consumable plants have been neglected over the years (Yang et al., 2016).

The Itai-Itai disease which means "ouch-ouch" disease occurred after World War II in Jinzu Valley, Toyama territory in Japan due to the continuous consumption of food and water contaminated with Cd (Hayat et al., 2019). This disease caused fractures and pains in the bones of the skeleton and also chronic health disorders resulting in the death of several victims (Nishijo et al., 2017). Other chronic health disorders caused by Cd include renal disorders, cardiovascular abnormality, and hormonal imbalances (Hayat et al., 2019).

2.3 Toxicant assessment and thresholds

Over the years, there have been reviews of allowable limits of Cd in ingestible products both in humans and animals. New threshold limits are aimed at reducing the health risks caused by the presence of heavy metals in the environment. In 1972, the World Health Organization (WHO) set the maximum allowable intake of Cd for adults at 60 – 70 µg/kg per day (Grant and Bailey, 1997). On the 30th of January, 2009, the European Food Safety Authority (EFSA) adopted a much lower threshold on Cd-contaminated food, wherein the allowable weekly intake of such products is 2.5 µg/kg body weight. This new amendment was relative to EFSA's statement on "tolerable weekly intake for cadmium", where it was stated that the new limit was adopted due to the recent risk assessment being carried out on Cd toxicity (European Commission, 2015). However, according to "Codex Alimentarius", the maximum Cd concentration in leafy vegetables such as spinach is 0.2 mg/kg (CODEX, 1995).

2.4 Bioavailability of metals in the soil

Concerning soil, bioavailability can be defined as the fraction of compounds that can freely move from the soil environment across the cellular membrane into an organism at a given time (Umeh et al., 2020, Semple et al., 2004). After the transfer of the contaminants into the organism, transformation, degradation, assimilation, or storage can occur within the organism (Naidu et al., 2008). Bioavailability is considered as being species-specific because different biological responses take place among varying receptors (Stokes et al., 2005). The total fraction of a contaminant that becomes bioavailable is dependent on various factors including the metal involved, contact time, and the environmental media.

Various chemical, biochemical and physical processes influence the availability of contaminants in the soil (Ehlers and Luthy, 2003, Umeh et al., 2020). By the process of adsorption, contaminants bind to the solid phase in the soil, while absorption or precipitation binds contaminants to the organic matter. The release of adsorbed contaminants into fluids can also be as a result of a change in water saturation, gas and water chemistry, and solid surface properties (Ehlers and Luthy, 2003), and this ultimately influences the level of bioavailable contaminants in plants.

2.5 Factors influencing uptake of cadmium by plants

The soil is classified as a dynamic system that is characterized by a high level of heterogeneity, where various processes determine the fate and transport of metals present within the soil matrix (Petruzzelli et al., 2020, Semple et al., 2004). Heavy metals contained in the soil can be present in five major pools (Petruzzelli et al., 2020), namely: (i) complex or simple soluble ions in the soil solution. (ii) exchangeable forms through the adsorption on mineral particles. (iii) adsorbed or complexed ions on organic matter. (iv) precipitation with carbonates, phosphates, and oxides or secondary minerals. (v) incorporation into the crystal lattice of clay minerals.

Different parameters such as pH, cation or anion exchange capacity, amorphous metal oxides, clay content, and soil organic matter influence the distribution of heavy metals across the five forms (Naidu et al., 2008, Petruzzelli et al., 2020). Retention and release of heavy metals in the soil influence the fraction which can eventually flow across the membrane of a plant. There is a need to understand how varying parameters influence the distribution of Cd in the soil.

2.5.1 Soil properties

2.5.1.1 pH

pH is one of the most important soil parameters that influence the sorption, desorption, mobility, and availability of heavy metals in the soil. At low pH, heavy metals exist in their ionic form and become more soluble, which results in higher bioavailability and uptake by plants. In acidic environments, Cd is largely present as Cd^{2+} ions, which is bioavailable for plants, while between pH 6 - 7, Cd exists in other forms such as $CdHCO_3^+$, $CdCl^-$ and hydrated $CdCO_3^0$ (Tudoreanu and Phillips, 2004, Olaniran et al., 2013). At high pH, Cd forms precipitate with carbonates and phosphates which are insoluble and become adsorbed to solid matrices within the soil (Cheng et al., 2020). A slight alteration in pH can result in drastic changes in solubility and bioavailability of Cd. As reported by Olaniran et al., (2013), with an increase in pH from 6 to 7 in 1.3 mM phosphate, the solubility of Cd was reduced by a magnitude of 8.8 fold. The soil pH is therefore a critical factor influencing the bioavailability and uptake of cadmium by plants.

2.5.1.2 Organic matter

The organic matter within the soil is composed of complex chemical forms which are important in determining the extent of bioavailability of a metal. Metals tend to adsorb to humic substances and when complexes such as fulvic acids (low molecular weight) are formed they become soluble, which leads to increased levels of the metal within the soil solution. When humic acids (high molecular weight) are formed, metals become retained in insoluble form within the soil (Petruzzelli et al., 2020).

Organic matter is one of the factors that influence metal mobility in soil and enrichment of the soil with organic matter helps to increase the sorption of Cd to the solid phase (Kubier et al., 2019, Bashir et al., 2009, Hettiarachchi et al., 2003). Mani et al., (2014), reported that in soils with varying concentrations of Cd (0, 5, and 10 mg/kg), Cd accumulated the least in spinach cultivated on the soils that were amended with compost (20 t/ha). Also, a combined application of organic manure and lime helped to decrease Cd concentrations by 61.3% in cultivated spinach shoots in comparison with those grown on only soil (Kumarpandit et al., 2017).

2.5.1.3 Redox potential

The activity of free electrons plays a role in influencing the speciation of metals within the soil. Via reduction and oxidation reactions, redox potential (Eh) is formed. However, the formation of redox potential is relatively slow, most especially when it occurs within the soil environment (Olaniran et al., 2013). High redox potentials occur in well-drained soils while low potentials are present in highly saturated soils (Petruzzelli et al., 2020). Cd exists in its cationic form (i.e. Cd^{2+}) in high redox environments but the formation of precipitates such as CdS and $CdCO_3$ occur under low redox conditions (Sebastian and Prasad, 2014, Kubier et al., 2019). The form in which Cd is present in the soil, therefore, has an effect on the uptake by plants.

2.5.1.4 Cation exchange capacity

Charge density on surfaces of soil particles varies from soil to soil and it enables the exchange of metal cations, thereby resulting in sorption or desorption of metals within the soil (Kubier et al., 2019). Cation exchange capacity (CEC) is influenced by the type and quantity of clay and by the organic substances in the soil (Petruzzelli et al., 2020). At low pH, there is a high level of protonation (i.e. formation of H^+) and, this results in competition with the metals in the soil, thereby making H^+ attach to exchangeable sites on soil surfaces and yielding a high level of metal cations in solution (Petruzzelli et al., 2015).

With higher CEC, there is higher sorption of divalent metals such as Cd in the soil (Selles et al., 1996). Soil texture, which is determined by the percentage of sand, clay, or loam fractions also influences the cation exchange capacity. Bashir et al. (2009), reported that with an initial Cd concentration of 50 mg/kg in the soil, spinach leaves cultivated on silt clay, silt loam, and sandy loam soils accumulated 28.4 mg Cd/kg, 32.6 mg Cd/kg, and 35.6 mg Cd/kg respectively.

2.5.2 Climatic conditions

Climatic conditions, such as rainfall patterns, temperature, and sunshine also influence the uptake of metals by plants. Previous studies reveal that with a temperature rise there is a corresponding increase in Cd uptake by plants (Fritioff et al., 2005, Pourghasemian et al., 2013). Cadmium concentration in baby leaf spinach (*Spinacia oleracea*) increased with a higher transpiration and atmospheric concentration of CO_2 (Chehine et al., 2014). Cultivation of spinach in water deficit conditions resulted in higher accumulations of Cd from the soil (Tack, 2017). However, it would be difficult to make a general conclusion as regards the impact of the watering regime on the uptake of Cd by spinach as there are currently limited studies on this factor.

2.5.2.1 Watering regime

Water plays a key role in the growth of plants, where several processes such as transport of nutrients and minerals, maintenance of plant structure, seed germination, biomass yield are influenced (Gonulal et al., 2021). An inadequate amount of water being applied below a plant’s demand, could lead to excessive stress and eventual death of the plant. Variations in water content in the soil could alter properties such as pH and redox potential, which could eventually influence the transfer of metals to plants (Tang et al., 2020).

Interestingly, the impact of varying water during cultivation is not only visible in the shoots of the plants but also below the ground. Watering pattern in the soil, either through natural or human intervention, influences the microbial community within the soil and ultimately the uptake of metals by plants (Hu et al., 2013). Also, increased exposure to Cd in the rhizosphere could inversely result in water deficiency in the roots (Moya et al., 1993), thereby causing other effects e.g. decrease in root area (Vaculík et al., 2012). This could therefore have an impact on the concentration of metals that could be transported to the above-ground biomass.

Only a few studies have been reported as regards the influence of the watering regime on the uptake of Cd by spinach. Tack, (2017) stated that with high volume of water being applied daily “daily high” and with increasing volume of applied water, the uptake of Cd by spinach was relatively reduced in comparison to other treatments with lesser water application. Rao and Marthur, (1994) showed that water uptake from soil depends on the plant’s demand and soil supply, which relatively influences the concentration of metal that could end up in the tissues of the plant. Determining the possible impact of different watering regimes on Cd uptake from soil and its accumulation in vegetables such as spinach is therefore important.

2.5.3 Plant physiology

Factors associated with plant physiology also play a role in the uptake of metals from the soil (Clemens et al., 2002). Variation in Cd uptake among leafy vegetables has been reported in various studies (Wang et al., 2009, Xue et al., 2014, Yin et al., 2016). Uptake and accumulation of metals vary not only from plant to plant but also among cultivars of the same species (Grant et al., 2008, Shen et al., 2021). In an experiment involving five cultivars of spinach in identical conditions, the least Cd concentrations were found in cultivar Bloomsdale in contrast with other cultivars, namely, Giant thick-leaved, Grodane, Mediana, and Spartacus which had higher Cd concentrations (Alexander et al., 2006). Also, Cd concentrations found in spinach shoots were within the range of 8.5 - 10.1 mg/kg and 6.1 - 6.6 mg/kg for high and low Cd cultivars respectively, under low Cd treatment (8.9 µmol Cd L^{-1}) (Yin et al., 2016). An alternative strategy for preventing Cd from entering the human food chain can therefore be the selection of cultivars with low-Cd uptake in their edible parts (Huang et al., 2015, Xin et al., 2013).

2.6 Estimating concentrations of metals in soil

Since varying factors including soil properties, soil chemistry, and soil-plant relationships can affect the bioavailability of metals in the soil, various soil-testing methods have been developed to determine different fractions of metals in the soil. Estimating concentrations of metals is often carried out via extraction protocols (single or sequential extraction processes) which enables a better understanding of the partitioning of metals within the soil (Rauret et al., 1999, Meers et al., 2007).

The chemical form of metal within the soil also influences its mobility, which then affects the potential uptake by plants. More soluble forms of metal can be easily extracted in comparison to adsorbed forms on organic matter or mineral particles. With the use of different extraction techniques, various forms of metal can thus be easily extracted. Time of equilibration, liquid to solid ratio, and molar concentration as specified by the extraction protocol are key parameters influencing the effective extraction of metals from the soil (Meers et al., 2007).

2.6.1 Soil pore-water sampling

Within the soil, metals can be present in water-soluble forms which tend to easily migrate from the soil matrix into the soil water solution. In order to assess the readily bioavailable fraction of metals, soil pore water can be collected and analyzed (Meers et al., 2007). There are numerous soil pore-water sampling methods, but the negative pressure Rhizon sampler (Figure 1) is highly used.

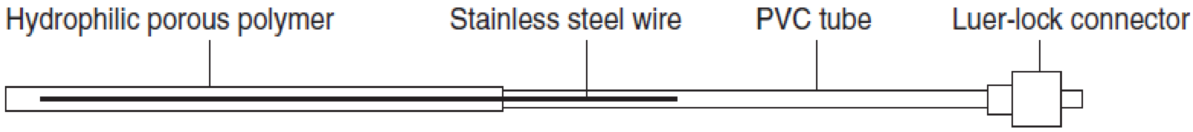


Figure 1. Schematic of a rhizon sampler (from 'Rhizon soil moisture sampler: operating instructions'; www.eijkelkamp.com)

2.6.2 Diffusive Gradient in Thin Films (DGT)

The DGT is a simple device that can be used for measuring the concentration of trace metals and for determining their bioavailable fractions in soils (Zhang, 2003). It is made of a plastic material with a base that is 2.5 cm in diameter. It consists of a window, cap, and piston (Figure 2a). The casing has a resin layer (0.4 mm), diffusive gel (0.8 mm), and a membrane filter, also referred to as a diffusive boundary layer (DBL) (0.14 mm). The resin layer is made of a chelex material which is infused with a hydrogel to accumulate the soluble metals from the soil. As depicted (Figure 2b), the resin layer is overlain by the diffusive gel and membrane filter. Metal ions present in the saturated soil have to diffuse through the membrane filter and diffusive gel to get to the resin layer. Acting as an infinite sink, the resin gel allows for the resupply of ions from the soil (Egene et al., 2018).

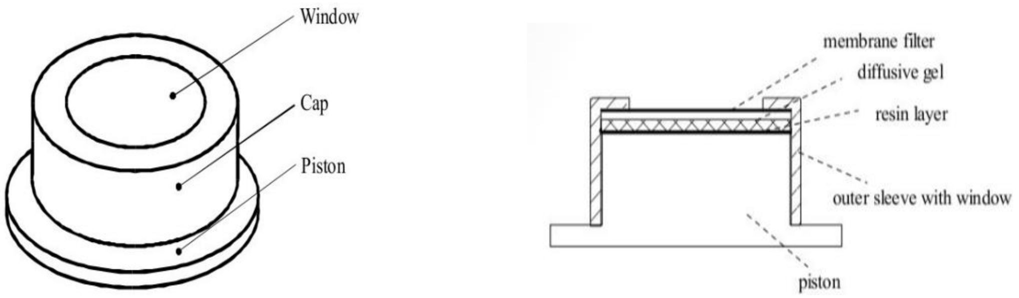


Figure 2. a) A typical DGT device b) Cross-sectional view of a DGT

Dissociation and migration of metals from the soil eventually result in the formation of a diffusive gradient within the diffusive layer of the device (Figure 3) (Sun et al., 2013).

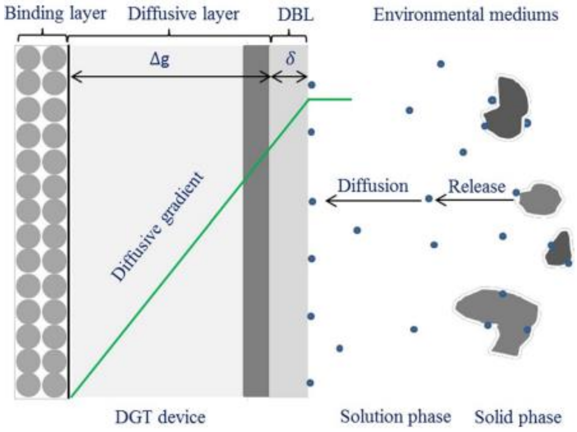


Figure 3. Diffusion of metal ions from the solid phase into the DGT device (Li et al., 2019a).

A ratio (R) can therefore be established between the concentration of metals on the resin layer (C_{DGT}) and the concentration of metals in the soil pore water (C_{sol}), this also depicts the soil kinetics in resupplying solutes to the DGT device (Egene et al., 2018).

$$R = \frac{C_{DGT}}{C_{sol}}, 0 < R < 1 \tag{1}$$

An R-value closer to 1 indicates a fast release of metals from the solid phase to the soil solution, while an R-value close to 0.1 depicts a slow release of metals to the soil solution (Egene et al., 2018).

2.6.3 Single chemical extractions

2.6.3.1 Inorganic salt-based extractions

Soil particles are generally negatively charged, which results in the adsorption of cations within the soil. An inorganic salt-based solution such as 0.01 *M* $CaCl_2$ can be used to determine the bioavailability of metals in soil samples. Some advantages of utilizing this extraction compound include: it has the same ionic strength as natural soil, no need for pH buffering, and Ca^{2+} ensures good coagulation of colloids in suspension (Houba et al., 2000). Using 0.01 *M* $CaCl_2$ enables metals that are weakly adsorbed to the soil to be extracted into the soil-water solution and metal content determination can therefore proceed at the laboratory.

2.6.3.2 Complexant-extractable metals

A mixture of ethylenediaminetetraacetic acid (EDTA) and Ammonium acetate (NH_4OAc) can be used as a complex extractant to determine the bioavailability of trace metals (Lakanen and Erviö, 1971, Meers et al., 2007). A major role of complex extraction is to imitate the root zone of plants in the soil. The function of EDTA and acetic acid is analogous to the complexation of metals by root exudates in the soil, while NH_4^+ has the potential to replace the exchangeable metal fractions. The acidity level (pH 4.65) during this extraction process is quite similar to the pH in the rhizosphere (Meers et al., 2007). Acid extractable and reducible fractions of metals in soil can be extracted with NH_4OAc-EDTA while oxidizable and residual fractions are not affected by this extraction process (Tack and Verloo, 1996).

2.6.4 Sequential Extraction

2.6.4.1 Modified Bureau Communautaire de Reference (BCR)

Initially, BCR proposed a standard procedure for the sequential extraction of soils and sediments (Quevauviller, 1998). However, the procedure was later modified because the solution of the reducible fraction was not sufficiently buffered, leading to low reproducibility (Rauret et al., 1999). No single extraction procedure can provide adequate information on the total metal concentration in the soil due to the distribution of metals in various forms and pools within the soil. The modified BCR (a three-stage sequential extraction) as proposed by the Bureau Communautaire de Reference, has been used in various experiments to further analyze contaminated soils, and data obtained from these analyses are been used to predict the mobility of metals within the soil (Caraballo et al., 2018, Sungur et al., 2014, Deng et al., 2019).

Through the adoption of this sequential extraction technique, extensive information can be derived on the mode of occurrence, source of contamination (Pueyo et al., 2008), physicochemical and biological availability, and transfer of trace metals in the soil (Sungur et al., 2014). Rather than determining the pseudo-total concentrations of heavy metals, understanding their speciation pattern and distribution in the soil is of greater importance (Sungur et al., 2014, Fernández-Ondoño et al., 2017). With the aid of the modified BCR, three steps of extraction are employed, namely: (i) extraction of exchangeable and weak acid-soluble fractions bound to carbonates, (ii) extraction of reducible fractions (bound to Mn and Fe oxides), and (iii) extraction of oxidizable fractions (bound to sulfides and organic matter) (Sungur et al., 2014, Pueyo et al., 2008).

2.6.5 Total metal content determination

Quantification of heavy metal content in the soil is carried out via various analytical methods which include digestion of the soil samples. A key protocol used in obtaining pseudo-total content of heavy metals in the soil is aqua regia digestion (Chopin and Alloway, 2007, Fernández-Ondoño et al., 2017). In an environmental matrix, aqua regia extraction is sometimes less preferred because it underestimates metal concentration such as Cd, Ni, Co, and Cr by up to 50% (Ščančar et al., 2000, Taraškevičius et al., 2013). However, in the topsoil (0 – 30 cm), where residual silicates are not in high concentrations, results derived from aqua regia digestion represent the total metal content (Martín et al., 2006). Aqua regia digestion is dependent on some factors such as the composition and distribution of the matrix (mainly organic matter) and the element to be analyzed (Liu et al., 2020, Sastre et al., 2002).

2.7 Spinach

Spinach (*Spinacia oleracea*) is one of the important vegetables in the Amaranthaceae family. Spinach can grow in temperatures between 5 – 30°C and also in freezing conditions (-9 to -6°C) (Koike et al., 2011). However, temperatures between 15 - 18°C result in the high growth of spinach (Parwada et al., 2020, Koike et al., 2011). Spinach tolerates frost conditions and can be planted throughout the entire year (Ogbaji et al., 2018).

Spinach can be grown on a wide variety of soil types, however, it gives a higher yield when grown on sandy loam soils compared to other soil types (Anwar et al., 2017). The vegetable has an initial growth rate that is quite slow but a high growth during the last 3 weeks before harvest (Bergquist, 2006). Soil pH between 6 – 7.5 is most preferred to cultivate the crop. Furthermore, spinach is classified as a moderately salt-sensitive crop (Shannon and Grieve, 1998). A hot climate, most especially when combined with a lack of water in the soil, could cause undesirable stress which may eventually lead to the death of spinach.

Stages of Spinach growth (Figure 4) can be generally classified into three, namely: (i) seedling growth stage (A – B) (ii) vegetative stage (C – E) (iii) flowering and seed formation stage (beyond E). Approximately 4 – 11 days are required for the development of its first true leaves (Bokov et al., 2020). However, germination, seedling growth, elongation of the root, photosynthesis, chlorophyll content, and metabolites in spinach are generally reduced when grown in stressed conditions such as saline soils (Kaya et al., 2002, Kim et al., 2021). With a daylight period of 10 – 12 hours, *S. oleracea* intensively grows and this favours the formation of big rosette leaves (Bokov et al., 2020). At the point of harvest i.e. 35 days of cultivation, spinach leaves can reach up to 7.5 – 10 cm in height (Parwada et al., 2020).

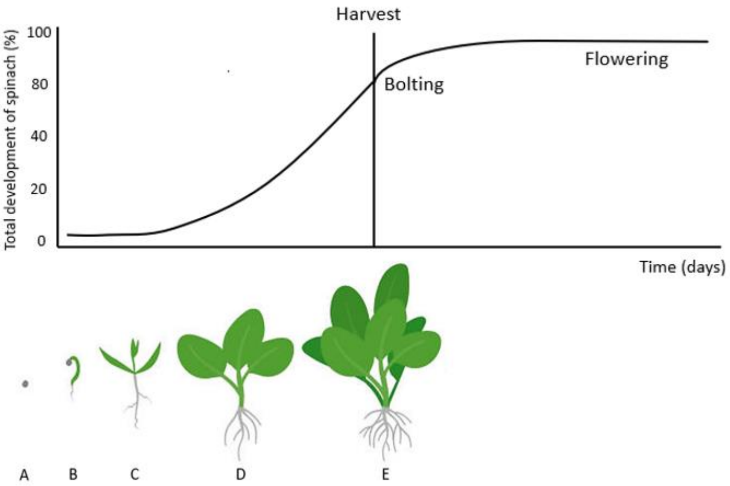


Figure 4. Cultivation stages of spinach (*Spinacia oleracea*). Image adapted from Noordijk et al., (2016). “A” represents spinach seed. “B” represents a developing seedling. “C” represents the development of 2 leaf rosette. D represents the formation of 3 leaves rosette. E represents the formation of 7 leaves rosette.

With a cultivation area of 4,540 Ha and a yield of 208,370 Hg/Ha, Belgium amasses 14% (94,600 tons) of the total production of spinach in Europe. Belgium currently ranks as the third highest producer of spinach after Italy (99,870 tons) and France (97,110 tons) (FAO, 2020). Various countries have benefited from the nutritional and economic power of cultivating, processing, packaging, storing, transporting and sales of spinach. Appendix 6, provides nutritional information on spinach. Spinach is comprised of some human-needed elements such as Fe, Ca, P, Mg, and Mn (USDA, 2020). Some health benefits of spinach include, regulation of blood pressure, maintenance of a healthy brain, boosting the immune system and prevention against the risk of cancers. It also contains chlorophyll which aids in the digestion of food (Verma, 2018).

2.8 Soil amendments for stabilizing metals in soil

Over the years, various studies have been carried out on how best to manage metal contamination in soils (Ashrafi et al., 2015, Xu et al., 2021). Soil stabilization is classified as a potential option in managing heavy metal contaminated soils. Figure 5, presents a classification of soil amendments in three major classes, namely: organics (animal manure, biosolids, biochar, etc), inorganics (lime, phosphate, and gypsum), and minerals (sepiolite, zeolite, and bentonite). More details are provided on the various classes of soil amendments.

2.8.1 Inorganic soil amendments

In recent years, clay has been introduced into the soil for the stabilization of heavy metals (Hamid et al., 2019). The negatively charged ions of clay materials allow for the adsorption of metals through direct bonding, ion exchange, or complexation (Ahumada et al., 2014). 2:1 clay mineral such as sepiolite has been reported to show strong sorption with Cd in contaminated soils (Padilla-Ortega et al., 2013, Hamid et al., 2021). Crystalline aluminum-silicate minerals such as zeolites are also known for their ability to immobilize trace metals and micro-elements due to their relatively high adsorptive capacity (Hamid et al., 2019). The application of inorganic amendments such as montmorillonite (Qin et al., 2020), lime (Shaheen and Rinklebe, 2015) have also been reported to reduce the bioavailability of metals in the soil. The addition of lime increases soil pH, thereby decreasing the solubility of Cd and consequently its uptake by plants (Hamid et al., 2019, Liu et al., 2014). Nonetheless, liming of contaminated sites requires a repetitive application of lime within 2-3 years (Sharma and Nagpal, 2018).

The addition of phosphate compounds has also been reported in various experiments for stabilizing heavy metals in the soil through the formation of metal-phosphate precipitates (Yang et al., 2018, Kastury et al., 2019). Through the incorporation of water-soluble and insoluble phosphate compounds, the mobility of metals in the soil is reduced by adsorption, precipitation, and complexation. The presence of apatite and rock phosphate in the soil also helps to decrease the uptake and accumulation of metals by plants (Chen et al., 2007). However, the application of phosphorus fertilizers can introduce some trace metals such as Cd into the soil (Bi et al., 2006, Li et al., 2020). Moreover, Hamid et al., (2019), states that the ionic strength of soil is invariably affected through the application of phosphate fertilizers, which reduces the sorption of Cd onto the soil thereby resulting in an increase of Cd in the soil solution.

2.8.2 Organic soil amendments

Materials rich in organic matter content such as crop residues and animal waste e.g. farmyard manure (Hamid et al., 2019) and poultry manure (Azeez et al., 2019, Ihnat and Fernandes, 1996) are used as sources of nutrients and for the immobilization of heavy metals in the soil. Stabilization of heavy metals by the action of organic wastes depends on some factors such as soil pH, nature of metal, soil type, and organic material properties (Hamid et al., 2019). With increased levels of organic matter in the applied waste, there is generally a corresponding decrease in the mobility and bioavailability of metals through the formation of organometallic complexes (Du et al., 2021, Mani et al., 2014).

The addition of organic amendments does not reduce metal concentration in the soil but only reduces their bioavailable fraction within the soil pore water. Indeed, materials such as biosolids, compost, and manures, contain high levels of humic substances which helps to reduce the bioavailability of metals through the process of adsorption or complexation to form stable complexes (Clemente et al., 2015). As a result of the large molecular size of humic acid, they become retained within the soil thereby reducing the potential uptake of metals by plants (Petruzzelli et al., 2020).

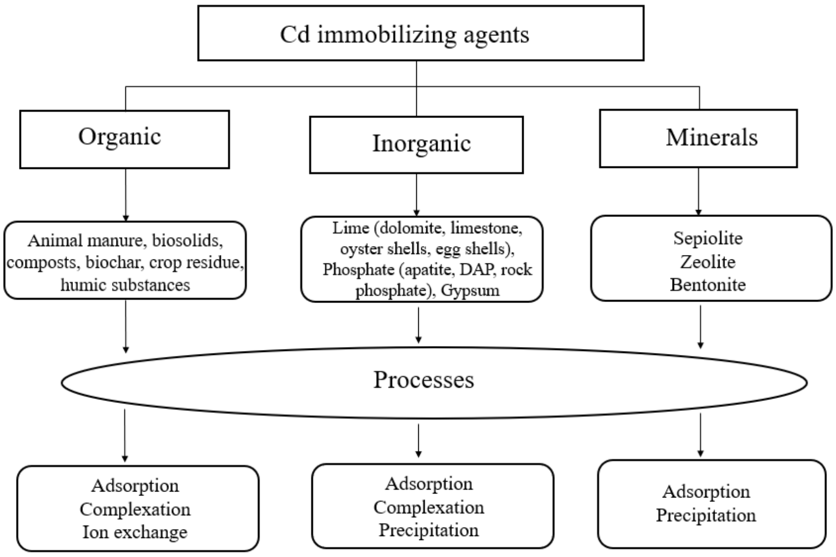


Figure 5. Classification of soil amendments and their various processes used in immobilizing Cd (Hamid et al., 2019)

2.9 Biochar as a soil amendment for stabilizing heavy metals in soil

Thermo-chemical conversion of biomass in an oxygen-limited environment, otherwise referred to as pyrolysis, results in the production of a carbon-rich material commonly referred to as biochar (Narzari et al., 2015, Ge et al., 2020). Pyrolysis can be further divided into different types, namely (i) fast pyrolysis, which operates at a temperature of about 500°C and produces biochar (12%), bio-oil (75%), and syngas (13%). (ii) slow pyrolysis (1 – 10°C min^{-1}), which produces biochar (35%), bio-oil (30%), and syngas (35%). Other processes that produce biochar includes, gasification and carbonization. (Narzari et al., 2015). Selection of biomass such as wood, rice straw, bamboo, spruce-tree, etc, for the production of biochar, depends on some factors which include, lignocellulosic content, pH value, energy content, particle size, moisture content, and volatility (Ge et al., 2020).

Biochar effectively contributes to numerous environmental and agricultural benefits (Tack and Egene, 2019, Beesley et al., 2010). Active functional groups, high pH, large surface area, and high CEC are key properties that influence metal retention unto biochar (Zhang et al., 2017). Due to the physical and chemical properties of biochar, metals can be potentially immobilized in the soil either by an increased level of sorption or by precipitation (Tack and Egene, 2019). The application of biochar can therefore influence the soil CEC, pH (Da Silva Mendes et al., 2021), organic carbon (Liu et al., 2018), and electrical conductivity and ultimately decrease bioavailable concentrations of metals in the soil (Guo et al., 2017, Nobaharan et al., 2022).

Biochar releases some important nutrients for plants, such as calcium (Ca), Mg, potassium (K), sodium (Na), and phosphorus (P) into the soil (Nobaharan et al., 2022). These minerals can eventually form mineral phases on the surface of biochar and increase the adsorption of metals from the soil solution (Rees et al., 2014). Biochar can immobilize metals through the processes of complexation, reduction, cation exchange, or precipitation (Figure 6). As a result of varying parameters in the production of biochar, there happen to be some differences in the interaction mechanisms between biochar and metals in the soil (Cheng et al., 2020). Adsorption of metals onto the surface of biochar is influenced by various factors such as the source of biomass, pyrolysis temperature, polarity, surface area, and target contaminant (Ahmad, 2014).

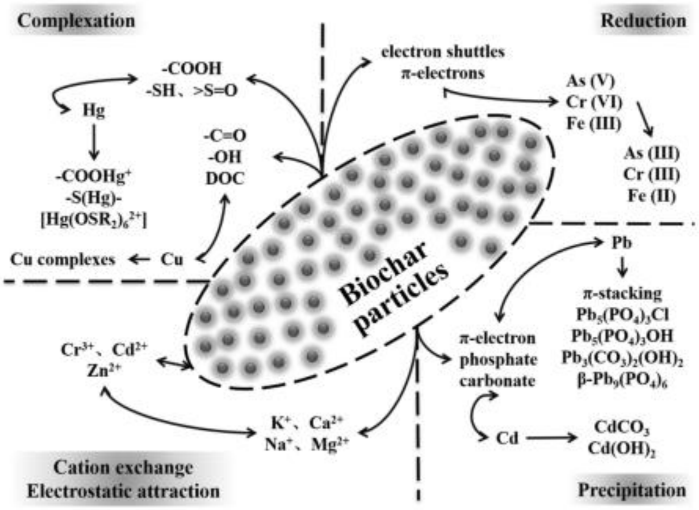


Figure 6. Possible interactions between metals and biochar in amended soils (Cheng et al., 2020).

Soluble fractions of Cd have been reported to be greatly reduced in soils amended with biochar (Egene et al., 2018). Soluble cadmium (Cd^{2+}) combines with alkaline ions present within the biochar such as $H_2PO_4^-$ and HPO_4^{2-} to form insoluble precipitates such as cadmium hydrogen phosphate i.e. $Cd(H_2PO_4)^+$. The application rate of biochar influences the uptake and accumulation of metals by plants. This was demonstrated by Egene et al., (2018), wherein soil with 2% and 4% biochar resulted in a decrease of 40% and 66% of Cd respectively in pore water concentration.

Different authors have shown biochar to be an effective solution in reducing the uptake of Cd in spinach. In a biochar-amended sewage-irrigated contaminated soil, Cd concentrations in cultivated spinach were found to be 5.42 mg/kg in the control (i.e. soil with no biochar) and 3.45 mg/kg in treatments with 5% biochar (Younis et al., 2015). Nobaharan et al., (2022), reported that with the application of biochar derived from rice straw, Cd concentrations in *S. oleracea* effectively decreased by 30.6% and 38.9% in comparison with the spinach plants grown on the control soil. In addition, artificially contaminated soil (50 mg Cd kg^{-1}) with 5% rice stalk biochar was reported to yield 0.8 mg Cd/kg soil in cultivated spinach in contrast to 1.5 mg Cd/kg in spinach grown on the control soil (Qayyum et al., 2019). In the current study, the effect of biochar application (2% and 4%, w/w) in decreasing Cd uptake by spinach was assessed.

3 MATERIALS AND METHODS

3.1 Soil sampling and biochar used

Soil samples were collected from an agricultural land in Tournai, Belgium. Various crops such as vegetable and tuber crops are typically grown on the field. With the aid of spades, 2 holes were dug (15 - 20 m apart) to about 30 cm below the soil surface to retrieve a representative sample of the top 30 cm layer. At the greenhouse, the soil samples were spread on a geotextile material and partially covered to achieve air drying. Large clods were crushed by hands. Air drying of soil lasted for 5 days, then homogenization of the soil was carried out. The soil was mixed to ensure homogeneity among soil samples collected from both holes on the farmland.

Biochar was purchased from GreenPoch company (Fleurus, HAINAUT, Belgium). The biochar was produced from the wood of spruce trees, via the process of pyrolysis (600 - 700°C). The biochar was oven-dried at a temperature of 60°C for 48 hours. The biochar was crushed by hand and sieved through a 4.8 mm mesh sieve. The biochar was mixed with the soil based on the percentage requirement of the treatments i.e. 2% (36 g) and 4% (72 g) w/w on a dry weight basis. Pots were filled with 1.8 kg of substrate i.e., soil with or without biochar (dry weight basis). The substrate was filled into 57 pots and divided into 3 treatments, having 4 replicates per sub-treatment. An equal volume of water i.e., 70% field capacity of raw soil (soil with no biochar) was applied during the equilibration period, which lasted for two weeks. After the equilibration period, nine pots (i.e., 3 replicates from each treatment) were removed from the greenhouse in order to carry out further analysis in the laboratory.

3.2 Nursery

About 20 g of soil was placed within the compartments of a nursery rack and 10 seeds were placed into each compartment. Transparent polythene was then used to cover the nursery rack to maintain humidity and also aid seed germination. 50 ml of water was applied during the germination period i.e., 2 weeks.

3.3 Water holding capacity

To determine the water holding capacity, 50 g of substrate from the three treatments were weighed onto filter papers which were placed into measuring cylinders. Then 50 ml of water was added to the substrate. The volume of water retained by the substrate was deduced by measuring the volume of leached water after 1 hour.

3.4 Moisture content determination

For the determination of average moisture content of substrate, approximately 10 g (dried sample) was weighed in triplicates and placed in an oven (VWR, Dry-line 115) at 105°C for 24 hours. After 24 hours, the oven-dried samples were retrieved and weighed. The moisture content (%) of the sample was then determined by dividing the mass of water lost, by the mass of the oven-dried soil. This was performed before and after equilibration including every extraction that was performed later on.

In addition, about 2 kg of air-dried soil was saturated with water and placed into pots to determine the moisture content at field capacity after 48 hours. A geotextile was placed above the pots to prevent excessive evaporation. The moisture content at field capacity was then determined by placing the pots in an oven (VWR, Dry-line 115) at 60°C for 48 hours.

3.5 Determination of cadmium content in fertilizer using aqua regia digestion

Two different types of fertilizers (Universele Mersele Engrais, COMP-O) were digested to determine their level of Cd concentration. Both had similar contents: Nitrogen (12%), Phosphorus (12%), and Potassium (17%). However, before applying the fertilizer directly to the treatments, the Cd level contained in the fertilizers was determined. This was done by initially crushing about 5 g of both fertilizers with the aid of a mortar and pestle, then approximately 0.5 g of crushed fertilizers was placed into 50 ml Erlenmeyer flasks, this was performed in triplicates for both fertilizers. About 2 – 3 ml of Milli Q water was then added to each Erlenmeyer. 7.5 ml HCl (picopure, 34 – 37% concentrated) and 2.5 ml of nitric acid (picopure, 67% concentrated) were then added to each Erlenmeyer and gently shaken together and left for about 1 hour. The flasks were then covered with watch glasses and placed on a heating plate (Ceran Hotplate 11A, Hannover, Germany) at 100°C and 50 W for 2 hours. The temperature was then increased to 150°C for another 1 hour. After digestion, the sample was filtered into a conical flask using a filter paper (Macherey-Nagel, Germany) with a diameter of 125 mm and pore size of 0.2 µm, then diluted to 100 ml using 1% nitric acid before analysis.

3.6 Analysis of cadmium in solution

Depending on the expected concentration of ions e.g. Cd in solution, the inductively coupled plasma-mass spectrometry (ICP-MS) (PerkinElmer, NexION 350D, Waltham, MA) or inductively coupled plasma-optical emission spectrometry (ICP-OES) (Thermo scientific, ICAP 7000 Series, Uk) was used for analysis. Prior to the measurement of samples on the ICP-OES or ICP-MS, the limit of detection (LOD) or the limit of quantification (LOQ) of samples are estimated. The LOD can be defined as the lowest possible concentration of an analyte within a matrix that can be detected (but not quantified) with a specific level of confidence. The LOQ, also referred to as the quantification limit, is the least possible concentration of an analyte that can be quantified within a matrix with acceptable accuracy and precision. LOD and LOQ depends on (i) the standard deviation (σ) of the measured element in blank samples and (ii) the slope (S) of the calibration curve obtained within the range of the LOD or LOQ (Shrivastava and Gupta, 2011). The ICP-OES and ICP-MS were employed when the limit of detection (LOD) was high (above 1 µg/L) or low (below 1 µg/L) respectively. In addition, for quality control (QC) of the analysis, an external sample with already known concentration of the analyte in a matrix was always measured. This implies that, the measurement of the QC was used to determine probable errors in the analysis of new samples.

3.7 Soil analysis

3.7.1 0.01 *M* calcium chloride ($CaCl_2$) extraction

Ten grams of the substrate was weighed into a 250 ml Erlenmeyer flask and 50 ml of the extraction solution (0.01 *M* $CaCl_2$) was added to it. The flasks were then placed on an agitating table (Gesellschaft fur Labortechnik mbH D-30938 Burgwedel, Germany) for 2 hours and later filtered with filter paper into polythene flasks. One drop of concentrated HNO_3 (pico-pure) was added to the filtrate and stored in the refrigerator (4°C) for further analysis.

3.7.2 Ammonium acetate-EDTA pH 4.65 extraction

Ten grams of substrate was weighed into a 250 ml Erlenmeyer while 50 ml of the extraction solution (0.5 M NH_4OAc + 0.5 M HOAc + 0.02 M EDTA, pH 4.65) was added to it. The Erlenmeyer's were then placed on the agitating table for 30 minutes and was filtered with filter paper into polythene flasks. One drop of concentrated nitric acid (pico-pure) was added to the filtrate and stored in the refrigerator (4°C) prior to analysis.

3.7.3 Saturated paste extraction and electrical conductivity

Saturated paste extraction was performed to determine the electrical conductivity. 400 g of soil samples were weighed into plastic buckets and Milli Q water was gently added to it and stirred to form a paste. This was then left to stand for 12 hours until the next morning. However, based on the extraction protocol provided by Van Ranst et al. (1999), the buckets were not ready for the next phase as the soil became oversaturated. Therefore, a small amount of soil was then added to respective buckets until the oversaturated water became soaked with the new soil, this was then left again for another 4 hours.

When the soil attained a proper paste form i.e., the surface of the soil looked shiny, while scooping a portion of the mixture with a spatula resulted in the paste running off the spatula, then the extraction was performed using a Büchner funnel. The filtrate was then retrieved and some portion of it was used for measuring the electrical conductivity using an electrical conductivity analyser (Consort, C6010, Belgium) while the remaining volume was used for measuring the Cd concentration on the ICP-MS.

3.7.4 Aqua regia digestion

To quantify the total Cd content in the soil samples i.e., soil with and without biochar, after equilibration, aqua regia digestion was performed (Ranst et al., 1999). With a ratio of 3:1, 7.5 ml of hydrochloric acid (pico-pure, 34 – 37% concentrated) and 2.5 ml of nitric acid (pico-pure, 67% concentrated) were added to 1 g of dried soil in 100 ml Erlenmeyer flasks. The flasks were covered with watch glasses and heated on a heating plate at 50 W and 150°C for 2 hours. After digestion was carried out, filtration of the solutions was performed using a filter paper in a 100 ml volumetric flask, while the Erlenmeyer's and watch glasses were rinsed with Milli Q water and the rinse water was added to the volumetric flasks. Milli Q water was added directly to the filter paper to wash off remnants of the solution and to also make up 100 ml in the volumetric flask. The filtrate was then stored in the refrigerator (4°C) prior to analysis.

3.7.5 BCR sequential extraction

In order to distinguish different fractions of Cd in the substrate, the modified BCR sequential extraction designed by Rauret et al. (1999) was carried out. The BCR extraction enabled the determination of Cd concentration distributed across different fractions of the soil i.e., the reducible fraction, the oxidisable fraction, and the residual fraction.

3.7.6 pH of substrate

Fifty ml of Milli Q water was added to 10 g of the substrate in 100 ml Erlenmeyer and placed on a shaker overnight for equilibration. The next day, the Erlenmeyer's were retrieved, shaken, and left to settle before inserting a previously calibrated pH meter (Metrohm, Switzerland). Also, pH-KCl was determined by mixing 10 g of soil sample with 25 ml of 1 *M* potassium chloride solution. The mixture was placed on the shaker for 30 minutes for equilibration, then the pH was measured (Ranst et al., 1999).

3.7.7 pH-stat experiment

This experiment was performed to understand how Cd is being released from the soil relative to variation in pH. With the aid of an automatic device (Metrohm 718 Stat Titrino, Switzerland) four pH levels (i.e., 4, 5.5, 7, and 8) were maintained for 12 hours using 0.1 M HNO_3 or 0.1 *M* NaOH. After maintaining the desired pH for 12 hours, the suspension was extracted using a syringe and centrifuged before decanting the supernatant. Due to the excessive time required to successfully perform a pH-stat experiment on all treatments, this experiment was carried out only on the blank soil i.e., soil without biochar. The supernatant was then placed into the refrigerator at 4°C before analysis.

3.7.8 Buffering capacity

10 g of soil samples were mixed with 50 ml of Milli Q water in 250 ml Erlenmeyer flasks and placed on the shaker overnight for equilibration. The next day, the samples were retrieved and pH values of 4, 5.5, 7, and 8 were attained in each flask via titration using a burette filled with either 0.1 *M* HNO_3 or 0.1 *M* NaOH. The final volume of acid or base added was determined when the pH of the mixture did not change by 1 unit for 10 minutes. The buffering capacity was then calculated using equation (2) (Hajnos, 2011):

$$\text{Buffer capacity} = \frac{dn}{d\,(pH)} \tag{2}$$

Where, *dn* is amount of acid or base added (moles) to change 1L of buffer solution and *d* (*pH*) is the resulting change in pH.

3.7.9 Diffusive gradient in thin films (DGT)

To obtain more information on the kinetics of metal release in the soil, DGT soil samplers (DGT research Ltd, Lancaster, UK) were deployed for 24 hours following the protocol provided by Zhang, (2003). Soil samples (with and without biochar) were saturated with Milli Q water at 100% field capacity for 24 hours. With the aid of a spatula, a small quantity of the saturated soil was smeared on the surface of the DGT device then the DGT was placed directly on the soil surface and gently pressed into the soil. After 24 hours, the DGT device was retrieved and the attached soil was washed off using Milli Q water. The resin layer was retrieved and placed into 1 ml of 1*M* HNO_3 for 24 hours. After 24 hours, 0.4 ml was retrieved and diluted 15 times then kept in the refrigerator at 4°C before analysis on the ICP-MS. Determination of the fluxes and concentrations at the interface of the DGT and the soil was calculated using the following equations (3 – 5) (Zhang, 2003).

$$\text{a) } M = \frac{Ce\ (V_{HNO3} + V_{gel})}{f_e} \tag{3}$$

Where *M* is the mass of metal amassed on the resin gel (µg) , *Ce* is the concentration of metals in 1*M* HNO_3 (µg/l), V_{HNO3} is the volume of HNO_3 added to the extracted resin gel (ml), V_{gel} is the volume of the resin gel, (typically 0.15 ml), and f_e is elution factor for each metal, typically 0.8.

$$\text{b) } F = \frac{M}{tA} \tag{4}$$

Where *F* is the flux of metal measured by the DGT (µg s^{-1} cm^{-2}), t is the deployment time in seconds, and A is the exposure area, typically 3.14 cm^2

$$\text{c) } C_{DGT} = \frac{F\Delta g}{D} \tag{5}$$

Where C_{DGT} is the concentration of metals on the resin gel layer (binding layer) (µg/L), Δg is the thickness of the filter membrane (0.14 mm) plus the thickness of the diffusive gel (0.8 mm), and D is the diffusion of coefficient of the metal in the gel (cm^2 s^{-1}).

3.7.10 Release kinetics

Cadmium release kinetics was determined by stirring a suspension of 10 g of soil (with and without biochar) in 50 ml of 0.01 M $CaCl_2$ on an agitating table, and sampling at different times i.e., after 1, 30, 60, 120, and 180 minutes. The pH and EC were measured directly in the soil suspension before transferring into centrifuge tubes. The tubes were then placed in a centrifuge (Eppendorf 5810R Series) and later filtered. About 10 ml of the filtered sample was poured into a polyethylene tube and the dissolved Cd was measured on the ICP-MS. Also, about 20 ml of the filtered sample was transferred into total organic carbon (TOC) glass bottles, then placed into a total organic carbon analyzer (TOC-VCSH, Germany) in order to measure the amount of TOC in each sample.

3.8 Cultivated plants and soils

After 2 weeks of equilibration, seeds were sown into all pots contained in each treatment. Each treatment had 16 pots with four replicates. Additional 2 weeks were provided to ensure proper germination of seeds, so commencement of treatment was 4 weeks after cultivation. For each treatment, the watering regime implemented was three-times watering per week (3XW), one-time watering per week (1XW), dry-wet watering (DWW), and wet-dry watering (WDW). Volume of water applied was calculated based on 70% field capacity of the control soil. After three and six weeks of treatment, plant height and no of flowers per pot were being noted of.

At the point of harvest i.e., ten weeks of cultivation, above-ground biomass from each pot was harvested. Plants were weighed and then dried using an oven (Memmert ULE 700, Germany) at 65°C for 72 hours. The dried plants were then again weighed and later crushed using a mortar and pestle before placing them in a milling machine (Fritsch 01692, 50 – 60 Hz, 740 W, Germany) in order to obtain a well-represented sample of the plants.

For determination of Cd concentration, 0.5 g of each plant was weighed into crucibles and was calcinated in a muffle furnace (Nabertherm, Germany) at 450°C for 2 hours. Then digestion was performed on a heating plate (Ceran Hotplate 11A, Hannover, Germany) at 150°C and 50 W, using 5 ml of 6M HNO_3 until the volume in each crucible was approximately 1 ml. 5 ml of 3M HNO_3 was later added and left to boil for 3 minutes before filtering into 50 ml volumetric flask while ensuring proper transfer from the crucible unto the filter paper. 1% HNO_3 was added directly onto the filter paper to quantitatively dilute the solution to the required mark in the Erlenmeyers. The samples were then placed into the refrigerator at 4°C for further analysis.

3.8.1 Rhizon sampling

Sampling of the soil solution was carried out using rhizon soil moisture samplers (SMS) (Eijkelkamp Agrisearch Equipment, Giesbeek, the Netherlands) which were placed in all 48 pots. Vacuum tubes were placed into the soil 24 hours after the watering of the pots was carried out. The vacuum tubes were then left in the pots for 24 hours to extract a sufficient amount of soil solution. Vacuum tubes were retrieved and transported for analysis. Then one drop of concentrated HNO_3 was added to each tube to prevent precipitation of metals to the walls of the tube. The tubes were then stored in the refrigerator at a temperature of 4°C for further analysis. Consequently, when the watering regimes started, rhizon sampling was carried out on a bi-weekly basis.

3.9 Postharvest soils

For the cultivated soils, 0.01 *M* $CaCl_2$ extraction procedure according to Van Ranst et al. (1999), was used for determining Cd concentration in 24 pots (8 pots per treatment) used in the experiment i.e., 3XW and 1XW for all treatments.

4 RESULTS

4.1 Soil and biochar characterization

Physicochemical properties of the soil and biochar used in the experiment were analysed and reported (Table 1). Following the soil classification provided by United States Department of Agriculture (USDA), the soil is classified as a sandy loam soil. The soil's moisture content at field capacity is 27%. The pH of the soil which is close to neutral suggests that adsorbed Cd in the soil could be present in forms such as $CdCO_3$ and $CdHCO_3$ (Olaniran et al., 2013). The cation exchange capacity (CEC) of the soil is typical for a sandy loam soil and it is expected to increase upon the addition of biochar. The higher the CEC, the higher the organic matter present in the soil which results in greater water retention ability (Ketterings et al., 2007). The biochar has a high level of total organic carbon (TOC) (83.4%). High TOC of biochar has been reported to facilitate soil fertility and promote biological processes within the soil (Wang et al., 2018, Sun et al., 2020).

Table 1. Physiochemical characteristics of the soil and biochar used (mean value ± standard deviation, n = 3)

Parameter	Units	Soil	Biochar
Sand	(%)	20.50	-
Clay	(%)	12.04	-
Loam	(%)	67.49	-
Texture class		Sandy loam	-
Field capacity	(%)	27 ± 0.03	-
Moisture content	(%)	20.9 ± 0.9	-
Total organic carbon (TOC)	(%)	0.88	83.4
H/C	(%)	-	0.38
Cation exchange capacity (CEC)	$Cmol_+/kg$	8.85	-
Electrical conductivity (EC)	(dS/m)	0.64 ± 0.02	0.28 ± 0.01
$pH-H_20$	-	6.82 ± 0.12	9.7
pH-KCl	-	5.9 ± 0.1	-
P	mg/kg	190	500
Mg	mg/kg	250	-
Na	mg/kg	14	-
Ca	mg/kg	1680	-
S	mg/kg	180	-
K	mg/kg	130	154
Cd	mg/kg	0.42 ± 0.03	0.1 ± 0.0
N	mg/kg	-	2210
Zn	mg/kg	-	66 ± 2
Mn	mg/kg	-	231 ± 13
Fe	mg/kg	-	1000 ± 0.0

4.2 Determination of easily extractable Cd in the soil using 0.01 *M* $CaCl_2$ extraction

After 2 weeks of equilibration, easily extractable Cd in soil treatments with 2% BC and 4% BC had significantly reduced ($P < 0.05$) by 36% and 52% respectively, in comparison to soil without biochar (Figure 7a). The influence of watering regime was quite evident in all treatments. Exchangeable Cd concentrations in the treatment with three-times watering (3XW) were lower in comparison to the treatments with one-time watering (1XW) (Figure 7b). There was a significant decrease in extractable Cd (mg/kg) ($P < 0.05$) in biochar amended soils compared to soils without biochar after equilibration and harvest. Determination of easily exchangeable concentrations of metals in the soil using 0.01 *M* $CaCl_2$, serves as an indicator of the metal pool that can be easily leached from the soil (Gandois et al., 2010). $CaCl_2$ extraction also acts as an equilibrium-based method to assess bioavailability of metals in soils (Ma et al., 2020).

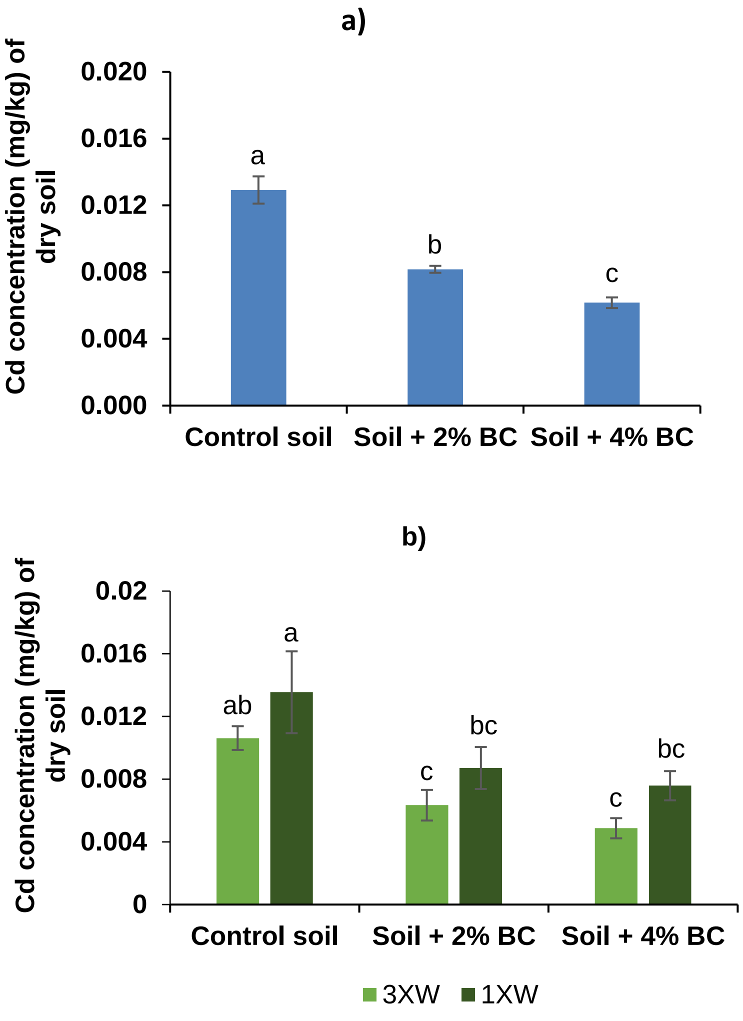


Figure 7. a) $CaCl_2$-extractable Cd concentrations (mg/kg) in soil (with and without biochar) after 2 weeks of equilibration (mean values, n = 3). Error bars indicate standard deviation. Bars with the same letter are not statistically different ($P > 0.05$). b) $CaCl_2$-extractable Cd concentrations (mg/kg) in soil

(with and without biochar) after 10 weeks of cultivation period (mean values, n = 4). Error bars indicate standard deviation. Bars with the same letter are not statistically different (P > 0.05).

4.3 Determination of Cd concentration in the soil using ammonium acetate-EDTA extraction

Water soluble, acid extractable, and reducible fractions of Cd (mg/kg) in EDTA-extracts was ~ 0.2 mg/kg in all treatments after 2 weeks of equilibration. This caused insignificant difference (P > 0.05) among the treatments (Figure 8). Concentrations of Cd in EDTA extracts were much higher in comparison with extracts of $CaCl_2$, as hypothesized by earlier studies (Ma et al., 2020, Römkens et al., 2009). EDTA extraction could provide good indication of metal bioavailability in soils (Meers et al., 2007).

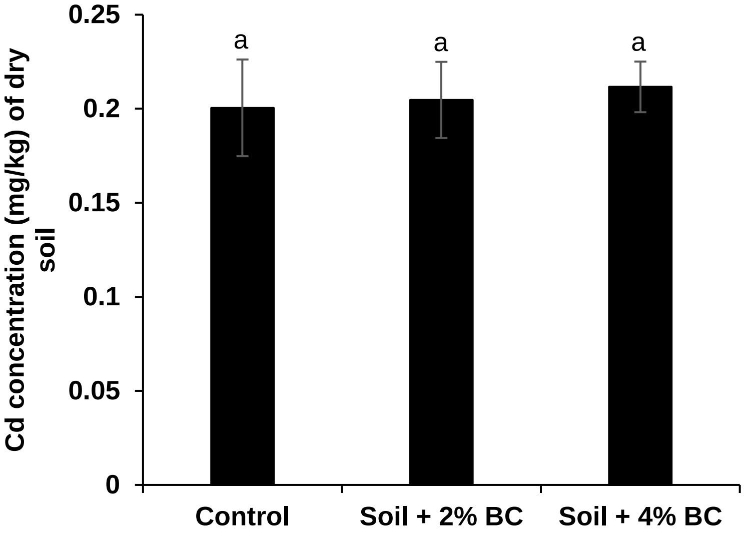


Figure 8. Cd concentration (mg/kg) released from the substrates by ammonium acetate-EDTA extraction solution (mean values, n = 3). Bars with the same letter are not statistically different (P > 0.05).

4.4 Pseudo-total Cd concentration in soils

Cd concentrations were measured to be 0.42 ± 0.03 mg/kg, 0.43 ± 0.03 mg/kg, and 0.44 ± 0.02 mg/kg for control soil, soil with 2% BC, and soil with 4% BC respectively. This revealed that the pseudo-total concentrations of Cd had no significant difference (P > 0.05) among soil treatments (with or without biochar). The measured concentrations of Cd is within the average concentration of Cd in a normal soil i.e. 0.01 to 1 mg Cd/kg (Kubier et al., 2019). This means that the soil has Cd concentrations within baseline contamination.

Furthermore, in order to determine the division of pseudo-total concentrations of Cd in various pools of the soil, the BCR protocol (Rauret et al., 1999) was implemented (Figure 9). The highest Cd concentration i.e., 0.280 ± 0.001 mg/kg, 0.270 ± 0.004 mg/kg, and 0.260 ± 0.001 mg/kg were found in the reducible fraction (F2) of the control soil, soil with 2%, and 4% BC respectively, while the least Cd concentration (mg/kg) was found in the oxidizable fraction (F3) for all soils (with or without biochar).

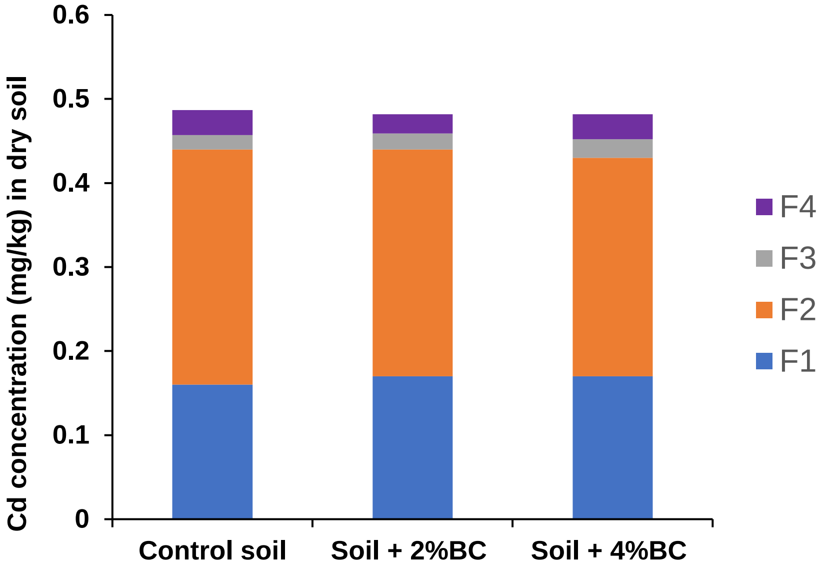


Figure 9. Fractionation of Cd in various pools of the substrate. F1 represents the exchangeable pool. F2 represents the reducible pool. F3 represents the oxidizable pool. F4 represents the residual pool.

The sum of Cd concentrations recovered from each step of the BCR, was however more than the pseudo total Cd concentrations earlier determined via the aqua regia digestion. This resulted in a recovery rate of 110%, 105%, and 101% for the control soil, soil with 2% BC, and soil with 4% BC respectively. These recovery rates are very reasonable and may not be significantly different from 100%. For effectiveness of the experiment, lake sediment was used as a reference material as suggested by the protocol. The recovery rates of the reference material were 82%, 126%, 52%, and 85% for F1 (exchangeable fraction), F2 (reducible fraction), F3 (oxidizable fraction), and F4 (residual fraction) respectively.

4.5 Saturated paste extraction

Average Cd concentrations (µg/L) measured during the saturated paste extraction were 1.50 ± 0.48 µg/L, 0.94 ± 0.21 µg/L, 0.43 ± 0.11 µg/L for the control soil, 2% BC treatment, and 4 % BC treatment respectively. The amount of Cd released from the control soil was significantly higher ($P < 0.05$) than the treatment with 4% BC but not significantly different from the treatment with 2% BC. Also, the lowest value for EC (0.64 ± 0.01 dS/m) was measured in the control soil, followed by the 2% BC treatment (0.66 ± 0.01 dS/m), while the highest EC was observed in the 4% BC treatment (0.68 ± 0.01 dS/m). There was no significant effect ($P > 0.05$) of biochar addition on the soil EC.

4.6 Soil pore water

Concentration of Cd (µg/L) in the soil pore water measured at 4 moments (Figure 10), revealed the impact of biochar on the availability of Cd within the soil solution. For each sampling of the rhizon during the cultivation period, Cd concentrations in the control soil was always higher in comparison with biochar amended soils. At the end of the experiment i.e., 10 weeks of cultivation, initial Cd concentration (µg/L) in soil pore water of the control soil increased within the range of 31% - 124%, while the initial Cd concentration (µg/L) in soil pore-water for treatments with 2% BC decreased within the range of 11% - 38%. A percentage decrease between 53% - 74% was observed for treatments amended with 4% BC.

Only extreme conditions i.e. 3XW and 1XW, are shown (Table 2 and Figure 10). This is because, change in Cd concentration (µg/L) in DWW was not significantly different ($P > 0.05$) from 3XW, while change in Cd concentration (µg/L) in WDW was not significantly different ($P > 0.05$) from 1XW. In all treatments, pH measurement on soil pore-water after 3 weeks of treatment was higher than pH after 6 weeks of treatment except for control soil under DWW condition (Appendix. 5).

Table 2. Cd concentration (µg/L) in soil pore water at extreme watering conditions (3XW and 1XW). Means and standard deviation are shown (n = 3). Values with the same letter are not statistically different ($P > 0.05$).

Treatment	**Watering regime**	**3 weeks of treatment**	**6 weeks of treatment**
Control soil	3XW	0.58 ± 0.22 a	0.62 ± 0.49 b
2% BC	3XW	0.38 ± 0.10 ab	0.34 ± 0.18 bc
4% BC	3XW	0.20 ± 0.08 bc	0.15 ± 0.09 c
Control soil	1XW	0.59 ± 0.16 a	1.05 ± 0.04 a
2% BC	1XW	0.20 ± 0.07 bc	0.27 ± 0.11 bc
4% BC	1XW	0.16 ± 0.14 c	0.22 ± 0.07 bc

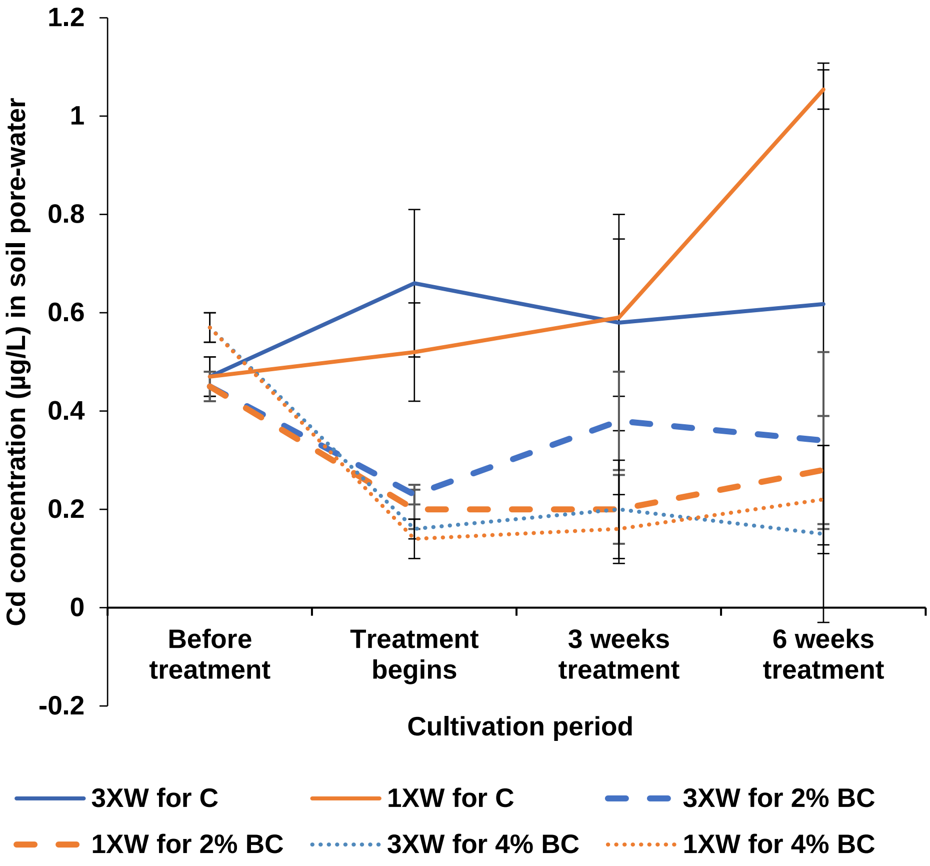


Figure 10. Cd concentrations in soil pore-water (µg/L) in soil (with and without biochar) at different timepoints under the wet condition (3XW) and dry condition (1XW). Error bars represent standard deviation (n = 4).

4.7 pH stat and buffering capacity

Cd release from the control soil at varying pH values (4 – 8) was determined (Figure 11). About 7.48 µg Cd/L was released at pH 4, while a reduction in Cd concentration to 0.23 µg/L was measured at pH 8. This is in line with various studies on Cd release from the soil at varying pH levels (Houben et al., 2013, Ur Rahman et al., 2021). This reveals that at low pH, the bioavailability and uptake of Cd by plants becomes higher in comparison with basic soils.

Cd release using the pH stat experiment could not be performed on the biochar amended soils and this prevented a comparison of Cd release among the treatments. However, buffering capacity of soil (with and without biochar) was performed. Alteration in pH of soils followed the same buffering pattern (Figure 12).

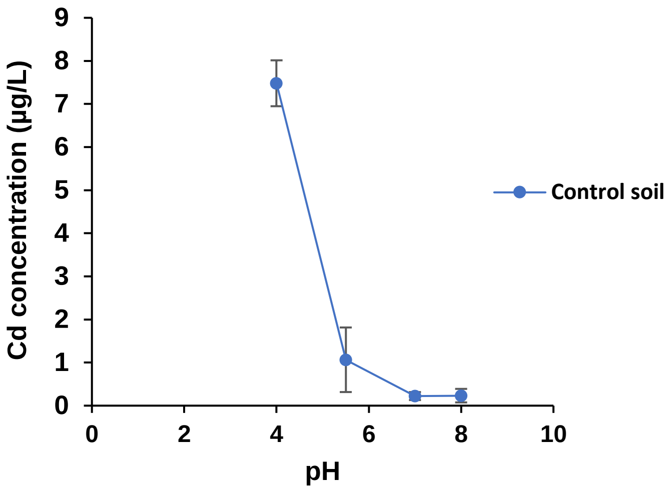
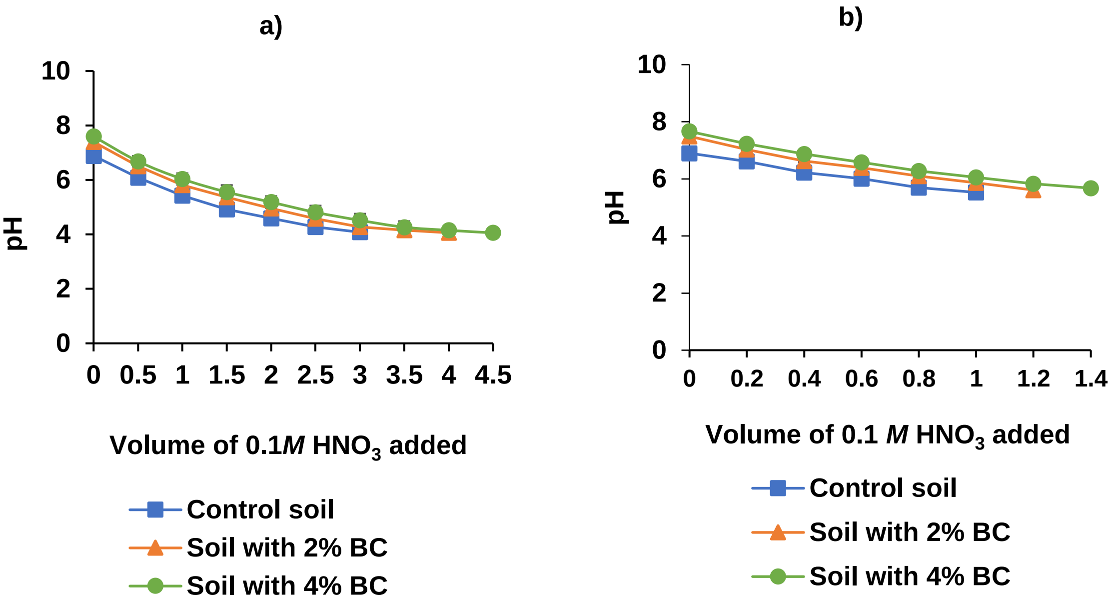


Figure 11. Release of Cd (µg/L) from control soil at varying pH.

During the reduction of average pH to acidic pH i.e., 4 in each treatment, the buffering capacity calculated was 2.17 ± 0.13 mol/L, 2.13 ± 0.11 mol/L, and 2.26 ± 0.19 mol/L for the control soil, treatment with 2% BC and 4% BC respectively. There was no significant difference (P > 0.05) between buffering capacity among the substrates. Nonetheless, biochar amended soils required more volume of titrate (0.1 *M* HNO_3) to achieve a lower pH such as 4 and 5.5, while the control soil required more titrate (0.1 *M* NaOH) to reach higher pH levels such 8.



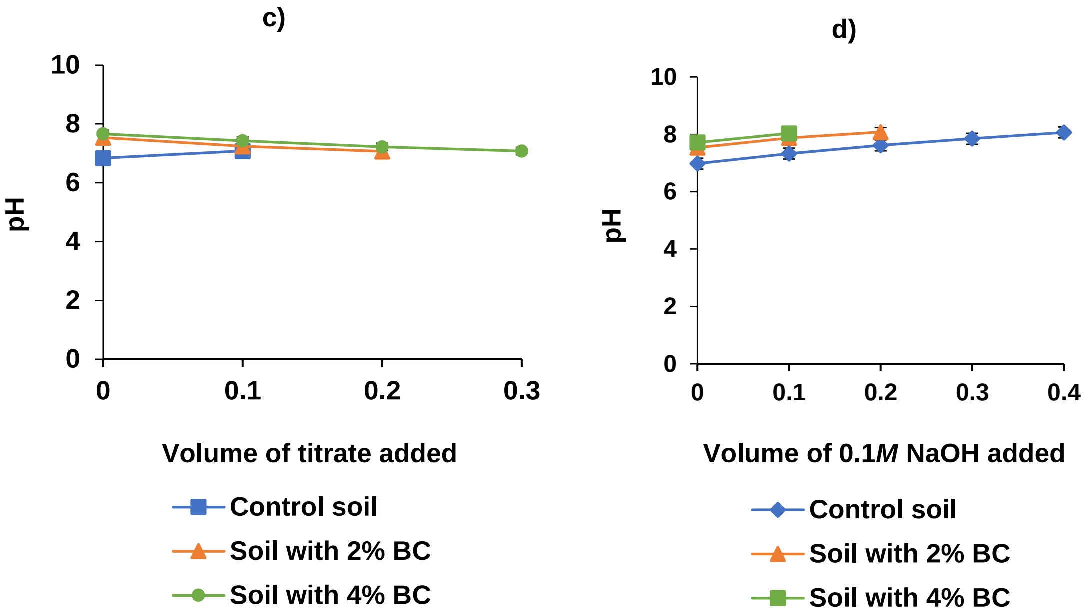


Figure 12. pH buffer curves for soils (with and without biochar) for different pH's a) pH 4 (b) pH 5.5 (d) pH 7 and (e) pH 8. Error bars signify the standard errors (n = 3).

4.8 Release kinetics

Cd release as a function of time (1 – 180 minutes) using 0.01 *M* $CaCl_2$ extraction solution revealed that BC amended soils significantly decreased ($P < 0.05$) the release of Cd (µg/L) from the soil (Figure 13). After 180 minutes of extraction time, the release of Cd into solution decreased by 46% and 58% in the 2% BC and 4% BC respectively in comparison to the control soil. Cd release from the control soil and 2% BC treatments followed quite similar pattern. After 60 minutes of extraction, the treatment with 4% BC showed a reduction in Cd that was being released into the soil solution.

With an increase in extraction time, there was an increase in the release of total organic carbon (TOC) and electrical conductivity (EC) for all treatments (Figure 13 c and d). TOC release from the control soil, soil with 2% BC and soil with 4% BC ranged between 13.6 – 23.5 mg/L, 12.9 – 24.2 mg/L, 19.4 – 24.2 mg/L respectively. Change in EC for the control soil, and treatments with 2% BC and 4% BC were within the range of 2.01 – 2.10 dS/m, 2.02 – 2.11 dS/m, 2.03 – 2.12 dS/m respectively. Variations in TOC and EC resulted in no significant difference ($P > 0.05$) among the treatments. Levels of pH for all treatments did not change more than 1 unit within the total extraction period. As expected, the treatments with biochar had a higher pH relative to the control soil without biochar.

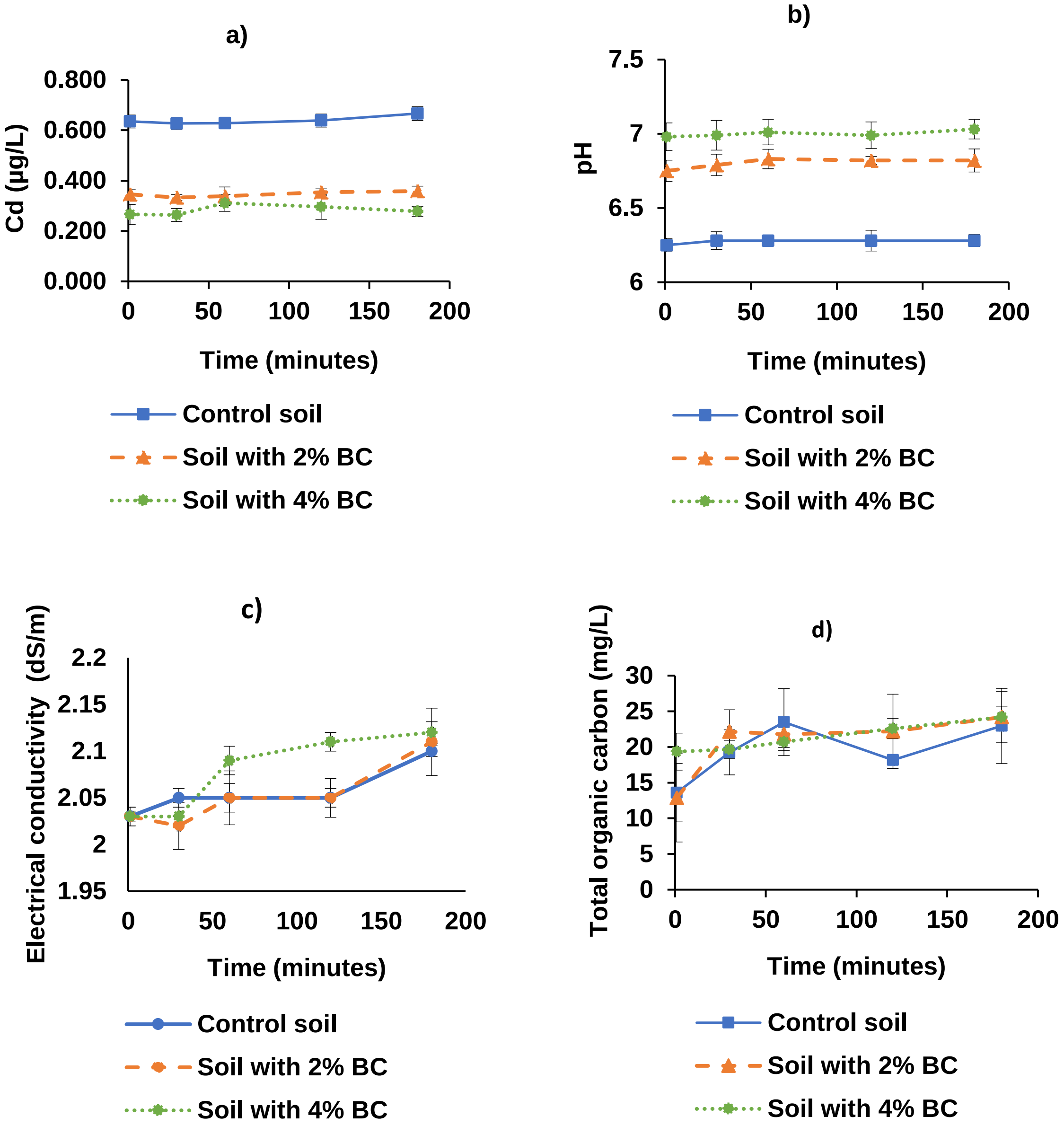


Figure 13a) Concentration of Cd (µg/L) released from each treatment with time. b) Change in pH of soil treatments with time. c) Change in electrical conductivity (dS/m) of soil treatments with time. d) Change in total organic carbon concentration (mg/L) of soil treatments with time. Errors bars represent standard deviation (n = 3).

4.9 Concentration of Cd measured by DGT

The highest concentration of Cd (0.36 ± 0.04 µg/L) measured on the surface of the DGT device (C_{DGT}) was obtained in the control soil while the lowest concentration (0.14 ± 0.04 µg/L) was found in the treatment with 4% BC (Table 3). In all treatments, the initial Cd concentration (µg/L) in soil pore-water (C_{sol}) was higher than the C_{DGT}. The R value, that which represents the ratio of resupply of metal from the solid phase, was 0.2, 0.3, and 0.8 for the treatments with 4% BC, 2% BC, and control soil respectively. Also, the effective concentration (C_E) of Cd directly relates to plant uptake (Egene et al., 2018). C_E was significantly higher ($P < 0.05$) in the control soil in comparison to biochar amended soils. Cd fluxes measured by the DGT device was 18.3 x 10^{-9} µg s^{-1} cm^{-2}, 8.86 x 10^{-9} µg s^{-1} cm^{-2}, and 8.44 x 10^{-9} µg s^{-1} cm^{-2} for the control soil, treatment with 2% BC and 4% BC respectively. A case of partial sustenance can be attributed to the three treatments because their R values were < 0.95. This implies that there is a supply of solute from the solid phase but it is insufficient to fully sustain the pore water concentration (Zhang, 2003). However, R values closer to 1 means a rapid resupply from the solid phase, while values closer to 0.1 signify a slow resupply of metal (Egene et al., 2018). This therefore suggests a slow resupply of Cd from the solid phase for the biochar treatments (2% BC and 4% BC) but a high resupply of Cd for the control soil

Table 3. Concentration of Cd in the pore water (C_{sol}) determined from initial rhizon extract, concentration of Cd measured by DGT device (C_{DGT}), effective concentration (C_E) measured by the DGT device, and the ratio (R) of C_{DGT} to C_{sol} . Means and standard deviation are shown (n = 3).

	C_{sol} (µg/L)	**C_{DGT} (µg/L)**	**C_E (µg/L)**	**R**	**Resupply of Cd**
Control soil	0.47	0.36 ± 0.04	3.49 ± 0.41	0.8	Rapid
Soil with 2% BC	0.45	0.16 ± 0.03	1.46 ± 0.4	0.3	Slow
Soil with 4% BC	0.57	0.14 ± 0.04	1.39 ± 0.36	0.2	Slow

4.10 Spinach

After 2 weeks of planting, there was less homogeneity in germination among the treatment with 4% BC, so two additional weeks were provided to achieve germination of new seeds. After 3 weeks of treatment (Plate 1 and Appendix 1), mean height of plants (cm) with 4% BC, was higher ($P < 0.05$) in comparison with the control soil for all watering regimes except for WDW regime. Also, the mean height of the plants with 2% BC (1XW and DWW) were significantly higher ($P < 0.05$) than that of the control soil. Bolting was observed among all treatments after 7 weeks of planting i.e. 3 weeks of treatment. This could be linked to stressed growing conditions of the plants which could also limit the plant's physiochemical properties.

Plate 1. Spinach after 7 weeks of planting (which includes 3 weeks of treatment). “A” Control soil (3XW). “B” Soil with 2% BC (3XW). “C” soil with 4% BC (3XW).

At the start of the second phase of treatment i.e., 8 weeks of planting, fertilizer was added once again to each pot due to gradual yellowing of plants. Yellowing of leaves was first observed among old leaves. This could be linked to nitrogen deficiency in the plant (Machado et al., 2020, Sawyer, 2010). Spinach is also reported to have a large demand for nitrogen during its vegetative growth (Nemadodzi et al., 2017, Zikalala et al., 2017). Despite the presence of biochar and fertilization at the start and mid-cultivation, all treatments still showed signs of yellowed leaves. Biochar has the potential to improve plant health, however due to its high carbon content, plant available nitrogen gradually gets depleted due to the microbial immobilization of nitrogen (Phillips et al., 2022).

Concentrations of major nutrients such as Mg, Ca, Zn, and Fe (mg/100 g,) were significantly higher ($P < 0.05$) in spinach (DW) compared to average concentrations in spinach leaves (DW) (USDA, 2020) (Appendix 3). Lower concentrations of potassium (K) and phosphorus (P) was observed across all treatments irrespective of the substrate or watering regime. This could be as a result of under-fertilization with K and P during cultivation (Prajapati and Modi, 2012).

After harvest i.e., 10 weeks of cultivation (Plate 2 and Appendix 2) which involved 6 weeks of treatment with biochar, there were signs of increased stress such as fallen stems and brown leaves among the treatments. Average biomass yield (Figure 14a) in harvested spinach for all treatments per watering regime was not significantly different ($P > 0.05$) using the post-hoc Duncan’s Multiple Range Test (DMRT).

Plate 2. Spinach after 10 weeks of planting (which includes 6 weeks of treatment). “A” Control soil (3XW). “B” Soil with 2% BC (3XW). “C” soil with 4% BC (3XW).

In the control soil and 2% BC treatment, biomass yield under DWW was significantly higher ($P < 0.05$) than 1XW and WDW. In the 4% BC treatments, the biomass yield under 3XW and DWW watering regimes, were significantly higher ($P < 0.05$) than treatments with 1XW and WDW. Highest biomass yield (i.e., 22 g, fresh weight) was observed under 2% BC (DWW) and 4% BC (DWW), while the lowest biomass yield (i.e., 10 g, fresh weight) was measured under the control soil (WDW) and 2% BC (WDW). Moisture content (%) in harvested spinach was obtained after determining the dry biomass yield (g) per treatment (Figure 14b). The highest moisture content (86%) was obtainable under treatment 4% BC (DWW), while 2% BC (WDW) had the least moisture content (71%). Table 4, shows the ratio between dry biomass yield (g) to fresh biomass yield (g). Dry biomass yield (g) of spinach cultivated under 2% BC and 4% BC treatment was not significantly different (P > 0.05) to those grown on the control soil.

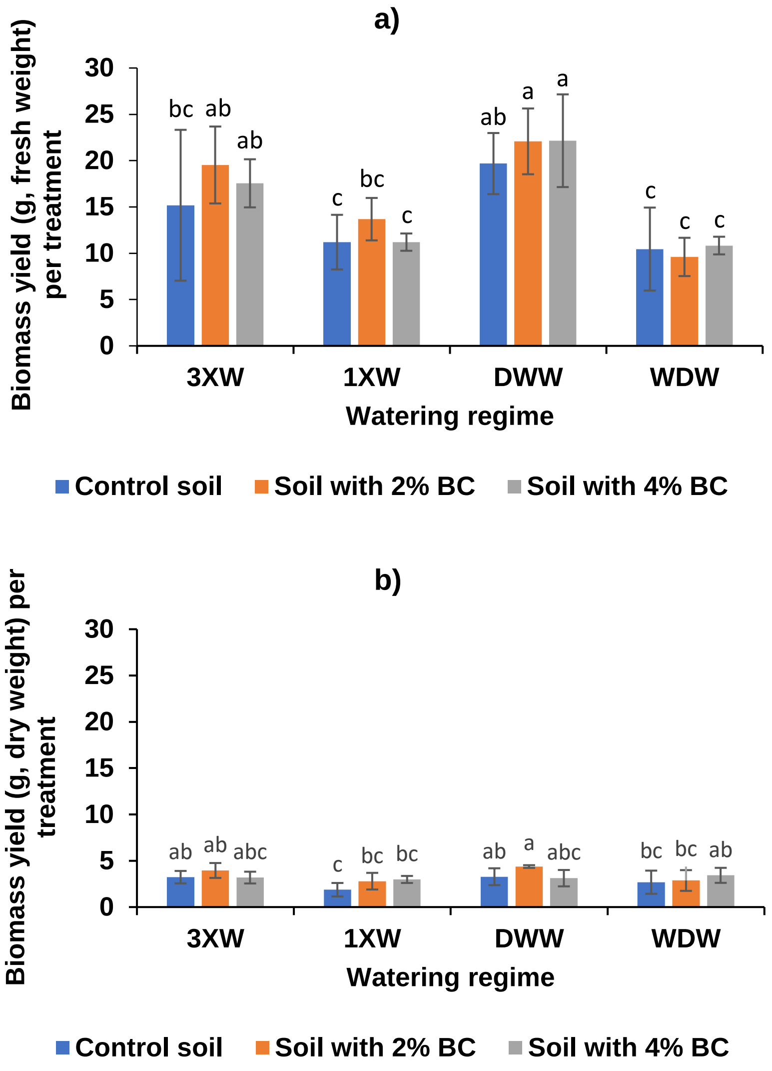


Figure 14. a) Biomass yield (g) of spinach (fresh weight) per treatment. b) Biomass yield (g) of spinach (dry weight) per treatment. Mean value (n = 4) and standard deviation are shown. Bars with the same letter are not significantly different (P > 0.05).

The ratio between dry and fresh biomass varied across the three treatments. Variations in this ratio is influenced by moisture content in plants, which is also influenced by the watering regime being applied. The highest ratio (0.31 ± 0.05) and least ratio (0.14 ± 0.02) were observed under 4% BC (WDW) and

4% BC (DWW) respectively. Watering regimes with 1XW were not significantly different ($P > 0.05$) from WDW conditions.

Table 4. Ratio of dry to fresh biomass yield (g) per treatment as a function of watering regime[a]. Averages sharing the same letter are not significantly different[b].

		Ratio of dry-fresh biomass yield per treatment	
	Control soil	**Soil with 2% BC**	**Soil with 4% BC**
3XW	0.27 ± 0.15 abc	0.20 ± 0.01 bcde	0.18 ± 0.03 cde
1XW	0.16 ± 0.02 de	0.20 ± 0.06 bcde	0.27 ± 0.03 abc
DWW	0.16 ± 0.02 de	0.20 ± 0.03 bcde	0.14 ± 0.02 e
WDW	0.25 ± 0.04 abcd	0.29 ± 0.08 ab	0.31 ± 0.05 a

[a] Means ± standard deviation

[b] Post-hoc Duncan's Multiple Range Test (DMRT) at 5% level of confidence

4.10.1 Cd accumulation in spinach

Total above ground biomass was analysed as a result of low biomass (≤ 5 g, fresh weight) of harvested spinach (which includes only stem and leaves) from the control soil (3 pots) and the treatment with 2% BC (1 pot). The total above ground biomass includes stem, leaves, flowers, and seeds.

Concentration of Cd in harvested spinach was evaluated using a one-way analysis of variance (ANOVA). Post-hoc analysis ($P < 0.05$) was performed using the DMRT in order to define mean pairs that were significantly different. Highest Cd concentration i.e., 0.71 mg/kg was observed under treatment with 2% BC (WDW), while treatment with 4% BC (3XW) resulted in the least Cd concentration i.e., 0.29 mg/kg. No significant difference ($P > 0.05$) in Cd concentration (mg/kg) for all treatments (with or without biochar) cultivated under 3XW regime (Figure 15).

Interestingly, Cd concentration (mg/kg) observed in the control soil under the 1XW was significantly lower ($P < 0.05$) in comparison to treatments with 4 % BC but not significantly different from that of 2% BC. This is a strange occurrence, as biochar amended soils were hypothesized to yield less uptake of Cd in cultivated plants. In addition, watering regime had significant effect ($P < 0.05$) in biochar amended soils compared to the control soil.

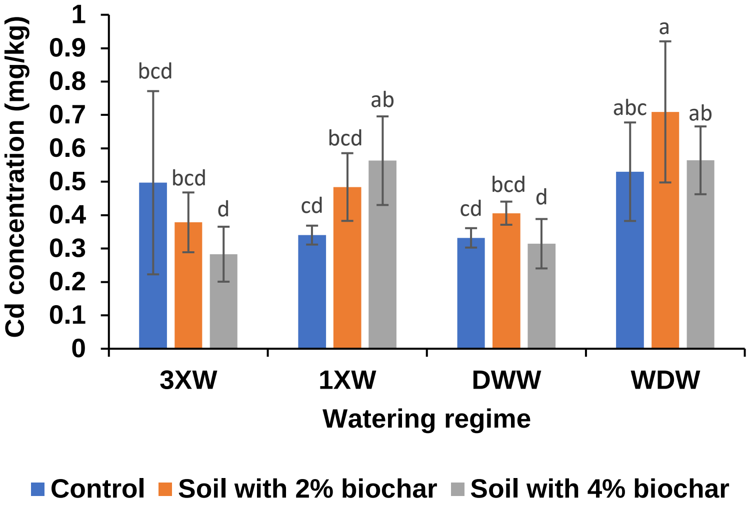


Figure 15. Cd concentration (mg/kg) in harvested spinach (fresh weight). 3XW is three-times watering per week, 1XW is one-time watering per week, DWW is dry-wet watering, WDW is wet-dry watering. Error bars represents standard deviation (n = 4). Bars sharing the same letter are not significantly different ($P > 0.05$).

4.10.2 Bioaccumulation in plants

The ratio between Cd concentration in plants (fresh weight) to Cd concentration in the soil (dry weight) can be used to obtain a bioaccumulation factor for the cultivated plants. Highest accumulation of Cd in spinach was observed under treatment with 2% BC (WDW), while the least was found in 4% BC (3XW). No significant effect of watering regime was observed within the control treatment (Table 5). With 2% BC treatment, Cd accumulation in plants cultivated under WDW was significantly higher ($P < 0.05$) in comparison to other watering conditions. 3XW and DWW conditions resulted in significantly lower ($P < 0.05$) Cd accumulation compared to 1XW and WDW under 4% BC treatment.

Table 5. Bioaccumulation factor (BAF) for all treatments. Values represent mean and standard deviations (n = 4). "3XW" is three-times watering per week. "1XW" is one-time watering per week. "DWW" is dry-wet watering. "WDW" is wet-dry watering. Mean values sharing the same letter are not significantly different[a].

		BAF in treatments	
	Control soil	**Soil with 2% BC**	**Soil with 4% BC**
3XW	1.183 ± 0.65 abc	0.88 ± 0.21 bcd	0.64 ± 0.19 d
1XW	0.81 ± 0.07 bcd	1.13 ± 0.24 bcd	1.28 ± 0.30 ab
DWW	0.79 ± 0.07 bcd	0.94 ± 0.08 bcd	0.71 ± 0.17 cd
WDW	1.26 ± 0.35 ab	1.65 ± 0.49 a	1.28 ± 0.23 ab

[a]Post-hoc Duncan's Multiple Range Test (DMRT) at 5% level of confidence

4.10.3 Total water added

For the first 30 days of cultivation, all pots were watered equally (Figure 16). Volume of water added (L) for the first two weeks of treatment was based on 70% field capacity of individual treatments. However, for the last 4 weeks of treatment, volume of water applied (L) was based on the field capacity of the control soil i.e., soil without biochar. A cumulative volume of 5.59 L, 3.35 L, 4.69 L, and 3.69 L of water was added to the treatments with 3XW, 1XW, DWW, and WDW respectively.

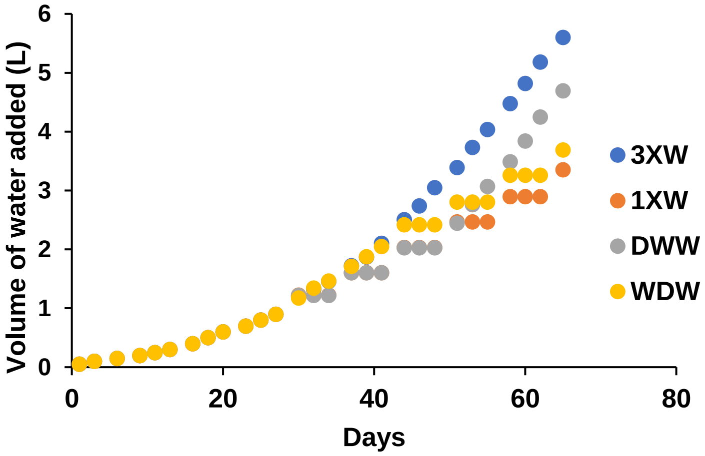


Figure 16. Cumulative amount of water applied (L) to all treatments. Data points represent mean value (n = 4). "3XW" is three-times watering per week. "1XW" is one-time watering per week. "DWW" is dry-wet watering. "WDW" is wet-dry watering.

5 DISCUSSION

5.1 Cadmium in soil and plant on the control soil

Cultivation of spinach on Cd-contaminated soil is expected to yield higher concentrations of Cd in spinach compared to soils having baseline concentrations of Cd. Nonetheless, farmers have claimed it could be difficult to meet the permissible limits of Cd in spinach i.e., 0.2 mg/kg (fresh weight) despite its cultivation on soils with baseline concentrations (Tack, 2022). Baldantoni et al., (2016), stated that Cd exceedance in spinach can be attributed to the fact that spinach naturally has a high tendency to accumulate Cd from the soil.

With respect to the limit provided by the European Commission (2015), the concentrations of Cd observed in this experiment for harvested spinach (FW) are considerably high i.e., > 0.2 mg/kg (fresh weight). However, as earlier stated, Cd determination in this study was based on the entire above ground biomass, whereas the permissible limit is based only on freshly harvested spinach leaves. Nonetheless, the measured concentrations of Cd in the spinach are still of concern as the soil used in this experiment was not contaminated, but with baseline concentration i.e., 0.42 mg Cd/kg. Consequences of exceeding Cd concentrations in harvested spinach will not only result in losses by the farmer but will also lead to contamination of the food chain. Therefore, it is important to know why Cd concentration continues to exceed permissible limits in spinach when cultivated on this soil.

Firstly, concentration of Cd in the pore water as observed throughout the experiment showed that the soluble Cd increased over time in the control soil. Cd concentration measured by the rhizon can also be linked to the pH of the soil pore-water. After 6 weeks of treatment, it was observed that average pH of the soil pore-water had decreased for all watering regimes except for DWW. This resulted in a significant increase ($P < 0.05$) in Cd concentration (µg/L) measured in the soil pore-water. A negative correlation ($R = -0.99$) between pH and Cd in the soil pore-water, as seen in the current study, aligns with the findings of Qi et al., (2018) and Olaniran et al., (2013), wherein Cd becomes more bioavailable with decreasing pH.

The impact of root exudates could be responsible for the variation in soil pH, where in organic acids are released within the rhizosphere. The release of exudates which potentially changes the pH in the soil can influence the availability, uptake and transfer of metals from the soil to cultivated crops (Reichman and Parker, 2005). Physicochemical properties, root morphology and microbial performance of the rhizosphere influence the bioavailability of Cd in the soil (Gong et al., 2022). As earlier mentioned, pH is one of the major factors affecting the uptake of Cd by vegetables such as spinach. Performing the pH-stat experiment on the control soil revealed that at an alkaline pH e.g. pH 8, lower concentrations of Cd could be released from the control soil, while higher concentrations could be released at an acidic pH e.g. pH 4. This suggests that under acidic conditions, Cd becomes more bioavailable and promotes absorption by the plant. Similar findings were found in previous studies (Park et al., 2011, Branzini and Zubillaga, 2012), wherein acidic soils had higher mobility and bioavailability of heavy metals in comparison to alkaline soils.

In addition, Cd concentration measured at the end of the treatment by the rhizon under wet condition i.e., 3XW was found to be quite similar to average Cd concentrations (0.64 µg Cd/L) measured from the release kinetic experiment. This also suggests that the release kinetic experiment using $CaCl_2$ as an extraction solution could predict potential bioavailability of Cd. A positive correlation (R = 0.58) observed between EC and Cd concentration in the soil corroborates the findings of Qin et al., (2021), wherein three different models (linear, logarithmic and quadratic) were used to obtain a positive correlation between EC and soil-Cd . Likewise, a correlation (R = 0.2) established between TOC and Cd concentration in the soil aligns with the results of Muehe et al., (2013). This implies that by increasing these parameters (EC and TOC) in the soil, the amount of adsorbed Cd is expected to increase, thereby reducing the concentration of Cd in the soil solution. By the end of the experiment, the initial Cd concentration (µg/L) in the soil pore water for the control soil had increased within the range of 31% - 124%, which could elevate the risk of exceeding Cd limit in cultivated spinach.

This increase in bioavailable Cd aligns with the result obtained by the DGT, wherein a R value of 0.8 for the control soil indicates a rapid resupply of Cd from the solid phase into the liquid phase. Concentrations of Cd measured by the DGT device was much lower in comparison to the concentrations in the soil pore-water. This is due to the fact that the DGT measures the concentrations of metal at the interface between the soil pore-water and the DGT device (Davison et al., 2000, Zhang, 2003). Significantly higher amount of Cd flux measured by the DGT in the control soil (1.83×10^{-8} µg s^{-1} cm^{-2}) compared to the amended soils predicts an increase in bioavailable Cd which potentially increases the risk of uptake by spinach. Concentration of metal flux from the soil measured by DGT is quite similar to the metal flux in the plants (Lehto et al., 2006).

Generally, the mobility and bioavailability of metals within the soil is dependent not only on the pseudo-total concentration but also on their fractionation in the soil (Lamb et al., 2009, Cui et al., 2016). The highest Cd concentrations (38% and 67%) measured in the first two pools of the soil using the BCR technique aligns with other studies (Alvarez et al., 2002, Pueyo et al., 2008), where the exchangeable and reducible fractions constituted a much larger percentage of metals compared to the last two fractions i.e., oxidizable and residual fractions. This suggests a potential increase in the soluble Cd once dissociation of Cd from these 2 pools occur. Sungur et al. (2014), revealed that the uptake of Cd relative to different pools of the soil is mostly dependent on the bioavailability of Cd in the exchangeable and reducible pool. However, the present study is in contrast with the findings of Deng et al., (2019), wherein the distribution of Cd in the soil was: residual > exchangeable = reducible > oxidizable. Having such fractionation of Cd in the soil as reported by Deng et al., (2019) could possibly result in a decrease in soluble Cd in comparison with the control soil used in the current study.

Finally, it can therefore be concluded that combining all these observations for the control soil such as high concentration of Cd in the exchangeable and reducible fraction of the soil, plus the increase in Cd concentration in the soil pore water with time. Also coupled with a high resupply of Cd from the solid phase into the soil solution, it is expected that cultivation of spinach (a plant with high affinity for Cd) on such a soil will likely lead to accumulation of Cd above permissible limits.

5.2 Effects of biochar

In the case of biochar amended soils, the easily extractable fraction of Cd (using 0.01 *M* $CaCl_2$) reduced significantly ($P < 0.05$) in comparison to soil with no biochar. This could be attributable to the large surface area of biochar which results in the adsorption of Cd. A decrease in easily extractable Cd is one of the conditions that potentially limits the availability and potential uptake of Cd from the soil (Sato et al., 2010). Biochar amended soils was also found to reduce NH_4NO_3 extractable Cd by 88.4% (Park et al., 2011). Extraction of metals using 0.01 *M* $CaCl_2$ solution has been studied to provide good predictions for the possible uptake of metals from the soil by plants (Nolan et al., 2005).

Despite having significantly decreased ($P < 0.05$) the soluble Cd, biochar application resulted in no significant decrease ($P > 0.05$) in Cd concentration in the exchangeable and reducible pool in the soil, using the BCR extraction protocol. A possible reason for this is that less Cd might have been incorporated within the biochar, which potentially resulted in equal release of Cd among all substrates, when a stronger extractant was used i.e., acetic acid and hydroxylammonium chloride. Since this experiment i.e., BCR, was performed only after 2 weeks of equilibration, the possible impact of ageing and watering regime on the extractability of Cd could not be determined. Similar findings were reported by Zahedifar, (2020), wherein the addition of 2% and 4% sugarcane bargasse-derived biochar had no significant influence on the exchangeable Cd in a soil with baseline concentrations of Cd.

However, Cd concentration in the oxidizable pool was in the order: 4% BC (0.022 mg/kg) > 2% BC (0.019 mg/kg) > control soil (0.017 mg/kg). This difference is attributable to the increase in organic matter content of soils amended with biochar. This result aligns with the findings of Park et al., (2011), who reported that the addition of chicken manure derived biochar resulted in an increase in the organic bound Cd within the soil. Sungur et al. (2014) likewise reported a significant positive correlation between soil organic matter (SOM) and Cd concentration in the oxidizable pool of the soil. Also, the high extractability of pseudo-total Cd obtained in the reducible fraction suggests that ~ 67% of total Cd could be associated majorly with Mn-Fe oxides. Cd concentration was not significantly different ($P > 0.05$) in the residual fraction for all treatments

At each sampling of the rhizons (Appendix 5), Cd concentration (µg/L) in soil pore water of the 2% BC and 4% BC treatment were always significantly lower ($P < 0.05$) to that of the control soil. This was similar to the findings of Park et al., (2011), wherein Cd concentrations measured in the soil pore water of biochar (1%) amended soils decreased by 42.7% in comparison to the control soil. Beesley et al., (2010) equally demonstrated that Cd concentrations (µg/L) in soil pore water reduced by 8.3% in soil amended with hardwood-derived biochar.

Low concentrations of Cd obtained by the rhizon samplers agree with the results of DGT. R values of 0.3 and 0.2 measured by the DGT device for treatments amended with 2% and 4% BC respectively indicates a slow resupply of Cd from the solid phase. Continuous adsorption of metal to biochar, coupled with a slow resupply of metal to the liquid phase would lead to lower bioavailability (Egene et al., 2018). Determination of metal concentrations using DGT serves as a method to indirectly measure the labile fraction of metals present in the soil (Lekgoathi et al., 2022). Least Cd fluxes measured by the DGT was in the treatments with 4% BC and 2% BC. Concentration of metal flux from the soil measured by DGT is quite similar to the metal flux in the plants (Lehto et al., 2006). However, despite the difference in Cd fluxes measured by the DGT, there was no significant decrease ($P > 0.05$) in Cd concentration in plants cultivated on biochar amended soils. This further suggests that factors other than Cd flux, influenced the accumulation of Cd in the plants. Under diffusion-limited conditions, metal concentrations measured by DGT can be directly used to determine the uptake and accumulation of metals by plants (Bidar et al., 2019).

Biochar application significantly increased ($P < 0.05$) the pH of the soil. Elevation in soil pH after biochar application can be as result of the increase in basic cations and subsequent dissolution of present hydroxides. Solubility of Cd is expected to be reduced with the addition of biochar. Increased pH levels will potentially decrease the bioavailability of heavy metals within the soil (Jien and Wang, 2013). Park et al., (2011) also stated that with an increase in pH, soil respiration could increase and therefore promote microbial life within the soil. Nonetheless, alterations in other factors also influences the mobility and bioavailability of heavy metals from the solid phase, this includes redox potential, cation exchange capacity, soil micro flora (Lekgoathi et al., 2022), organic matter and silica content (Merdy et al., 2009).

The use of biochar has been proven to improve soil biological and physiochemical properties and eventually improving plant yield (Mandal et al., 2020, Trakal et al., 2017). However, in the present study, plant biomass yield (g) was not significantly different ($P > 0.05$) among soil treatments (with or without biochar). Insignificant yield (g) in biomass across treatments could also have an effect on Cd concentrations measured in the plants. Reduction in metal uptake from the soil, which results in lower accumulation in plants is related to the degree of metal immobilization and dilution factor (i.e., increase in plant biomass) (Park et al., 2011).

In addition, the soil-plant concentration factor, otherwise referred to as BAF, could also be looked into. Application of biochar did not cause major decrease in concentration factor in harvested spinach (Table 5). Despite causing the immobilization and reduction of bioavailable fractions of metals, application of biochar has been reported to increase the bioaccumulation of metals in plants (Mackie et al., 2015, Rees et al., 2014, Li et al., 2019b). This could be caused by the effect of biochar on Cd concentrations around the roots. With the addition of 5% biochar, fraction of Cd bound to the roots of cultivated rice decreased by 44%, thereby increasing soluble Cd from 6.7 to 45.7% (Li et al., 2019b). This suggests there could be a negative effect of biochar application on Cd concentration in the rhizosphere. A reduction in bioavailable concentrations of heavy metals in the soil does not guarantee a lower accumulation in the shoots (Chaignon et al., 2009, Bravin et al., 2009).

In summary, addition of biochar as a soil amendment to the soil was able to significantly decrease the easily extractable Cd from the soil and likewise the concentrations of Cd in the soil solution. Also, biochar successfully reduced the rate of Cd release from the soil with time, coupled with a decrease in Cd resupply from the solid phase. However, biochar did not significantly reduce Cd concentration in the exchangeable and reducible fraction of the soil. Since, no significant decrease in Cd concentration was observed in spinach grown on biochar amended soils, application of this biochar is not sufficient to attenuate the risk of exceeding permissible limits of Cd on this soil.

5.3 Impact of watering regime

Watering regime had more impact on Cd concentration in the soil pore water for the control soil compared to biochar amended soils. In the control soil, the highest concentration (1.05 µg Cd/L) was observed under the 1XW regime while the lowest concentration (0.62 µg Cd/L) was obtained under the 3XW regime. This agrees with the hypothesis of Tack (2017), who stated that an increase in water application during cultivation leads to a decrease in dissolved concentrations of Cd due to its adsorption unto the soil matrix. Significantly lower biomass yield in the treatments with 1XW and WDW can be attributed to water stressed conditions. With inadequate irrigation, stomatal (pores on leaf surface that help in the exchange of water vapour and carbon dioxide) density are highly affected (Du et al., 2015). Also, photosynthetic rates and stomata conductance has been reported to be high in sandy loam soils (Lekgoathi et al., 2022).

Watering regime had an influence on the easily extractable fraction of Cd in all treatments. In conditions with more water being applied e.g., 3XW, the release of Cd was significantly lower compared to concentrations released in treatments with low watering e.g., 1XW. Also, variations in water application had an effect on Cd concentrations (mg/kg) in both biochar amended soils. For 4% BC treatment, 3XW and DWW resulted in significantly lower ($P < 0.05$) Cd concentrations in spinach compared to 1XW and WDW, while for the 2% BC treatment, Cd concentration in spinach cultivated under the WDW condition was significantly higher ($P < 0.05$) in comparison to other watering regimes. Despite having dry conditions throughout the experiment, Cd concentrations under 1XW were not significantly higher than Cd concentration under the DWW condition.

Interestingly, there was a higher accumulation of Cd in spinach cultivated on biochar amended soils in comparison to the control soil, most especially in the 1XW and WDW regimes. A relationship between the ratio of dry and fresh biomass yield (g) of spinach could provide more insight. Table 4 reveals higher ratio of dry to fresh biomass yield (g) i.e., 0.20 ± 0.06 and 0.27 ± 0.03 for the treatments with 2% BC and 4% BC respectively in comparison with the control soil (0.16 ± 0.02). An increase in this ratio could be a result of water stressed conditions in the biochar amended pots. Similarly, Gonulal et al., (2021) demonstrated that an increase in ratio of dry to fresh biomass yield (g) was significantly higher in less irrigated treatments in comparison with treatments with 100% irrigation. Water stressed conditions in "decreasing" treatments could be responsible for an increase in ratio of dry to fresh biomass yield (g) of cultivated spinach (Tack, 2017). This therefore suggests an increase in water stress under the 1XW for the biochar treatments compared to the control soil. This could potentially promote more uptake of Cd from the biochar amended soils. Impact of biochar application on available water capacity is not always positive (Hardie et al., 2014).

Finally, it can be concluded that there was no significant effect ($P > 0.05$) of watering regime on the uptake of Cd from the control soil. However, variations in watering regimes had a significant impact ($P < 0.05$) on the uptake of Cd in biochar amended soils. In water stressed conditions (1XW and WDW), the uptake of Cd was higher in harvested spinach. Regular supply of water during cultivation is therefore expected to decrease Cd levels in spinach.

6 CONCLUSIONS

In conclusion, Cd concentration (mg/kg) in spinach cultivated on the control soil most times exceed permissible limits set by the European Commission (i.e., 0.2 mg/kg FW) because of the continuous increase in Cd concentration in the soil pore water, coupled with a high concentration of Cd in the exchangeable (~ 38%) and reducible fraction (~ 67%) of the soil, plus a rapid resupply of Cd from the solid phase into the soil solution. Despite being a soil having just baseline concentrations of Cd, planting of spinach (a plant with high affinity for Cd) on this soil will likely result in Cd concentrations above 0.2 mg/kg (FW). Addition of biochar (2% and 4%) as a soil amendment successfully reduced the rate of Cd release from the soil with time, coupled with a decrease in Cd resupply from the solid phase. Moreover, biochar amended soils were observed to have significantly lower ($P < 0.05$) concentration of Cd in the soil pore water and easily extractable fraction of the soil. However, the exchangeable and reducible fraction of Cd in the soil was not different from that of the control soil. Hence, the application of this particular biochar as a soil amendment to this soil will not alleviate the risk of exceeding Cd concentration in harvested spinach. Lastly, variation in watering regime had no significant impact on the uptake of Cd by spinach grown on the control soil. However, for 2% BC treatment, Cd concentration in spinach cultivated under WDW condition was significantly higher ($P < 0.05$) compared to those cultivated with other watering regimes, while for 4% BC treatment, Cd concentration in spinach cultivated under the 3XW and DWW conditions were significantly lower ($P < 0.05$) than those cultivated under the 1XW and WDW regimes.

7 RECOMMENDATIONS

The application of biochar as a soil amendment on this soil requires much more understanding in order to achieve lower Cd concentrations in cultivated spinach. Some recommendations to future studies include:

i) **Proper analysis of the rhizosphere**. As earlier stated, transfer of Cd from the soil by the roots to the above ground biomass is influenced by the pH and microbial life of the rhizosphere. Root exudates tend to alter the solubility of metals thereby increasing their bioavailability in the soil solution. Cd accumulation by the roots of spinach could therefore provide more insight into the decrease in total Cd content from the substrate.

ii) **Micronutrient management.** Cd and some nutrients such as Zn and Fe, have been reported to have an effect on one another when present in the soil. Therefore, the possibility of micronutrient management could decrease the uptake of Cd (while enhancing the uptake of nutrients e.g. Zn and Fe) by spinach. However, caution is to taken as regards this modification, as excessive application of nutrients such as Zn in the soil could result in contamination of the soil thereby deteriorating the biological and physicochemical properties of the soil.

iii) **Cultivation of different cultivars and/or species of spinach.** Due to the variations in plant genotype and physiology, different cultivars and specie of spinach tend to accumulate Cd uniquely. This could not only establish differences in the accumulation of Cd but could also provide a possible option of cultivar selection by the farmer.

iv) **Type of biochar used**. As reported by various studies, the extent to which biochar functions as a soil amendment, depends on the type of feedstock used in producing it. Currently, manure-derived biochar, most especially that which is produced from cow manure, has been reported to yield great decrease in concentrations of Cd measured in cultivated vegetables. Therefore, this option could be made use of in subsequent research

v) **Duration of research.** Insignificant decrease in concentrations of Cd measured in spinach grown on biochar amended soils could also be attributed to the time allocated for the experiment. Ageing, plays a key role in determining the impact of biochar addition in reducing the uptake of metals from cultivated soils. It is therefore expected that an increase in cultivation time could cause significant differences in uptake of Cd from the soil.

vi) **Field versus greenhouse cultivation.** Cultivation could also be carried out on the field so as to compare both planting conditions on the uptake of Cd by spinach. However, major environmental conditions such as rainfall and sunshine should be duly monitored so as not to disrupt the experimental set up.

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APPENDICES

APPENDIX 1

Spinach plants after 7 weeks of planting (which includes 3 weeks of treatment)

CONTROL SOIL

3XW

1XW

DWW

WDW

2% BC TREATMENT

3XW

1XW

DWW

WDW

4% BC TREATMENT

3XW

1XW

DWW

WDW

3XW - three-times watering per week. **1XW** – one-time watering per week. **DWW** – dry-wet watering i.e., one-time watering for the first three weeks then three-times watering for the last three weeks. **WDW** – wet-dry watering i.e., three-times watering for the first three weeks then one-time watering for the last three weeks. **BC -** biochar

APPENDIX 2

Spinach plants at harvest (10 weeks of cultivation, which includes 6 weeks of treatment)

CONTROL SOIL

3XW

1XW

DWW

WDW

2% BC TREATMENT

3XW

1XW

DWW

WDW

4% BC TREATMENT

3XW

1XW

DWW

WDW

3XW - three-times watering per week. **1XW** – one-time watering per week. **DWW** – dry-wet watering i.e., one-time watering for the first three weeks then three-times watering for the last three weeks. **WDW** – wet-dry watering i.e., three-times watering for the first three weeks then one-time watering for the last three weeks. BC - biochar

APPENDIX 3

3.1. Concentration of nutrients, mg/100 g, dry weight (DW) basis in spinach leaves

mg/100g (DW)					
Ca	Mg	K	Zn	Fe	P
1151.16	918.61	6488.37	6.26	31.51	567.78

3.2. Concentration of nutrients measured in above ground biomass

3XW	Nutrients	Control soil (mg/100 g, DW)	Soil with 2% BC (mg/100 g, DW)	Soil with 4% BC (mg/100 g, DW)
	Ca	1519.89	1515.01	1618.92
	Mg	859.55	697.48	684.31
	K	2869.1	3656.7	4428.3
	Zn	10.32	9.62	6.92
	Fe	44.34	28.03	23.01
1XW				
	Ca	1890.82	1686.57	1491.54
	Mg	1167.9	913.29	694.64
	K	3070.9	4063.9	4090.5
	Zn	11.51	13.01	11.54
	Fe	42.61	36.02	30.14
DWW				
	Ca	1743.21	1279.1	1573.91
	Mg	1034.1	706.87	764.25
	K	2983	3124.4	5330.5
	Zn	45.3	10.71	15.82
	Fe	52.87	20.88	22.7
WDW				
	Ca	1842.58	1804.09	1549.82
	Mg	1095.6	838.8	613.35
	K	2886.4	3745.9	3441.5
	Zn	41.527	11.15	10.62
	Fe	72.24	28.66	22.77

APPENDIX 4

Watering pattern implemented during the experiment. Week 1 to week 4 represents the period before application of the different watering regimes. Week 5 to week 10 represents the period when the watering regimes was implemented.

WATERING PATTERN

	March 2022																	
	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Treatments	**Week 1**							Week 2							Week 3			
3x per week	🟦			🟦				🟦			🟦				🟦			🟦
1x per week	🟨			🟨				🟨			🟨				🟨			🟨
Dry-Wet	🟩			🟩				🟩			🟩				🟩			🟩
Wet-Dry	🟪			🟪				🟪			🟪				🟪			🟪

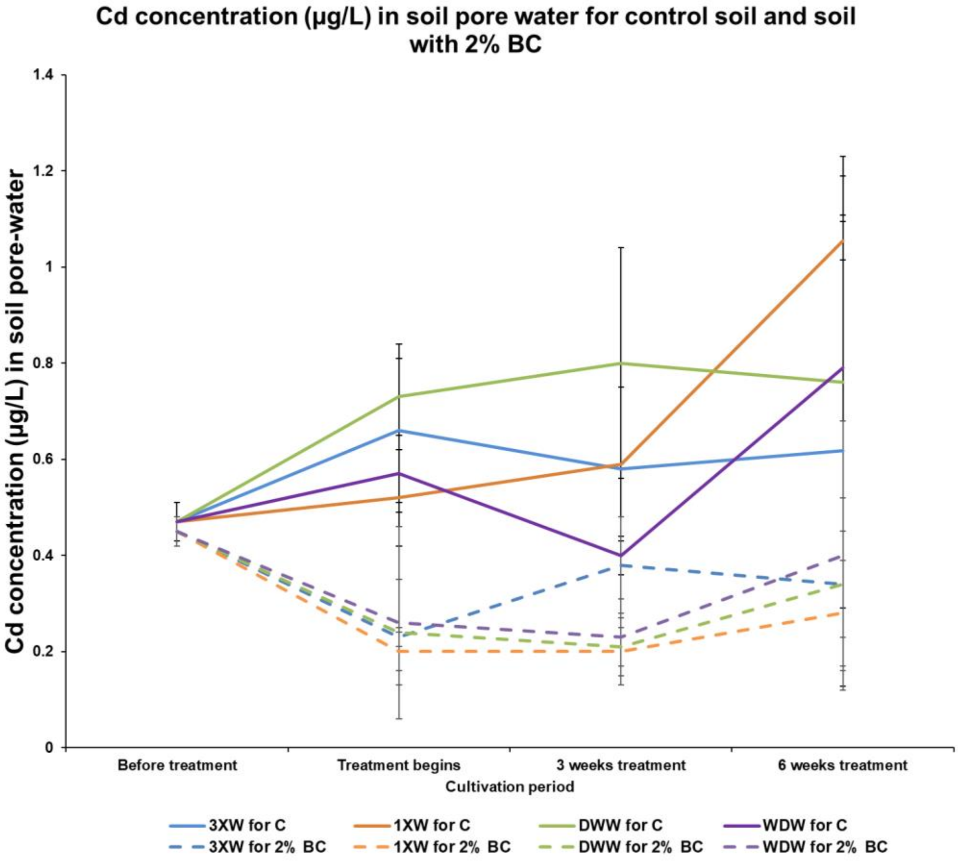
	April 2022																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Treatments				Week 4							Week 5							Week 6							Week 7					
3x per week				🟦			🟦				🟦		🟦		🟦			🟦		🟦		🟦			🟦		🟦		🟦	
1x per week				🟨			🟨				🟨							🟨							🟨					
Dry-Wet				🟩			🟩				🟩							🟩							🟩					
Wet-Dry				🟪			🟪				🟪		🟪		🟪			🟪		🟪		🟪			🟪		🟪		🟪	

	May 2022																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Treatments		Week 8							Week 9							Week 10				
3x per week		🟦		🟦		🟦			🟦		🟦		🟦			🟦		🟦		
1x per week		🟨							🟨							🟨				
Dry-Wet		🟩		🟩		🟩			🟩		🟩		🟩			🟩		🟩		
Wet-Dry		🟪							🟪							🟪				

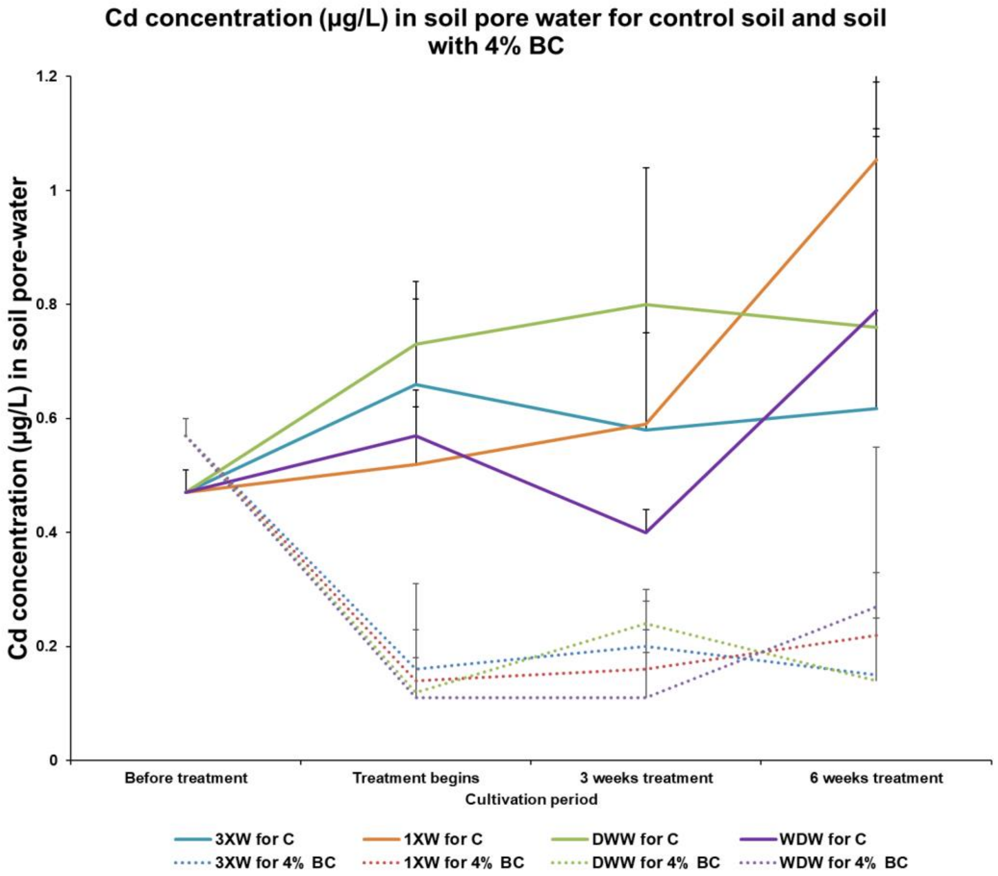
APPENDIX 5

Change in pH of soil pore-water after 3 weeks and 6 weeks of treatment. Means and standard deviation are shown (n = 4).

	Watering regime	3 weeks treatment	6 weeks treatment
Control soil	3XW	6.75 ± 0.04	5.99 ± 0.01
	1XW	6.81 ± 0.01	-
	DWW	6.69 ± 0.03	7.05 ± 0.04
	WDW	6.91 ± 0.04	6.40 ± 0.03
Soil with 2% BC	3XW	7.52 ± 0.02	6.92 ± 0.04
	1XW	7.29 ± 0.02	7.00 ± 0.03
	DWW	7.19 ± 0.04	6.89 ± 0.05
	WDW	7.09 ± 0.01	7.06 ± 0.01
Soil with 4% BC	3XW	7.42 ± 0.04	7.21 ± 0.03
	1XW	7.25 ± 0.01	7.15 ± 0.04
	DWW	7.59 ± 0.04	7.31 ± 0.03
	WDW	7.53 ± 0.02	7.03 ± 0.04



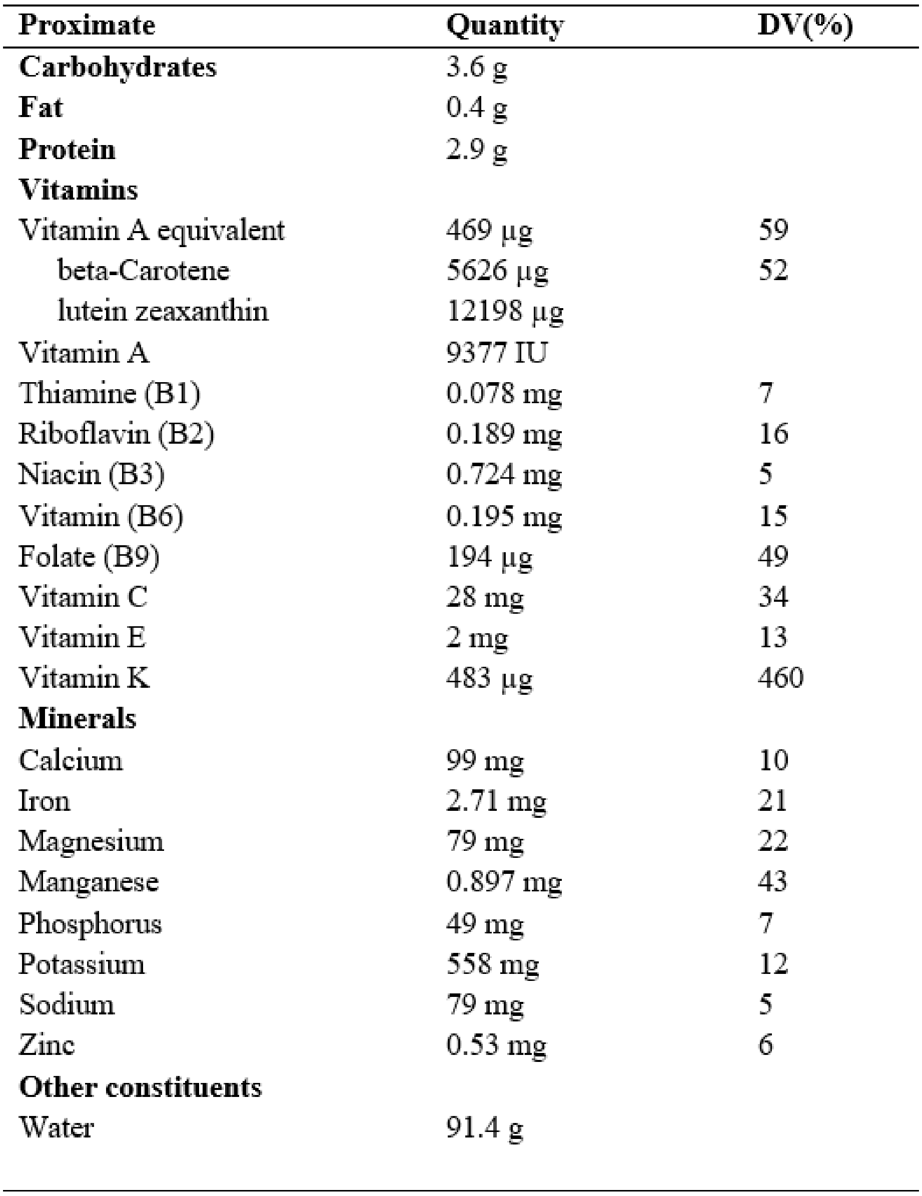
Error bars represent standard deviation. Average concentrations are shown (n = 4)



Error bars represent standard deviation. Average concentrations are shown (n = 4)

APPENDIX 6

Nutritional value per 100 g of raw Spinach (*S. oleracea*) (FW). Source: USDA FoodData Central



Proximate	Quantity	DV(%)
Carbohydrates	3.6 g	
Fat	0.4 g	
Protein	2.9 g	
Vitamins		
Vitamin A equivalent	469 µg	59
beta-Carotene	5626 µg	52
lutein zeaxanthin	12198 µg	
Vitamin A	9377 IU	
Thiamine (B1)	0.078 mg	7
Riboflavin (B2)	0.189 mg	16
Niacin (B3)	0.724 mg	5
Vitamin (B6)	0.195 mg	15
Folate (B9)	194 µg	49
Vitamin C	28 mg	34
Vitamin E	2 mg	13
Vitamin K	483 µg	460
Minerals		
Calcium	99 mg	10
Iron	2.71 mg	21
Magnesium	79 mg	22
Manganese	0.897 mg	43
Phosphorus	49 mg	7
Potassium	558 mg	12
Sodium	79 mg	5
Zinc	0.53 mg	6
Other constituents		
Water	91.4 g	

DV % = percentage of dietary value