HUMIC SUBSTANCES AS SOIL AMENDMENTS IN COAL MINE RECLAMATION

by

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ABSTRACT

Coal is mined for energy generation around the world, resulting in extensive disturbances to soil health and vegetation, and producing large amounts of waste. Heavy metals enter the environment through coal industry activities before being transmitted to the food chain. Reclaiming post mining sites covered by sandy soils with low nutrient and high heavy metal concentrations for agricultural uses is very challenging. Using coal waste derived humic substances as soil amendments may enhance soil reclamation outcomes due to their great potential as a soil conditioner, plant growth biostimulator, and heavy metal adsorbent.

This research was undertaken in three greenhouse and laboratory experiments at the University of Alberta that ran for three months each, and two field experiments on a former underground coal mine (Shendong mining area) in China that ran for two years. The overall objective was to assess potential of a coal waste derived humic substance product called nano humus as a soil amendment for mined sandy soils in combination with other materials.

Direct application of nano humus at the beginning of each growing season at 150 g/m^2 was a suitable reclamation strategy. The beneficial effect of nano humus was expressed in year two in the field. As a soil conditioner, nano humus positively changed most soil variables, particularly soil cation exchange capacity (38 %), total organic carbon (49 %), and available nitrogen, phosphorus, and potassium (29 to 64 %). As a plant growth biostimulator, nano humus significantly enhanced the total biomass of alfalfa (*Medicago ruthenica* L.) by 749 %, barley (*Hordeum vulgare* L.) by 250 %, and sea buckthorn (*Fructus Hippophae* L.) by 147 %. As a heavy metal adsorbent, nano humus removed 89 % of cadmium from contaminated water after 15 minutes and 93 % after 24 hours at a high metal concentration of 100 mg/L; it reduced 25 % of thallium, 18 % of cadmium, and 3 % of arsenic from contaminated soils after two years. Beneficial effects were more pronounced with combined fertilizer and arbuscular mycorrhizal fungi than with sole applications of each.

Our findings confirmed the great application potential of a lignite derived humic substance as a plant growth stimulator, soil conditioner, and heavy metal adsorbent in coal mine reclamation and remediation. The pronounced performance of combined applications with arbuscular mycorrhizal fungi and fertilizer provided insights for future reclamation strategies. Our research has global implications as it can be applied in other sandy soil regions facing similar reclamation challenges in the world where industrial released heavy metals pose a great risk to the environment and public health substantially.

PREFACE

This dissertation is an original work by Yihan Zhao. All experiments and data analyses were implemented by Yihan Zhao under the supervision of Dr. M. Anne Naeth.

Chapter II has been submitted to Plos One, as Y. Zhao and M.A. Naeth, “Lignite derived humic substances and inorganic fertilizer are effective soil amendments for barley in sandy soil.” Y. Zhao conducted experiments, data analyses, and wrote the original draft. M.A. Naeth administrated the project and resources, supervised, and edited and reviewed drafts.

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Chapter V has been published as “Y. Zhao and M.A. Naeth. 2022. Lignite Derived Humic Products And Cattle Manure Biochar Are Effective Soil Amendments In Cadmium Contaminated And Uncontaminated Soils. Environmental Advances, 8, 100186. Doi: 10.1016/j.envadv.2022.100186.” under a Creative Commons Attribution License (CC BY 4.0). Y. Zhao conducted experiments, data analyses, and wrote the original draft. M.A. Naeth administrated the project and resources, supervised, and edited drafts.

Chapter VI has been submitted to Chemosphere, “Cd(II) And Zn(II) Adsorption By Three Carbon Based Materials For Remediation Of Contaminated Water.” Y. Zhao conducted experiments, data analyses, and wrote the original draft. M.A. Naeth administrated the project and resources, supervised, and edited drafts.

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I. Background

1. Introduction

Coal has dominated the world's energy mix for centuries due to its relatively abundant reserves, wide geographical distribution, and high affordability (World Coal Institute, 2005). It is mined on most continents, in over 100 countries (Skipka & Theodore, 2014). Currently, it is the second largest energy source in the world, providing nearly 40 % of the world's electricity production (World Energy Council, 2013). World coal production was 7.3 billion tonnes at the end of 2017, 43 % in China, 10 % in India, 10 % in the United States, 7 % in Australia, and 1 % in Canada (Natural Resources Canada, 2019). Based on current global production rates, coal reserves are expected to last for at least 115 years, much longer than oil and gas reserves (World Energy Council, 2013).

Considerable coal mining activity has begun and continued in recent decades around the world; however, an increasing number of coal mines have been closed in recent years, and many others have been historically abandoned. More coal mines are expected to close due to exhaustion of mineral resources, and due to some countries and jurisdictions working towards fossil fuel reduction and/or elimination. Coal extraction produces large amounts of waste and extensive disturbance to the environment (Bian et al., 2010; Kuter et al., 2014; Huang et al., 2015). The total mined area in the world is approximately 57,277 km^2 (Maus et al., 2020), leaving a tremendous challenge for reclamation.

Effective reclamation practices are essential to meet regulatory requirements and to ensure much needed land is available for further human use after mine closure. Reclamation of coal mines requires providing and/or building a suitable soil, remediating any contaminants, and revegetating. Organic amendments are often needed in soil building and soil reclamation, as there is often not enough suitable soil salvaged to fill the mined areas, particularly in older mined areas where soil salvage was not required by legislation. Although the use of coal waste derived materials as soil amendments have been introduced as a low cost biological reclamation technique, if, how, and why they work is not well understood. The research presented in this thesis investigated the appropriate land reclamation implementation and biological impacts of a coal waste derived humic product called nano humus, in combination with a beneficial mycorrhizal population and inorganic fertilizer. Nano humus was compared directly to a more common agriculture waste derived biochar in heavy metal remediation of water and soils.

2. Literature Review

2.1 Coal mining

Coal is an organic rich, combustible sedimentary rock (World Coal Institute, 2005), regarded as a fossil fuel, which is a later stage of peat formed over long time periods through compaction, pressure, and elevated temperature influences (Taylor et al., 1998). The coalification process can take millions of years at depths of up to seven kilometres. Coals are ranked depending on their physical and chemical properties. Low ranked coals are typically moist, soft, and friable, with low carbon and energy content. High ranked coals are characterized by a black vitreous lustre, and are dry, hard, and strong, with high carbon and energy content (World Coal Institute, 2005).

Coal is mined for energy generation around the world. Coal mining is the process used to obtain coal from the ground. The mining method, either under ground mining or surface mining, is greatly influenced by coal deposit geology. Under ground mining accounts for approximately 60 % of the global coal production; 95 % of coal production is from under ground mining in China (Bian et al., 2010). The majority of coal in Canada is produced from surface mining, with few under ground mines in operation (World Energy Council, 2013). Under ground mining has two common methods, longwall and room and pillar mining; surface mining methods are either strip mining or open pit mining depending on the depth and thickness of the coal seam from the surface (World Coal Institute, 2005). The lifetime of a mining project varies from a few years to hundreds of years. Final mine closure and decommissioning normally take less than five years. Post closure management might take longer, from a decade to perpetuity. This is the final step to maintain, monitor, and assess effectiveness of ongoing reclamation.

2.2 Environmental impacts of coal mining

Despite coal mining contributing significantly to global energy generation and the global economy, it poses considerable environmental concerns. Both surface and under ground coal mining produce large amounts of waste and extensive disturbances to the environment. Surface subsidence, soil loss and degradation, vegetation removal and destruction, heavy metal contamination of soil and ground water, loss of biodiversity, hydrologic disruptions to surface and ground water, and greenhouse gas emissions are considered the main environmental consequences of mining activities (Bian et al., 2010; Kuter et al., 2014; Huang et al., 2015).

Subsidence is sinking or shifting of the ground surface after mine collapse over time (Dontala et al., 2015). Surface subsidence from under ground mining is a common problem around the world, with an estimated 0.2 hectares of subsiding land occurring with every ten thousand tonnes of coal mined (Bian et al., 2010). Surface coal mining typically requires the temporary use of a large area, and the land disturbance might be extensive (World Coal Institute, 2005). These land disturbances can amplify the damage on soil, vegetation, and water in the mining area

Coal mining can cause structural and functional changes in mined soils, such as increased soil compaction, decreased soil water holding capacity, reduced fertility, altered soil structure and nutrient distribution, loss of soil microbial diversity, and toxicity (Tripathi et al., 2009; Dontala et al., 2015; Feng et al., 2019; Guo et al., 2020). The mining process damages or eliminates above ground vegetation, can lead to reduction of vegetation cover, limitation of vegetation growth, and loss of biomass (Huang et al., 2015). The impact on hydrology is mostly caused by lowering the ground water table, changing the flow of ground water and streams, causing water loss or contamination, and modifying watersheds (Bian et al., 2010; Dontala et al., 2015).

Coal mining is one of the major sources through which heavy metals enter the environment before being transmitted to the food chain (Musilova et al., 2016; Sidhu et al., 2019). Heavy metals exist in coal refuse and fly ash, and they can be released into the soil as a result of coal industry activities, such as production and accumulation of coal gangue, migration and sedimentation of wastewater, and coal transportation (Li et al., 2018). Both surface and under ground coal mining can generate acid mine drainage which can dissolve toxic metals and move into surface and ground waters, causing contamination for decades or even hundreds of years after mine closure (Ahemd & Tahlawi, 2011; Omwene et al., 2018). Excessive release of heavy metals may pose great environmental and public health risks. Heavy metals, unlike organic pollutants, are not biodegradable and can accumulate in living organisms (Fu & Wang, 2011). Due to their mobility and toxicity, heavy metals have been considered priority pollutants in many parts of the world (Volesky & Holan, 1995; Qaiser et al., 2007).

The emission of greenhouse gases from coal mining operations is of environmental concern. Carbon monoxide (CO), carbon dioxide (CO_2), nitrous oxides (NO_X), and sulfur oxides (SO_X) can be released into the air due to spontaneous coal combustion and methane leaking (Bian et al., 2010). Methane with a 21 times stronger greenhouse effect than carbon dioxide, is discharged into the air during under ground coal mine activities, with emissions predicted to rise by 20 % in the next decade (Dontala et al., 2015).

2.3 Coal mine reclamation and technical challenges

Reclamation has increased in conceptual complexity over time, requiring greater effort for sustainability and commonly requiring socially, economically, and environmentally acceptable techniques. Mine reclamation has been an important part of sustainable development strategies in many countries. Reclamation is defined slightly differently in different regions of the world. Generally, it means the process of converting disturbed land to its former or other productive uses. Remediation, which is a part of reclamation, is the process of removing, reducing, or neutralizing substances, wastes, and hazardous materials on contaminated sites. The obligation of reclamation and remediation is to achieve equivalent capability with stable, non-erodible, favourably drained soil conditions and to prevent or minimize negative environmental effects. End land uses of closed mining sites are based on the local needs of the area or country.

With increasing population pressure on global land resources, extensive areas of post mining lands are reclaimed for agriculture upon closure (Palogos et al., 2017). Although sandy soils are among the most extensive soils throughout the world, covering a 900 million ha(Driessen et al., 2001), they have received limited research attention. In northern China in the transitional zone between Mu Us desert and the Loess Plateau, the majority of the post coal mining lands are covered by sandy soils with low organic matter and nutrient concentrations. Sandy soils with low organic carbon and low nutrient retention pose a challenge for vegetation establishment. Although inorganic fertilizers can provide essential mineral elements to obtain better growth, they easily leach in sandy soils. Various physical, chemical, and biological methods have been suggested around the world, such as addition of topsoil, mulches, superabsorbents, organic amendments, mycorrhizal fungi, and biosolids (Maiti, 2012; Kumar et al., 2018; Pinto et al., 2018; Cozzolino et al., 2021). However, our knowledge of their application and success in reclamation in mined sandy soils is limited.

Heavy metal remediation from aqueous solutions is usually achieved by employing technologies such as chemical precipitation, ion exchange, membrane filtration, and electrochemical (Masindi & Muedi, 2018). However, these conventional methods of treating heavy metals have great limitations in efficiency and cost (Nguyen et al., 2013). For example, chemical precipitation requires large quantities of chemicals to reduce metals, generating hazardous sludge that requires further treatment (Barakat, 2011). To reduce heavy metal bioavailability in soils, conventional remediation technologies have been used, such as soil excavation, thermal desorption, electrokinetics, and soil washing with chemicals. Soil excavation just transports contaminants elsewhere (Sidhu, 2016). Thermal desorption, conducted by heating contaminated soil to volatilize metals, is very expensive, labour intensive, and limited to small areas (Awa & Hadibarata, 2020). Electrokinetics, which involves application of high voltage to contaminated soil to remove metals is time consuming with low efficiency (Dhaliwal et al., 2020). Soil washing with water, chemicals, and other fluids, is very expensive and environmentally disruptive (Li et al., 2019). High price, low efficiency, and large environmental disturbances are key concerns hindering widespread application of these techniques in coal mine reclamation.

2.4 Humic substances

Low cost materials derived from agricultural waste, industrial by-products, natural materials, and/or modified biopolymers have been suggested for use in land reclamation, as they are usually readily available, cost effective and can help reduce waste accumulation. Humic substances showed great potential in land reclamation, particularly contaminant remediation due to many advantages, including low cost, high efficiency, easy operation, and high availability.

Humic substances are the most stable component of soil organic matter and are characterized as complex and supramolecular self-assembled mixtures of diverse heterogeneous hydrophobic and hydrophilic compounds (Piccolo, 2002). They are formed through aerobic and anaerobic decomposition of (mostly) plant detritus and secondary synthesis reactions (Thorn et al., 1989). Humic substances as by-products of microbial metabolism are typically the oldest and major component of soil organic matter, at approximately 60 % by weight (Stevenson, 1994). Humic acid (alkali soluble), fulvic acid (alkali and acid soluble), and humin (insoluble in alkali and acid) are three fractions of humic substances that are based on solubility in alkali or acid (Meng et al., 2017). They could be employed as soil conditioners to improve soil fertility by modifying soil physiochemical environments (Kalbitz et al., 2000; Bronick & Lal, 2005; Bezuglova et al., 2017), as natural biostimulators to enhance plant growth (Chen et al., 2004; Tahir et al., 2011; Ciarkowska et al., 2017), and by using them as heavy metal remediators to reduce metal bioavailability (Havelcová et al., 2009).

Researchers found humic materials can improve soil properties. They could improve soil quality by affecting soil structure and porosity through an effect on particle aggregation (Bronick & Lal, 2005). Since they are the main source of available organic carbon, they can shape soil biotic communities (Kalbitz et al., 2000), and thus determine soil health and fertility. Ibrahim & Goh (2004) assessed the utilization potential of humic substances as a soil amendment for reclamation and remediation at a former gold mine in Manitoba Canada where the soil is highly acidic and contaminated with copper. They found humic substance amendment increased soil pH from 3.5 to 5, increased cation exchange capacity and soil organic carbon, and promoted macro aggregate formation in mine tailings. Improvement of soil physical, chemical, and biological properties was most obvious in poor soils. A three year study reported that a lignite derived humic substance product improved soil properties in sand and medium textured soils and the increment was more obvious in the sand textured soils (Ciarkowska et al., 2017). Bezuglova et al. (2017) found humic substances led to higher availability of soil mobile phosphorus and numbers of microorganisms through plant root exudation.

The overall mechanism of humic substances on plant growth is not well understood. It is clear that the effects of humic substances on plant growth are dynamic, non-linear, cross interrelated processes that make it complex and require interdisciplinary investigations (Canellas & Olivares, 2014). Some researchers suggest that humic substances may have a direct influence on promoting plant growth by affecting the uptake and transport of humic substances associated with physiological processes in plant tissues, such as cell membrane permeability, oxygen uptake, respiration, photosynthesis, hormonal activity, and root cell division in elongation zone (Cacco & Dell'Agnola, 1984; Chen & Aviad, 1990; Nardi et al., 2002; Türkmen et al., 2004). Others suggest humic substances may indirectly influence plant growth by affecting microbial growth, organic matter mineralization and solubilisation, and availability of micro elements (iron, zinc, manganese) and some macro elements (potassium, calcium, phosphorus) (Chen & Aviad, 1990; Ayuso et al., 1996; Sharif et al., 2002). These mechanisms may be interconnected.

The stimulating effects of humic substances on plant growth have been reported in numerous publications. Ciarkowska et al. (2017) found that plant biomass was promoted. Humic substances tripled root biomass and quadrupled shoot biomass in plants growing in sand textured soils and increased biomass by 2.5 times in plants growing in medium textured soils relative to plants growing in a control. Tattini et al. (1990, 1991) reported that humic substances increased the root to shoot ratio of olive plants, indicating that overall plant health was promoted. Rengrudkij & Partida (2003) observed positive effects of humic substances on avocado tree root development. Tahir et al. (2011) found humic substances increased wheat by 10 % of plant height and 18 % of shoot biomass.

Although the mechanisms are not yet completely understood, it is widely reported that humic substances serve as exogenous auxins directly influencing plant physiology, particularly primary and lateral root elongation and root hair formation (Casimiro et al., 2003; Saini et al., 2013; Nardi et al., 2021). Many researchers hypothesized that the occurrence of auxin or auxin like components in humic substance structures exerts positive effects on root growth (Nardi et al., 2002). Active indole-acetic acid (IAA) concentrations have been identified in humic substance structures by different methods. Pizzeghello et al., (2001, 2002) used immunoassays and found indole acetic acid in humic substances from different extraction origins. Trevisan et al. (2010) identified indole acetic acid using genetic and molecular biology techniques. Some researchers suggested humic substances may trigger plants to produce auxin (Jeon et al., 2018; Shah et al., 2018; Nardi et al., 2021, 2022). The mechanism by which auxin or auxin like activities are induced by humic substances is still being studied; very little is known and needs further investigation.

Nevertheless, auxins have been confirmed as a main control signal to lateral root formation (Casimiro et al., 2003). Trevisan et al. (2010) used specific inhibitors of auxin transport (or action) and proved humic substances associated auxin activity was a main factor that promoted plant lateral root formation since they observed activation of an auxin synthetic reporter DR5::GUS and enhancement of transcription of IAA19. Zandonadi et al. (2010) found an inductive effect of humic substances on specific auxin action molecular targets. They found that humic substances induced root development stimulation and root plasma membrane H^+-ATPase synthesis activity was elicited by expression of the early auxin responsive gene IAA5 and IAA19. Hager (2003) reviewed the mechanism of humic substance induced auxin activity on lateral development. The final target of auxin activity, activation of H^+ pumping ATPase at the plasma membrane, could enhance H^+ ions in cell wall compartments. Increased H^+ pumping decreases cell wall pH and activates pH sensitive enzymes and proteins in the cell wall, loosening cell walls and triggering cell elongation. Schmidt et al. (2007) found a significant increase in root hair length, root density, and cell proliferation in root tissue following humic substance treatments, leading to an increased absorptive root surface area. Mora et al. (2012) found humic substances induced incensement in secondary root numbers, root thickness, and root biomass.

Many studies have shown that enhancement of H^+-ATPase in plant roots may promote shoot development due to the nitrate (NO_3^-) root to shoot distribution which can serve as a pseudo hormonal signal which eventually promotes shoot growth. Humic substances, especially low molecular fractions, could elicit auxin like activity and support nitrate uptake by decreasing pH at the surface of roots, thus facilitating H^+/NO_3^- transport (Muscolo et al., 1999). This is because low molecular weight humic substances may have flexible conformational structure and could be associated with more efficient bioactive humic component diffusion to cells of plant roots (Nardi et al., 2002, 2007). The change of NO_3^- may result in the root to shoot distribution of certain active cytokinins and polyamines (principally putrescine) concentrations in plant shoots.

Humic substances are considered suitable amendments for heavy metal remediation in soils, waters, and other systems (Klučáková & Pavlíková, 2017). For example, Pusz (2007) demonstrated the positive potential of using humic substances (raw lignite, 70 % humic acids) as an amendment in copper smelter reclamation to rebuild soils and decrease the plant available concentrations of heavy metals. This ability in heavy metal remediation can be attributed to the high content of oxygen containing functional groups. The amphiphilic, heterogeneous character of humic substances result in complex reactions between humic substances and metal ions (Klučáková & Pavlíková, 2017). Humic substances can bind to heavy metal ions effectively in different ways from a purely electrostatic, nonspecific interaction of metal cations with a net negative charge on the surface of a humic particle, to specific interactions in formation of complexes and chelates with functional groups (Havelcová et al., 2009; Chen et al., 2011; Xu et al., 2013; Meng et al., 2017). The binding strength of heavy metals to humic substances may depend on metal types (Shaker & albishri, 2014). Klučáková & Pavlíková (2017) evaluated heavy metal ion (Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}) adsorption efficiency of lignite derived humic substances and found a very high adsorption efficiency, from 80 to 100 %.

Humic products are commercially available in many countries, such as Canada (Black Earth®), China (Nano Humus®), Germany (Novihum®), and Poland (Rekulter®). The majority of commercial humic products are derived from leonardite or lignite wastes of mining process (Chen et al., 2004) that have relatively high humic contents (Taylor et al., 1998). Whether these products are functionally the same is questionable due to differences in particle size, extraction source, and chemical composition. Efforts have been made to investigate whether biological effects of humic substances from different extraction sources, chemical composition, and molecular weight are identical. Muscolo et al. (2013) compared results of eleven studies on seed germination, plant growth, and action mechanisms of humic substances from different origins with different plant species. The findings showed that different origins of humic substances may have similar effects when plant materials and experimental conditions were not very different. However, Rose et al. (2014) pointed out that the extraction source has a strong influence on efficacy of humic materials on plant growth after conducting a meta-analysis of 94 published papers.

The majority of humic studies were conducted in greenhouses or growth chambers or cells with controlled conditions, thus the field efficacy of humic materials remains uncertain. Although the positive effects of humic materials have been widely reported by greenhouse studies, some of the recent field studies reported opposite findings. Hartz & Bottoms (2010) highlighted humic materials are ineffective in enhancing plant nutrient uptake, biomass, and yield in silt loam soils in the field. Another field study by Mahoney et al. (2017) also reported insignificant promoting effects on plant height, seed production, and yield in silt loam and clay loam textured soils with relatively high soil organic matter (> 3 %). This published evidence mentioned the potential ineffectiveness of humic materials in nutrient rich soils, and it is questionable whether humic materials can work in nutrient poor sandy soils under field conditions.

2.5 Humic substances in combination with arbuscular mycorrhizal fungi

The combined use of humic substances and soil biota has generated great research interest. The use of microbial inoculants in combination with humic substances leads to improved plant development and yield (Canellas et al., 2013; da Piedade Melo et al., 2017; da Silva et al., 2017). Rhizosphere microbial activity is a principal factor determining availability of nutrients to plants and significantly influences plant health and productivity (Jeffries et al., 2003). Mycorrhizae are normally the major organisms for soil-plant interactions and can enhance water and nutrient acquisition (Smith & Read, 2008). In undisturbed natural environments, most plant species can build a beneficial association with mycorrhizal fungi, resulting in a fungus-root structure called mycorrhiza which originated 460 million years ago (Fulton, 2011). Arbuscular mycorrhizal fungi (AMF) are common soil microorganisms that build mutualism symbiotic relationships with the roots of most plant species, facilitating uptake of mineral nutrients from the soil in exchange for carbohydrates, and thus become an important plant root component (Smith & Read, 2008; Brundrett, 2009). They are estimated to form mycorrhizal symbioses with 74 % of plant species (van der Heijden et al., 2015) and can be found in almost all habitats and climates.

The significant ecological presence of AMF can be attributed to special coiled hyphal structures which can enhance micronutrient acquisition and plant water relations by increasing nutrient and water exchange surface area (Fulton, 2011). AMF form arbuscules and highly branched hyphal structures inside cortical root cells after penetrating into and between the outer cells of roots and develop an extensive network of filaments (extraradical hyphae) that grow in surrounding soil. The filament network of AMF could potentially serve as a biological barrier against heavy metal movement to plant shoots. AMF was considered more efficient than other fungi in heavy metal sequestration. Joner et al. (2000) found *Glomus mosseae* (the largest genus of AMF) was over ten times more efficient in heavy metal adsorption than *Rhizopus arrhizus* (non-tolerant fungi). Investigations on its effects in heavy metal contaminated soils are not abundant and have inconsistent results (Khan, 2001; Vogel-Mikuš et al., 2006).

AMF could improve soil properties by increasing aggregate stability (Rillig, 2004; Cozzolino et al., 2013) and plant-water relations with the help of the AMF filament network which enables plants to search water and nutrients deeper and wider in the soil profile (Toro et al., 1998; Wang et al., 2009; Treseder, 2013). AMF can interact with phosphate solubilizing bacteria and contribute to nutrient cycling and plant nutrition (Toro et al., 1998). This was evidenced by Wang et al. (2009) who found available nitrogen, phosphorus, and potassium adsorption by plants increased 5.3, 18.7, and 3.2 %, respectively, after inoculation with AMF. This is particularly important for acquisition of immobile nutrients that do not readily move through the soil, such as inorganic phosphate (Augé, 2001; Whipps, 2004; Wu, 2011). In a coal mine reclamation study inoculation with AMF increased plant survival 15 %, plant cover 9 %, and total root length 90 % within 13 months relative to non-mycorrhizal controls (Bi et al., 2007).

AMF inoculation method was assumed to affect effectiveness of inocula on revegetation. The methods of introducing AMF propagules (inoculum) into soils are targeted and non-targeted (Berruti et al., 2016). Targeted methods include inoculating pot soils with AMF, adding inocula to holes or furrows when transplanting or seeding, and application of seed coating products. Non-targeted methods include broadcast (scattering and mixing inocula with soil), hydroseeding, and irrigating a mixture of inocula and water. Emam (2016) assessed both targeted and non-targeted methods (pre-inoculation of transplants versus broadcast) in a greenhouse and at a grassland mine reclamation site. Results showed that both application methods improved plant growth; however, the targeted method (pre-inoculation) had more impact on increasing the biomass of native plants. AMF inoculation in field studies commonly uses targeted methods by placing inocula in planting holes or ditches before seeding or transplanting (Bi et al., 2007; Du et al., 2008). Greenhouse studies are commonly conducted by mixing inocula in potted soils when seeding (Wang et al., 2009; Caser et al., 2019).

Humic substances are important to development of soil biota including AMF (Gryndler et al., 2005). Research has shown humic substances enhance plant root biological activity (watercress), root cell proliferation, root length elongation (maize), and root branching (rockcress) (Piccolo et al., 1992; Canellas et al., 2002; Casimiro et al., 2003), which may increase contact between plants and AMF hyphae (Gryndler et al., 2009). This increased contact may stimulate mycorrhizal colonization and enhance plant growth. However, studies on the synergistic effects of humic substances and AMF on plant growth and soil properties are very limited. Most AMF studies were conducted in greenhouses (65 %) and growth chambers (4 %), with only 24 % in the field (Berruti et al., 2016). Field environments are much more complex than that of controlled indoor environments. How plants will respond to the combination under field conditions is not known.

2.6 Humic substances in combination with fertilizer

The global demand for fertilizer has increased dramatically, with the estimated demand for nutrients (nitrogen, phosphorus, potassium) for fertilizer use at 185 million tonnes in 2016 and increasing to 198 million tonnes in 2021 (FAO, 2019). Since sandy soils often have low nutrient contents, chemical fertilizers have been widely used to provide essential mineral elements to obtain maximum crop yield. One of the most important challenges with traditional fertilizer is their low nutrient utilization efficiency for nitrogen, phosphorus, potassium, and micronutrients typically lower than 58 %, 31 %, 51 %, and 10 %, respectively (Pathak et al., 2003). Soils with low organic matter and cation exchange capacity have low capacity to hold nutrients provided by inorganic fertilizer, which means nutrients will leach deep into the soil and be lost, resulting in limited benefit to plant growth (Reid & Naeth, 2005a, 2005b). Excessive chemical fertilizers will be wasted, adversely influencing soil physical, chemical, and biological properties and contaminating ground water (Savci, 2012; Khan et al., 2018).

Sandy soils are particularly prone to nutrient losses due to their high infiltration capacity, low water holding capacity, and low nutrient retention capacity (Von Uexkull, 1986). To amend sandy soils, a combination of humic substances with inorganic fertilizer were suggested (Sharif et al., 2002; Turgay et al., 2011; Suman et al., 2017). Their combination tended to work as nutrient carriers, supplying nutrients in both rapidly available forms and slow released complexed forms. Humic substances could work as a nutrient release controller by improving soil structure and cation exchange capacity. This is particularly important for sandy soils where nutrients are easily leached over time. Their combined effect in sandy soils remains unknown although the combination has generated great research interest.

3. Knowledge Gaps

Most humic research was conducted in either greenhouses or growth chambers or cells with controlled conditions, with only a few studies conducted under field conditions. Knowledge of their efficacy in field applications is scarce and remains uncertain, which have questioned the economic viability of applying humic products for reclamation. The inconsistent results can be attributed to different experimental conditions, soil conditions, application rates, and application methods (Jindo et al., 2020). Chen et al. (2004) demonstrated that a typical application rate (2-3 kg/ha) of commercial humic products is ineffective. Hence, an appropriate soil application rate is a critical question to answer. Appropriate application timing is another essential point as they may influence the agronomical response (Canellas et al., 2015).

To our knowledge, no previous research has assessed the effect of application timing of humic materials in mined sandy soils, despite the fact that some researchers have realized its importance for practice (Canellas et al., 2015; Jindo et al., 2016). In these studies plant response was mostly investigated in a single phase of vegetation growth. It is still unclear if a positive response at an early stage will remain throughout the entire vegetative life cycle. Rose et al. (2014) reviewed more than 94 published humic studies and mentioned the majority of studies had an experiment duration of no more than 6 months and plant growth measurements were mostly during the early plant growth stage. Data are scarce on long term effect of humic materials on the entire vegetative cycle of plant species.

Despite the combined use of humic materials with other inputs, such as AMF and inorganic fertilizers, being theoretically strong, studies on their synergistic effects are very limited, especially in heavy metal contaminated sandy soils. Although humic materials are a strong candidate for heavy metal remediation of water and soil, less attention has been devoted to remediation. More research will be needed to determine whether humic materials are effective in heavy metal remediation and what the underlying mechanisms are. Such knowledge may provide a new and economical alternative technique for future remediation.

We do not know whether humic products from the same extraction source (lignite) have the same chemical characterization, and we do not know whether they are functionally the same. Knowledge of direct comparisons with other carbon rich organic materials such as biochar is also very limited. These knowledge gaps have hampered insights into realization of the full potential of humic materials and their widespread applications in land reclamation.

4. Thesis Structure And Research Objectives

To address some of the existing knowledge gaps in use of humic products for land reclamation, our research was conducted in a University of Alberta greenhouse, under field conditions (Shendong coal mine, China), and in the University of Alberta Land Reclamation laboratory. The overall research objective was to investigate the potential of a lignite derived humic product called nano humus as a soil amendment in coal mine reclamation and remediation. Specific objectives were to determine the most appropriate application rate and timing of nano humus; to investigate plant and soil response to a single use of nano humus, and in combination with arbuscular mycorrhizal fungi and/or inorganic fertilizer; to assess use of nano humus in removal of heavy metals from contaminated soil and water and compared with humic powder (another lignite derived humic product) and a cattle manure derived biochar; to provide a better understanding of underlying mechanisms. The thesis is written in paper format with chapters as follows.

- Chapter II: A greenhouse experiment was conducted using sandy soils to investigate effects of nano humus with and without inorganic fertilizer on plant growth, and to determine the most appropriate application rate of nano humus prior to field applications.
- Chapter III: A field experiment was conducted over two consecutive years in China at Shendong coal mine, a former under ground coal mine, to assess the effect of nano humus application timing on plant growth and soil properties and to identify the most appropriate application timing for future practice.
- Chapter IV: A field experiment was conducted in China at Shendong coal mine over two consecutive years to assess the effect of nano humus application, alone and in combined applications with arbuscular mycorrhizal fungi, and/or inorganic fertilizer, on soil variables, soil metal removal, and plant growth.
- Chapter V: A greenhouse experiment was conducted to assess plant response to two lignite derived humic substance products, nano humus and humic powder, and a cattle manure biochar, as soil amendments in loamy sand and silt loam textured soils, with and without cadmium contamination.
- Chapter VI: A laboratory batch experiment was performed at University of Alberta to investigate the heavy metal removal capacity of nano humus in contaminated metal solutions in comparison to humic powder and cattle manure biochar.

II. Lignite Derived Humic Substances And Inorganic Fertilizer Are Effective Soil Amendments For Barley In Sandy Soil

1. Introduction

Sandy soils are among the most extensive soils throughout the world, covering 900 million ha or 7 % of the land surface (Driessen et al., 2001). With increasing population pressure on global land resources, sandy soils are being cultivated more intensely. Since sandy soils often have low nutrient contents, chemical fertilizers have been widely used to provide essential mineral elements to obtain maximum crop yield. The global demand for fertilizer has increased dramatically, with the estimated demand for nutrients (nitrogen, phosphorus, potassium) for fertilizer use increasing from 185 million tonnes in 2016 to 198 million tonnes in 2021 (FAO, 2019). Excessive fertilizer application has led to leached nutrients from croplands, adversely influencing soil physical, chemical, and biological properties, and contaminating ground water (Savci, 2012; Khan et al., 2018). Sandy soils are particularly prone to nutrient losses due to their high infiltration capacity, low water holding capacity, and low nutrient retention capacity (Von Uexkull, 1986). Thus there is an increasing demand for effective and environmentally friendly materials to amend nutrient poor sandy soils, enhancing nutrient retention and reducing chemical and synthetic fertilizer inputs.

Humic substances as natural biostimulators have been widely discussed for agricultural uses (Rengrudkij & Partida, 2003; Ciarkowska et al., 2017). They are complex, heterogeneous mixtures of polydispersed materials, bound by noncovalent intermolecular interactions, such as Pi (π-π, CH-π), vander Waals, charge transfer, and hydrogen bonding (Nebbioso & Piccolo, 2011; Piccolo, 2016). They are naturally formed in soils, waters, sediments, and organic geological deposits (Schnitzer & Monreal, 2011) through biochemical and chemical reactions during decomposition and transformation of plant and microbial residuals (humification) (Thorn et al., 1989). The majority of humic substance commercial products are derived from leonardite or lignite (Chen et al., 2004), a young and low ranked coal type that has relatively high humic contents (Taylor, Teichmüller, Davis, et al., 1998). They are often applied directly to soil in combination with inorganic fertilizer.

Humic materials are known to improve soil fertility by influencing physical, chemical, and biological conditions (Kalbitz et al., 2000; Bronick & Lal, 2005; Bezuglova et al., 2017), which in turn increase water and nutrients, especially at the soil surface. Although mechanisms are not yet completely understood, humic substances are reported to potentially directly influence plant growth through uptake and transport physiological processes in plant tissues, such as cell membrane permeability, oxygen uptake, respiration, photosynthesis, hormonal activity, and root cell division (Chen & Aviad, 1990; Nardi et al., 2002; Türkmen et al., 2004). Humic materials may indirectly influence plant growth by affecting microbial growth, organic matter mineralization and solubilization, and availability of microelements and some macroelements (Ayuso et al., 1996; Sharif et al., 2002). These mechanisms are likely interconnected, and the effect of humic substances might be due to complex dynamic, non-linear, cross interrelated processes requiring more investigation (Canellas et al., 2002).

The value and potential of humic materials in agriculture are uncertain, with some studies showing stimulation effects on plant growth (Tattini et al., 1990; Adani et al., 1998; Tahir et al., 2011), and others without impact (Feibert et al., 2003; Hartz & Bottoms, 2010). Various factors in the crop field might influence results, such as climate fluctuations, weather variability, soil type, water conditions, and field management. Thus, investigations are needed to evaluate effects on plant growth under controlled conditions. The main objective of our study was to assess effects of a lignite derived humic substance product, nano humus, with and without inorganic fertilizer, on barley growth parameters in sandy soil, under controlled greenhouse conditions. This study contributes to understanding the chemical characterization of the humic product and development of theoretical assessment on its application in sustainable agriculture.

2. Materials and Methods

2.1 Materials

We used barley (*Hordeum vulgare* L.) in our study as it is a common agricultural crop species around the world. In 2019, world barley production was 159 million tonnes and ranked fourth in quantity for produced cereal crops (FAOSTAT, 2019). It matures rapidly and is highly adaptable to unfavourable environments where other cereals cannot grow well, such as cold, drought, and poor soil quality (Gürel et al., 2016; Ligaba & Katsuhara, 2010). Barley seeds were procured from a local commercial farm (Tribend Ranch Limited). Before the experiment, a seed germination test was conducted in the experimental soil to determine seeding density. Seed germination began after 5 days and was 36 % within 7 days. Seeding density was 15 seeds per pot for the experiments. Since the selected plant species is a cosmopolitan species with no conservation interest, there were no necessary permits and/or licenses for the collection of plant or seed specimens. The experiment on plants, including collection and disposal of plant material, was conducted at the University of Alberta greenhouses following relevant institutional, national, and international guidelines and legislation.

The experimental sandy soil was collected near the town of Devon (53°24'27.27"N, 113°45'34.94"W). The collected soil was completely mixed so all pots would have the same soil. Three composite soil samples were delivered to a local commercial laboratory for testing. Total soil carbon was determined by combustion (Carter & Gregorich, 2008); available nitrate and sulphate by calcium chloride extraction (Laverty & Bollo-Kamara, 1988); and available phosphorus and potassium by modified Kelowna extraction (Ashworth & Mrazek, 1995). Particle size (sand, silt, clay) was determined by hydrometer after treatment with calgon (Carter & Gregorich, 2008). Soil pH and electrical conductivity (1:10 soil water suspension ratio) were determined using a pH and conductivity meter (Oakton 300 Series, Oakton Instruments, IL, USA). Mean total carbon was 4.5 %; available nutrients were 18.4 mg/kg nitrate, 18.9 mg/kg phosphate, 90.3 mg/kg potassium, and 206.7 mg/kg sulphate. Soil texture was loamy sand with 84.3 % sand, 8.8 % silt, and 6.8 % clay. Soil pH was neutral at 7.3; electrical conductivity was 0.7 dS/m.

Nano humus® is a lignite derived humic substance commercial product. The product is a black powder, with recommendations to dissolve in water before applying on the soil surface. The recommended application rate for agriculture use in sandy soil is equivalent to 5 g per plant pot; at a ratio of 1:100 powder to water. It was applied once in week 3. The main elements are carbon, hydrogen, nitrogen, and oxygen. It contains 83.2 % organic matter, 50 % humic acid, and 1 % micronutrients (copper, iron, zinc, aluminum, manganese, boron); macronutrients include 0.86 % nitrogen, 1.11 % phosphorus (P_2O_5), 5 % potassium (K_2O), 0.29 % sulfur, and 0.34 % magnesium; with 4 % silica sand and ash by weight. Nano humus pH was 8.98 and electrical conductivity 0.08 dS/m (Oakton 300 Series, IL, USA). The surface chemical characteristics of nano humus was determined by Fourier transform infrared (FTIR) spectroscopy (Thermo Scientific Nicolet, model iS50, Madison, WI, USA).

General purpose fertilizer (Plant Prod®) was used, containing 20 % total nitrogen, 20 % available phosphoric acid, and 20 % soluble potash. It was applied at concentrations of 100 mg/L and 250 ml per pot as recommended by the manufacturer. It was applied once with an injector in week 3.

2.2 Experimental design and greenhouse procedures

The experiment was conducted from November to February (110 days), under controlled greenhouse conditions set to 22 °C with 16 hours photoperiod. A complete randomized experimental design was implemented with ten treatments, replicated six times for a total of 60 experimental units (10 treatments x 6 replicates); there were 5 measurements per replicate. The 10 treatments comprised sole application of nano humus at four rates (NH1, NH2, NH3, NH4), sole application of fertilizer (F), combined application of nano humus and fertilizer (F+NH1, F+NH2, F+NH3, F+NH4), and an untreated control (CON). NH1 = 2.5 g nano humus 100 mg/L, NH2 = 5 g nano humus 100 mg/L (recommended rate), NH3 = 7.5 g nano humus 100 mg/L, NH4 = 10 g nano humus 100 mg/L, F+NH1 = 20-20-20 fertilizer with 2.5 g nano humus (100 mg/L), F+NH2 = 20-20-20 fertilizer with 5 g nano humus (100 mg/L), F+NH3 = 20-20-20 fertilizer with 7.5 g nano humus (100 mg/L), F+NH4 = 20-20-20 fertilizer with 10 g nano humus (100 mg/L), CON = untreated.

Pots were 20.32 cm diameter and 13.97 cm height, with a tray under each to alleviate soil and nutrient loss from drainage by pouring back the drainage after each watering. Pots were each filled with approximately 1 gallon of sandy soil, to approximately 5 cm from the top, then randomly placed in trays on a greenhouse bench. Seeds (15 per pot) were placed at 1 to 2 cm depth in each pot, then covered lightly with soil. One week after germination completion, barley was thinned to 5 plants per pot. Barley seedlings were watered when pots were lighter than the weight of moistened pots.

2.3 Plant measurements

Plant height was measured before applying fertilizer and nano humus treatments (week 3), then every week thereafter. At the end of the experiment in week 15, plant height, root length, seed numbers per head, and shoot and root biomass were determined. Plant height was measured with a ruler from the soil surface to the tallest living leaf of each plant in each pot. Roots and shoots were collected separately for each sampling plant. Shoots were cut at the soil surface and placed in paper bags. Roots were collected from the soil and dry soil was carefully removed by gentle rubbing and shaking. Roots were then washed gently with tap water and non-root material was removed using tweezers. Longest and shortest roots for each individual plant were measured with a ruler and mean length determined. Roots and shoots were oven dried at 80 ºC for 48 hours then weighed to determine dry biomass. Seeds were counted in each individual seed head and averaged for each plant.

2.4 Statistical analyses

Statistical analyses were conducted with R software (version 3.6.1), and significance was accepted at $p < 0.05$ for all tests. One-way multivariate analysis of variance (MANOVA) was performed to investigate treatment effects on plant height over time (weeks 3 to 15, n = 30);

treatment effects on multiple growth variables (plant height, shoot biomass, root length, root biomass) in the final week 15 (n = 30). Assumptions of univariate and multivariate normality (QQ plot), linearity (scatter plot matrix), low multicollinearity (Pearson's correlation test), homogeneity of covariances (Box's M-test), and variances (Levene's test) were determined before MANOVA computation. Pillai's Trace was the multivariate statistics method. When statistical significance occurred, a univariate one-way ANOVA (type II sum of squares) was conducted for each dependent variable (n = 30). For significant factors, Tukey's honest significant difference test was performed. Since the sample size of seed numbers in the final week was unequal, which may cause errors (total sum of square ≠ sum of square of effects + error), it was removed from MANOVA and analyzed separately by a one-way ANOVA (type III sum of squares) to assess treatment effects on seed production (n = 30). Assumptions of normality (QQ plot, Shapiro-Wilk test), and equal variances (Levene's test) were determined prior to running the test. Least square means were used for pairwise comparisons. Multiplicity adjustments were conducted with Tukey's honest significant difference adjustments.

3. Results

3.1 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) analysis of nano humus was performed to determine surface functional groups. Infrared spectroscopy frequency ranges, the appearance of vibration for functional groups were characterized according to Socrates (2004) and Hesse et al. (2005). Nano humus was enriched in aromatic carbon and phenolic groups; the results were supported by the spectra (Figure 2.1). Nano humus was characteristic of hydrophilic and hydrophobic components. The broad and rounded band in the range of 3,200 to 3,500 cm^{-1} (-OH stretching) formed the hydrophilic components. Alkyne (-CΞCH) appeared as a few weak bands from 2,260 to 2,100 cm^{-1} and aliphatic hydrocarbon compound (C-H bending) at 1,981 cm^{-1} suggested hydrophobic components. Nano humus was enriched in aromatic carbon, with bands at 1,371 and 1,559 cm^{-1} (C=C stretching) indicating aromatic structures. Strong bands of symmetric C-O stretching were observed at 1006 and 1030 cm^{-1}. Background FTIR spectra indicated the presence of ambient water (~3,600 cm^{-1}) and carbon dioxide (2,324 cm^{-1}).

3.2 Treatment effects on shoots

Treatment effect was significant on barley height for all weeks combined (weeks 3 to 15) (p=0.0001). Treatment effect was significant from weeks 4 to 8 throughout the jointing stage (p <

0.05), but not significant over the boot stage from weeks 9 to 11 ($p > 0.05$). Statistical significance increased noticeably (p values decreased) during the heading stage (weeks 12 to 15); being greatest in week 15 ($p = 1.3\times10^{-8}$). Combined application of nano humus and fertilizer yielded taller seedlings than sole application and no treatment (Figure 2.2). Although mean plant heights did not vary significantly among nano humus application rates, treatment of F+NH2, F+NH3, and F+NH4 showed slightly higher values over the study period. In particular, the slight stimulation effect of F+NH2 treatment was noticed at the early growth stage (early stem elongation), F+NH4 treatment at the mid growth stage (later stem elongation), and F+NH3 at the late growth stage (heading stage).

In week 15, effects of combined application on height were more obvious (Table 2.1), increasing 7 % relative to the control. Greatest height enhancement was with fertilizer only (9 %), closely followed by F+NH3 (8 %). Barley height and shoot biomass were less affected by sole application of nano humus (NH1, NH2, NH3, and NH4) relative to the control. However, combined application provided a marked stimulation effect on shoot biomass, producing significantly higher biomass than the control and sole application of nano humus. A mean 80 % enhancement was evidenced by combined treatments F+NH1, F+NH2, F+NH3, and F+NH4. Shoot biomass was similar with all four nano humus rates, either with or without fertilizer.

3.3 Treatment effects on roots

All treatments of nano humus, with or without fertilizer, increased barley primary root length and root dry biomass in sandy soil after 15 weeks (Table 2.1). The beneficial effects on barley root growth varied slightly among four rates of nano humus. Longer roots were found in all nano humus treatments. Sole application of nano humus generally increased primary root length by 16 to 25 % relative to the control, and 15 to 24 % relative to fertilizer alone. Mean root length of barley with fertilizer alone (15.29 ± 0.52 cm) was similar to that of the control (15.17 ± 0.72 cm), indicating fertilizer may not influence barley primary root elongation. This was further confirmed by comparing root lengths between groups of nano humus with and without fertilizer, showing a lack of significant differences at each of the four rates. A clear enhancement on lateral root formation was observed in barley seedlings with nano humus alone, fertilizer alone, and their combinations, which led to greater root biomass. Among all studied growth parameters, the strongest beneficial effect was found on root biomass production by combined application and nano humus alone. Root biomass increased 92 % relative to the control with combined treatments, 49 % with nano humus alone, and 68 % with fertilizer alone. The greatest promoting effect was with the combined treatment of F+NH2, resulting in a 124 % increase.

3.4 Treatment effects on seed production

Barley reached maturity in week 15, with greater seed production with combined treatments of nano humus and fertilizer over corresponding sole applications (Table 2.1). Although sole application was generally not statistically different from the control, application of nano humus increased mean seed numbers (per head) by 2 to 11 %; fertilizer increased it by 11 %. The enhancement effect of combined application was greatest, by 17 % on four rate means. This is particularly notable for F+NH1, F+NH2, and F+NH3 which was significantly greater than the control, increasing by 55 %, 37 %, and 37 %, respectively.

4. Discussion

In our study, the most distinct promoting effect of nano humus as a sole application was on root systems. Larger roots are associated with greater capacity for nutrients and water uptake, which is particularly important for sandy soils with limited nutrient conditions and water holding capacity (Ehdaie et al., 2010; Wang et al., 2016). This may be because the only contact organ for humic substances in the soil is roots. Our results agree with those of other studies showing beneficial effects of humic materials on root development in various nutrient poor growth media, including sandy soils. For example, Ciarkowska et al. (2017) found humic materials tripled root dry biomass in coarse textured soils (77 % sand, 20 % silt, 3 % clay) and increased root biomass 2.5 times in medium textured soils (35 % sand, 51 % silt, 14 % clay) relative to plants in the control. Eyheraguibel et al. (2008) reported a significant increase in root dry biomass (36 %) and root length (23 %) in hydroponic conditions after humic material application. Schmidt et al. (2007) found a significant increase in root hair length, root density, and cell proliferation in root tissue growing in humic substances. Mora et al. (2012) found humic substances induced increments in secondary root numbers, root thickness, and root biomass under hydroponic conditions.

Seed production is a key factor determining agricultural production. The enhanced seed production we saw in our study after humic substances application was also evidenced by Machiani et al. (2019) who reported a 10 % increase in common bean seed production and a 16 % increase in fennel with application of humic substances in silty clay soils. An equivalent of 84 % peanut seed production increase in loamy clay soils was found by Moraditochaee (2012).

When combining nano humus with inorganic fertilizer, the stimulation effect on plant biomass and seed production was more obvious than with sole application. Our finding was consistent with Suman et al. (2017) who found the combined application significantly enhanced plant height (6 %), total biomass (7 %), leaf area index (3 %), chlorophyll content (5 %), and fruit yield (20 %) over sole application of fertilizer in sandy loam soils. Similarly, Sharif et al. (2002) reported a 20 to 23 % increase in shoot biomass and a 32 to 39 % increase in root biomass in silty clay loam soils with the combined addition of humic materials and inorganic fertilizer. However, there was little information on the combined effect of humic substances and fertilizer on seed production in the literature, making this a significant contribution from our study.

Although there were no statistically significant differences between the four rates of nano humus, they all showed beneficial effects on barley growth, especially on root growth and seed production. The recommended nano humus application rate by the manufacturer is equivalent to 5 g per plant pot at a ratio of 1:100 powder to water. This rate is now known to be suitable for sandy soils from our study, but requiring more study for other soils.

Humic substances, as a group of chemical compounds with supramolecular structure, can chemically interact with plants cell membranes after penetration (Kulikova et al., 2013, 2016) and therefore modify plant function. Functional groups are considered the most important characteristics of humic materials. The beneficial effects of nano humus are likely attributed to the presence of various organic functional groups, from polar (hydroxyl, phenolic) to nonpolar (aliphatic, aromatic), which are responsible for its hydrophilic and hydrophobic properties. Ojwang & Cook (2013) investigating chemical interactions between humic fragments and biomembranes, found surface adsorption occurs via H bonds and then humic fragments enter the cell and modify membrane structural homogeneity. Hydrophilic structures of nano humus (phenolic -OH groups) were detected by FTIR. They are likely to constitute the interaction between nano humus and barley cell membrane surfaces. Other polar functional groups in nano humus structure may also contribute to the interaction and penetration. Hydrophilic components in the structure of humic materials could trigger nitrogen metabolism related enzymatic activities after penetration and therefore positively affect root growth and biomass production (Vaccaro et al., 2009; Aguiar et al., 2013; Garcíaa et al., 2016).

Humic substances exert auxin-like activities directly influencing plant physiology, particularly root hair formation, and lateral root development (Canellas et al., 2002; Casimiro et al., 2003). The presence of auxin-like material in humic structure was reported by genetic and molecular biology techniques (Trevisan et al., 2010, 2011) and gas chromatography mass spectrometry (Canellas et al., 2002). The auxin activity could enhance H+ ions in cell wall compartments by activation of H+ pumping ATPase at the plasma membrane, thereby decreasing pH at the root surfaces. This may activate pH sensitive enzymes and proteins in the cell wall, loosening cell walls and triggering cell elongation (Hager, 2003).

This explains the promoting effect of fertilizer with nano humus on barley seed production. Humic fragments facilitate H^+-ATPase in barley roots and induce root to shoot distribution of nitrate. In the heading stage, a vital growth stage for all crops, this involves nutrient reserve transportation (Dante et al., 2014; Sehgal et al., 2018). Therefore, barley accumulated available nutrients in sandy soil provided by nano humus and inorganic fertilizer and then translocated that to seed heads for seed formation controlled by humic substance induced hormonal signals. The effect on seed development was seen in a previous study that reported improvement of sunflower seed yield after applying humic materials (Emam & Awad, 2017).

Without external sources of nutrients, nutrient deficiency might occur and become one of the most limiting factors affecting barley growth in sandy soils. Nano humus, a lignite derived humic material, contains essential nutrients in complex nitrogen-phosphorus-potassium matrices which ensure nutrient ion assimilation and nutrition. However, similar to other humic products, nano humus does not contain sufficient nitrogen-phosphorus-potassium for barley growth. The combined application of inorganic fertilizer and nano humus serve as nutrient carriers in both water soluble and complexed forms and alleviate nutrient deficiencies in sandy soils. A significantly better response of barley was observed in sandy soils with both nano humus and fertilizer relative to sole application.

Humic materials serve as an adsorption and retention complex for nutrient elements (Brannon & Sommers, 1985) such as nitrogen, phosphorus, potassium, zinc, magnesium, calcium, sodium, iron, copper, and manganese (Dursun et al., 2002). After surface application of inorganic fertilizer, rapidly available forms of nitrate, phosphate, and potassium infiltrated into sandy soils. However, they are likely to leach from the sandy soil over time, especially with an application of relatively high concentrations of nitrogen, phosphorus, and potassium at one time. It is important to slow release of nutrients and enhance their utilization. Functional groups in humic structure, such as phenolic hydroxyl groups, can bind mineral ions in soils and build metallic complexes (Piccolo et al., 1996; Zanin et al., 2019). This implies that plants growing in soils might benefit from humic materials by forming stable complexes with micronutrients, such as iron-humic substances (Colombo et al., 2013; Cieschi & Lucena, 2018). Iron complexation can increase phosphate availability in soils as its bridges can bond phosphate to humic substances (Urrutia et al., 2014). This process plays a vital role as phosphate is one of the most important elements limiting plant growth due to its relative immobility (Morgan & Connolly, 2013). This is particularly important for growing barley in sandy soils with limited essential nutrients. When an external source of nutrients is added to the soil, such as chemical fertilizer, humic materials can slow the release of nutrients and improve fertilizer utilization efficiency. These abilities have been evidenced in various studies (Selladurai & Purakayastha, 2015; Chen et al., 2017; Suman et al., 2017). The enhanced nutrient retention is likely to reduce leaching from sandy soil. This, in turn, makes the plant grow better for almost all plant growth parameters when applying combined treatments relative to sole application of fertilizer. Sandy soil amended with nano humus could be an effective management option for reducing nutrient leaching and improving nutrient availability in production agriculture. Future research on the effect of humic materials on plant nutrient uptake and nutrient availability will be needed to validate this.

5. Conclusions

Our study provided insights on theoretical assessment of application of a lignite derived humic substances product, nano humus, with and without inorganic fertilizer to sandy soils. Sole application of nano humus markedly promoted barley primary root length, root biomass, and seed production, the latter a significant contribution from our study. Although four rates of nano humus were not significantly different, they all showed beneficial effects on barley growth. Applying nano humus at recommended rates (equivalent to 150 g/m^2) is suitable for sandy soils. Sole application of inorganic fertilizer primarily benefitted height and shoot biomass development. Combined treatments of nano humus and inorganic fertilizer resulted in the greatest beneficial effect on barley growth over corresponding sole applications. The concomitant use of humic substances together with inorganic fertilizer is suggested as an effective soil amendment for enhancing agricultural plant growth in sandy soil regions.

Table 2.1 Mean and standard errors (SE) of height, shoot biomass, root length, and root biomass by treatments in week 15. Means in columns with different letters are significantly different.

Rate	Height	Shoot biomass	Root length	Root biomass	Seed
	cm	g/plant	cm	g/plant	number/head
			Mean ± SE		
CON	46.57 ± 0.77bc	0.65 ± 0.04b	15.17 ± 0.72b	0.14 ± 0.01c	12.24 ± 0.70c
F	50.99 ± 0.93a	1.04 ± 0.07a	15.29 ± 0.52b	0.23 ± 0.02ab	13.53 ± 0.83bc
F+NH1	49.82 ± 0.93ab	1.12 ± 0.07a	18.79 ± 0.62a	0.23 ± 0.02ab	18.86 ±0.86a
F+NH2	49.87 ± 1.00ab	1.17 ± 0.07a	17.77 ± 0.58ab	0.31 ± 0.02a	16.74 ± 0.74ab
F+NH3	50.24 ± 0.78ab	1.22 ± 0.05a	20.07 ± 0.65a	0.27 ± 0.02ab	16.67 ± 0.83ab
F+NH4	49.24 ± 0.89ab	1.17 ± 0.07a	18.63 ± 0.59a	0.24 ± 0.02ab	15.43 ± 0.86abc
NH1	46.32 ± 1.02bc	0.74 ± 0.04b	18.47 ± 0.57a	0.22 ± 0.02abc	13.47 ± 0.78bc
NH2	44.85 ± 0.94c	0.59 ± 0.04b	17.58 ± 0.83ab	0.19 ± 0.02bc	12.38 ± 0.89c
NH3	44.88 ± 0.84c	0.62 ± 0.04b	17.76 ± 0.55ab	0.20 ± 0.01bc	12.62 ± 0.89c
NH4	47.04 ± 0.81abc	0.75 ± 0.05b	18.95 ± 0.61a	0.21 ± 0.02bc	13.25 ± 0.80bc

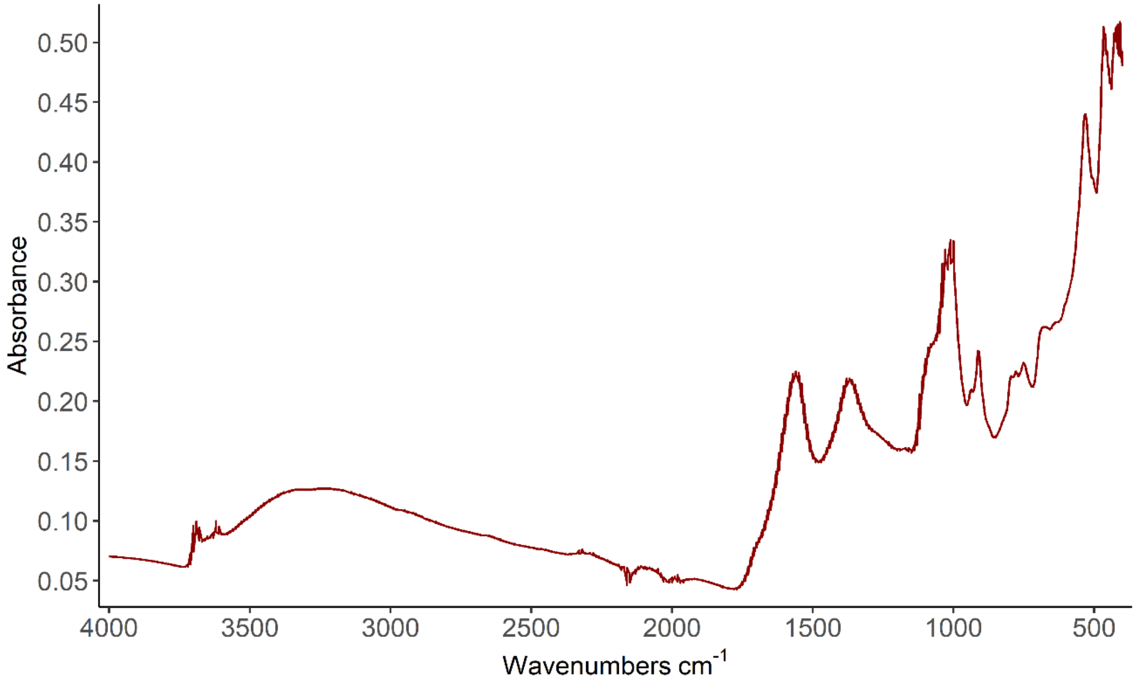

Figure 2.1 Fourier transform infrared spectroscopy (FTIR) spectrum of nano humus.

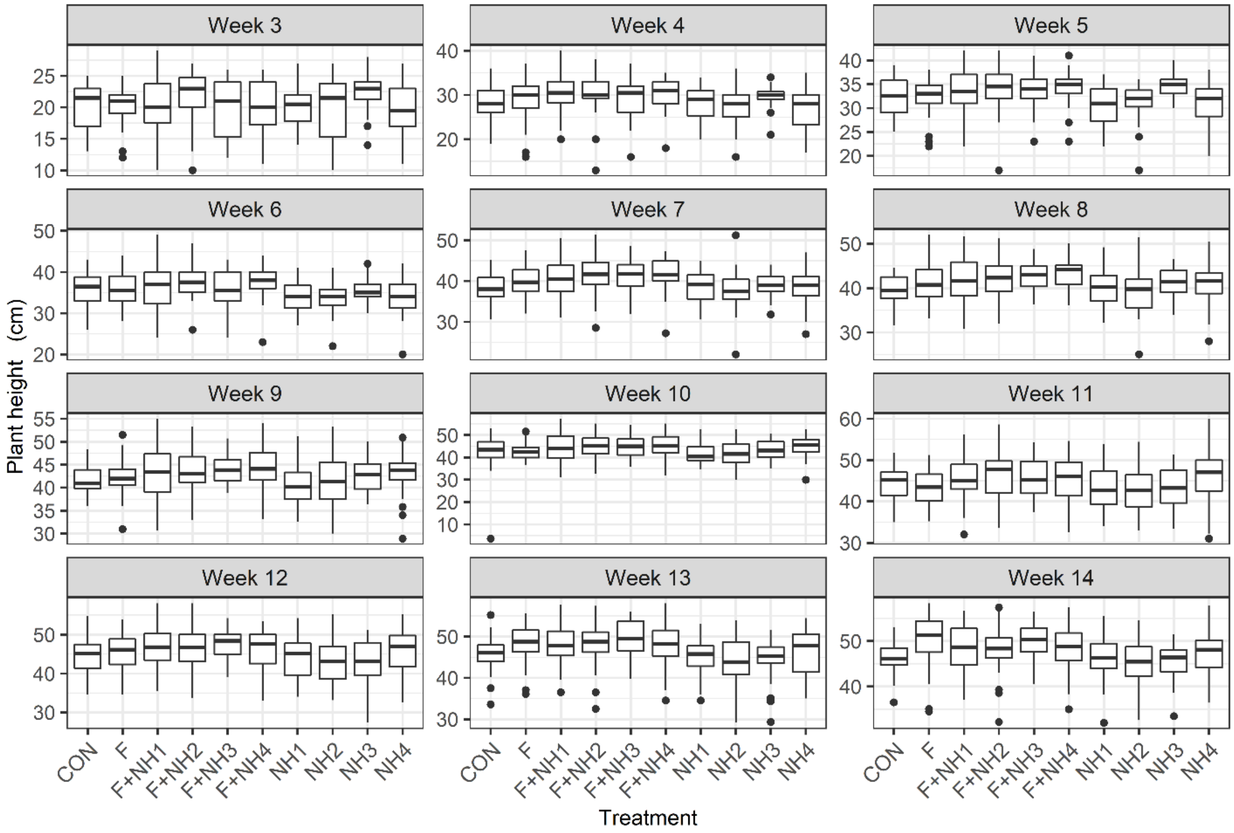

Figure 2.2 Grouped boxplots for mean plant height by treatments from weeks 3 to 14.

III. Application Timing Optimization Of Lignite Derived Humic Substances For Three Agricultural Plant Species And Soil Fertility

1. Introduction

Coal is mined for energy generation worldwide, and its extraction through opencast and underground mines produces large amounts of waste and extensive disturbances to the environment. Surface subsidence, soil degradation, vegetation destruction, soil and ground water contamination, and loss of biodiversity are the main environmental consequences of mining activities (Bian et al., 2010; Kuter et al., 2014; Huang et al., 2015). With increasing pressures from the burgeoning human population on land resources, extensive areas of post mining lands must be reclaimed for agriculture upon closure. In the transitional zone between Mu Us desert and Loess Plateau in northern China, most post mining lands are covered by sandy soils with low organic matter and nutrient concentrations. Due to high infiltration capacity, low water holding capacity, and low nutrient retention capacity, sandy soils are especially prone to nutrient losses (Von Uexkull, 1986), making crop production very challenging.

Various physical, chemical, and biological methods for land reclamation have been suggested around the world, such as addition of topsoil, mulches, organic amendments, biosolids, and mycorrhizal fungi (Maiti, 2012; Kumar et al., 2018; Pinto et al., 2018; Cozzolino et al., 2021). Use of humic substances as soil organic amendments is a low cost biological technique that has received considerable attention due to the great potential of these materials as natural biostimulators and soil conditioners (Rengrudkij & Partida, 2003; Tahir et al., 2011; Ciarkowska et al., 2017). Humic substances are complex, supramolecular, self-assembled mixtures of diverse heterogeneous compounds (Piccolo, 2002) that occur naturally in soils, waters, sediments, and organic geological deposits (Schnitzer & Monreal, 2011). Commercial humic products are mostly derived from leonardite or lignite wastes from mining (Chen et al., 2004), and their use helps reduce the accumulation of industrial wastes.

The effect of humic materials for agricultural uses remains uncertain, with some studies reporting positive effects (Tahir et al., 2011; Ciarkowska et al., 2017; Mosa et al., 2020), and others reporting no effect (Feibert et al., 2003; Hartz & Bottoms, 2010; Mahoney et al., 2017). These inconsistent results can be linked to experimental conditions, soil conditions, application rates, and application methods (Jindo et al., 2020). To our knowledge, no previous research has studied the effect of application timing of humic materials in mined sandy soils, despite some researchers suggesting its importance for practice (Canellas et al., 2015; Jindo et al., 2016). Knowing the appropriate timing of humic material applications under field conditions is critical for practice, as timing may influence agronomic plant response (Canellas et al., 2015). The majority of humic studies were conducted in greenhouses or growth chambers and cells with controlled conditions, with few under field conditions.

Lack of information on the effect of application timing with consecutive field observations has hampered insight into realization of the full benefits of humic materials in land reclamation and sustainable agriculture. Thus, this study was undertaken to ascertain appropriate application timing of a lignite derived humic product called nano humus for coal mine reclamation. The study investigated soil and plant response to nano humus over the entire plant growth cycle, of three agricultural species, in two consecutive years, in the field on a former underground coal mine in the transitional zone between Mu Us desert and Loess Plateau in China.

2. Material and Methods

2.1 Site description

The research site was located in Shendong mining area (39.31249°N, 110.2097°E), one of the largest former underground coal mines in northern China, covering approximately 72.4 km^2 area. The mining area is located 1,200 m above sea level on the Ordos plateau, which is part of the Mu Us Desert - Loess Plateau transitional zone. The climate is semi-arid, with mean annual temperature 8.5 °C (-15.9 to 29.8 °C in a year). The non-frozen period is usually from early April to early October. Mean annual evaporation (2,300 mm) is almost six times greater than annual accumulated precipitation (420.3 mm). Approximately 60 to 70 % of precipitation falls between July and September. Ground water in this area is mainly recharged by atmospheric precipitation. Soils were sand texture (90 % sand, 2 % silt, 8 % clay), containing 0.83 % total organic carbon, 35 mg/kg available nitrogen, 2 mg/kg available phosphorus, and 21 mg/kg available potassium. Soil was neutral with pH 7, electrical conductivity 0.3 dS/m, and cation exchange capacity 2.88 cmol(+)/kg. Since slopes can influence soil and plant variability, three study sites were established on a 500 m^2 unvegetated flat area.

2.2 Humic materials

Nano humus, a lignite derived humic product, was obtained from Wuhan Shangyuan Environmental Protection Co, Ltd (Beijing, China). Nano humus is a black, fine grained substance with recommendations to dissolve in water (1 % suspension concentration by weight) before applying on the soil surface. Our nano humus contained approximately 83.2 % organic matter, 50 % humic acid, and 1 % micronutrients (copper, iron, zinc, aluminium, manganese, boron); 0.86 % nitrogen, 1.11 % phosphorus (P_2O_5), 5 % potassium (K_2O), 0.29 % sulphur, and 0.34 % magnesium as macronutrients; with 4 % silica sand and ash by weight, pH 9, and electrical conductivity 0.08 dS/m (Oakton 300 Series, IL, USA).

2.3 Experimental design and procedures

During the 2018 and 2019 growing seasons (May to August), a field experiment was conducted in a randomized complete block design with three replications (three blocks) to determine the effects of nano humus application timing (5 timings), plant species (3 species), and experiment year (2 years) on soil chemical properties and plant growth. Experimental blocks were 5 m x 15 m, each containing 15 treatment plots (1 m x 1 m) randomly assigned within the block.

There were five application timings for each growing season considering the total number of applications and duration between applications; a single application in May, two applications in May and June, two applications in May and July, two applications in May and August, and a control (untreated). To avoid confounding effects, the total amount of nano humus was fixed to 150 g/m^2 at 1 % concentration for each year based on producer recommendations and our pre-experiment tests); applying 150 g/m^2 for the single application, and splitting into 75 g/m^2 for each of the two applications.

Alfalfa (*Medicago ruthenica* L.), barley (*Hordeum vulgare* L.), and sea buckthorn (*Fructus Hippophae* L.) were studied as they are economically useful agricultural and reclamation species that are widely used in many regions with high adaptability to dry and sandy areas (Li & Schroeder, 1996; Erice et al., 2010; Gürel et al., 2016). Alfalfa seeds (57 % pure live seed), barley seeds (76 % pure live seed), and sea buckthorn seedlings (100 % live seedling) were obtained from local seed and nursery companies. Alfalfa, a perennial plant species, was seeded in May 2018 at 160 kg/ha (2700 seeds per treatment plot). Barley, an annual plant species, was seeded at 30 kg/ha (150 seeds per treatment plot) in May 2018 and 2019. Sea buckthorn, a hardy perennial shrub, was planted as seedlings (approximately 30 cm tall, 65 g) at 160,000 plants/ha (16 seedlings per treatment plot) in May 2018.

A sprinkler system was installed on the sites in May 2018 and managed by Shendong coal group during the experiment. Plants were watered every day for the first two weeks, every two days in June, and once a week in July and August when precipitation occurred more frequently; this was repeated for two years. Unexpectedly, alfalfa in blocks 1 and 2 died due to a severe spring drought in April 2019 together with damage to some sprinklers. Alfalfa in block 3 remained in the second growing season for vegetation assessment. Barley and sea buckthorn were not affected.

2.4 Vegetation and soil measurements

Vegetation was assessed at the end of each growing season in August. Plant height, root length (main and lateral roots), and dry biomass (shoot, root, total) were determined for alfalfa; plant height, root length (mean of the longest and shortest fibrous root), and dry biomass (shoot, root, total) for barley; plant height, root length (main and lateral roots), stem diameter, and dry biomass (shoot, root, total) for sea buckthorn. Five plants were measured and sampled individually for each treatment plot along two intersecting diagonal lines to represent each treatment. Plant height was measured with a ruler from the soil surface to the highest live leaf. Root length was measured with a ruler from root crown (soil surface) to root tip after digging the whole root system from the soil. Stem diameter was measured at the widest part (approximately 5 to 15 cm above the ground) with a digital caliper. Samples were oven dried at 80 °C for 48 hours, to determine root, shoot, and total dry biomass.

At the end of each growing season (end of August), soil samples were taken from the upper 15 cm of surface soil for each treatment plot with a hand shovel. Samples were kept cool until they were sent to a commercial laboratory for analyses. Soil pH and electrical conductivity were measured electrometrically at 1:10 soil water suspension ratio. Cation exchange capacity was measured by exchange with calcium acetate (pH = 8.2) (Chapman, 1965). Total organic carbon was determined by combustion, available nitrogen by potassium chloride extraction method (Carter & Gregorich, 2008), and available phosphorus and potassium by modified Kelowna extraction (Ashworth & Mrazek, 1995) These soil parameters were measured for each sample as they represent agricultural soil productivity and were assumed the most influenced by addition of humic substances.

2.5 Statistical analyses

Statistical analyses were performed with R software (version 3.6.1); significance was accepted at $p < 0.05$. Application timing, year, and plant species were considered fixed factors; experiment block was considered as a random factor. Their effects upon measured soil and vegetation parameters were incorporated with analysis of variance (ANOVA) separately. Before running the test, data were confirmed for normality (QQ plot and Shapiro-Wilk test) and equal variances assumptions (Levene's test). Tukey's Honest Significant Difference test was then performed for pairwise comparisons. Principal component analysis (PCA) was conducted to investigate soil and vegetation response to application timing. The variance of the variables is approximated by the length of the arrows and their correlations are approximated by the angles between arrows. The relationship of plant biomass and close soil parameters (less than 90 degrees on PCA biplot) was further examed by Pearson product-moment correlation. The correlation coefficient (r) was used to measure the dependence between two variables.

3. Results

3.1 Effect of application timing on soil properties

Soil properties exhibited no significant changes with nano humus application in year one, regardless of application timing (Table 3.1). After two years of repeated application of nano humus, soil pH generally increased by 0.3 to 0.6 units. Soil available phosphorus and available potassium were significantly increased, with greatest improvement from the single application at 63 % and 96 %, respectively, relative to the control. Splitting into two applications showed less prominent effects. There was no significant change in electrical conductivity, total organic carbon, and available nitrogen.

3.2 Effect of application timing on plant growth

Plant and soil parameters were used to assess the differences among application timings for two growing seasons (Figure 3.1). Barley biomass had a significant positive correlation with soil available phosphorus ($p < 0.01$, $r = 0.45$) and potassium ($p < 0.001$, $r = 0.53$); alfalfa ($p < 0.0001$, $r = 0.78$) and sea buckthorn biomass ($p < 0.0001$, $r = 0.64$) had a significant positive correlation with soil available potassium.

All four nano humus application timings showed positive effects on plant growth of the three species. Applying nano humus all at one time in May was substantially better for plant growth than splitting into two applications, especially for barley and sea buckthorn. Plant growth was less affected by the duration between the two applications.

In the first growing season, alfalfa growth in sandy soils responded most positively to the single application of nano humus, with no significant influence from other application timings timings (Table 3.2). Effects of a single application on root biomass, plant height, and lateral root length were significant, showing 78, 36, and 28 % increases, respectively, relative to the control. In the second growing season, all nano humus treatments significantly stimulated plant growth parameters, particularly pronounced in total biomass with 5 to 11 times increases. The difference between application timings was not significant.

The effect of nano humus on barley growth in sandy soils was not obvious in the first growing season with small variations among application timings (Table 3.3). Although all nano humus treatments stimulated barley growth in the following experiment year, only the single application resulted in significant growth enhancement over the control, producing markedly higher root biomass (360 %), total biomass (250 %), shoot biomass (201 %), height (52 %), and root length (14 %). Two separate applications showed less obvious effects with no difference among three durations between applications (1, 2, 3 months).

The positive effect of nano humus on sea buckthorn growth became noticeable in the second growing season (Table 3.4). All nano humus treatments significantly improved sea buckthorn lateral root length, stem diameter, and biomass, with greatest benefits from the single application. The single application enhanced shoot biomass 164 %, total biomass 147 %, lateral root length 111 %, root biomass 109 %, main root length 63 %, stem diameter 44 %, and height 37 % relative to the control. There was no obvious difference between the three durations of two applications.

4. Discussion

All amended soils eventually received the same quantity of humic substances (150 g/m^2) before plant harvesting, which was the most appropriate application rate in sandy soil that we determined from a previous study. The single application means soils received all humic substances (150 g/m^2) at once, while spitting the treatment into two application times halved the amount for each application (75 g/m^2). Thus the effect of the application may be triggered with a specific threshold amount at a specific stage of plant development. The more positive plant response could be linked to the higher rate of humic substances at an early development stage that resulted from the single time application. This may be because the biological response of plant growth to humic materials is rate dependent (Rose et al., 2014) at a key phase in plant physiological development. Researchers reported enhanced plant growth with higher application rates of humic materials than lower rates within a specific range of rate. Arjumend et al. (2015) compared wheat (Triticum aestivum) growth with a single time application of a humic material at different rates (0, 50, 100, 150, 200 mg/kg). They found that higher rates (> 150 mg/kg) significantly increased plant height, root length, biomass, and leaf chlorophyll, whereas lower rates (0, 50, 100 mg/kg) had no effects. Cavalcante et al. (2013) found application rate of humic materials (0, 0.4 0.8, 1.19, 1.59 ml per seedling) had a significant impact on yellow passion fruit (Passiflora edulis), with highest rates (1.59 ml) having greatest stimulation on height, biomass, and leaf chlorophyll. According to these studies, humic materials improved plant development through altering soil nutrient status.

The positive effect of humic applications on plant development of alfalfa, barley, and sea buckthorn can be explained by the elevated nutrient availabilities in soil environments, since we identified strong correlations between plant biomass and soil available phosphorus and potassium. Soil potassium and phosphorus are essential nutrients to plants, and their availabilities are important for agricultural species in sandy soils, with phosphorus being one of the most vital nutrients limiting plant growth due to its relative immobility (Morgan & Connolly, 2013). Humic materials are biologically and chemically stable with a low mineralization rate (Qualls, 2004), hence the release of nutrient contents from humic materials into the soil profile could be relatively slow. Due to the presence of oxygen containing functional groups, humic materials can increase soil available nutrients that naturally exist in non-available forms through formation of stable complexes with metal micronutrients (Urrutia et al., 2014; Cieschi & Lucena, 2018). Early application of nano humus at the beginning of the growing season could stimulate these processes and provide adequate available nutrients for plant uptake at a time when it was most needed by plants.

Nano humus at a rate of 150 g/m^2 contains approximately 13 kg/ha nitrogen, 17 kg/ha phosphorus, and 75 kg/ha potassium. Phosphorus and potassium recommendations for alfalfa are up to 185 kg/ha and 336 kg/ha, respectively, depending on soil conditions; with the best timing in early spring before alfalfa initial growth (Lissbrant et al., 2009). Barley requires less than 67 kg/ha of phosphate and 90 kg/ha of potassium (Mahler & Guy, 2007), most of which will be used in early plant development before stem elongation (Yara, 2018). Sea buckthorn can utilize 200 to 300 kg/ha of phosphate and 200 to 250 kg/ha of potassium, with recommendations to apply in the fall or prior to planting (Li & McLoughlin, 1997; Li & Beveridge, 2003). Only a single time application at the beginning of the growing season may have met nutritional requirements of the three plant species at early stage developments, which is a major determinant of later biological developments and reclamation success.

5. Conclusions

Our study was the first to investigate the effect of soil application timing on growth of three plant species of an industrial waste generated humic substance called nano humus in mined sandy soil in the field. Application timing had significant impacts on soil available phosphorus and potassium, and plant biomass development after two years of application. A single time application increased soil available phosphorus (63 %) and potassium (96 %), which resulted in greater total biomass of alfalfa (749 %), barley (250 %), and sea buckthorn (147 %) relative to untreated controls. The impact of a split application was generally not significant regardless of the duration (1 to 3 months) between applications. A single time application would be a more suitable land reclamation strategy in sandy soil regions, which could provide better nutritional conditions in soils and consequently better plant growth than splitting the application amount into multiple times. We recommended a single time application of nano humus (150 g/m^2) at the beginning of each growing season in sandy soil regions for future practice. These recommendations can be extrapolated to sandy soils in other parts of the world.

Table 3.1 Changes in soil parameters under different application timings. Means in columns with different letters are significantly different.

Application timing	pH	Electrical conductivity	Cation exchange capacity	Total organic carbon	Available nitrogen	Available phosphorus	Available potassium
	/	dS/m	cmol(+)/kg	%	mg/kg	mg/kg	mg/kg
After one growing season							
Control	7.03b	0.30a	2.88a	0.83a	35.31a	2.05a	21.02a
May	7.15b	0.29a	2.97a	0.81a	36.95a	2.00a	17.42a
May and June	7.30ab	0.31a	2.72a	0.87a	35.08a	1.98a	21.22a
May and July	7.52a	0.30a	3.17a	0.82a	38.78a	2.07a	21.08a
May and August	7.31ab	0.31a	3.00a	0.77a	34.52a	2.00a	18.06a
After two growing seasons							
Control	7.31b	0.10a	2.93a	0.20a	33.11a	1.63b	30.56c
May	7.62a	0.10a	3.11a	0.26a	27.92a	2.65a	60.04a
May and June	7.94a	0.10a	3.01a	0.18a	24.89a	1.53b	51.11ab
May and July	7.93a	0.10a	3.07a	0.21a	32.72a	2.00ab	49.11ab
May and August	7.94a	0.10a	2.87a	0.23a	31.24a	1.75b	45.11b

Table 3.2 Alfalfa mean height, main root length, lateral root length, shoot biomass, root biomass, and total biomass with different application timing. Means in columns with different letters are significantly different.

Application timing	Height	Main root length	Lateral root length	Shoot biomass	Root biomass	Total biomass
	cm	cm	cm	g/plant	g/plant	g/plant
			After one growing season			
Control	29.51b	29.06a	6.49b	0.85ab	0.45b	1.31ab
May	40.22a	29.79a	8.31a	1.61a	0.80a	2.38a
May and June	29.30b	21.63b	5.15b	0.78ab	0.34b	1.13ab
May and July	25.44b	22.67b	5.54b	0.69ab	0.32b	1.01ab
May and August	23.37b	24.86ab	7.46ab	0.27b	0.19b	0.46b
			After two growing seasons			
Control	57.91b	67.09b	16.91b	2.24b	3.05b	5.29b
May	90.73a	98.69a	35.33a	30.34a	14.57a	44.91a
May and June	108.98a	92.90ab	44.92a	37.03a	25.40a	62.43a
May and July	92.02a	90.22ab	25.68ab	14.17a	14.76ab	28.93ab
May and August	100.04a	110.12a	30.84ab	20.85a	13.16ab	34.01ab

Table 3.3 Barley mean height, root length, shoot biomass, root biomass, and total biomass with different application timing. Means in columns with different letters are significantly different.

Application timing	Height	Root length	Shoot biomass	Root biomass	Total biomass
	cm	cm	g/plant	g/plant	g/plant
			After one growing season		
Control	20.13b	8.30a	0.39a	0.15ab	0.55a
May	25.49a	7.96a	0.58a	0.23a	0.81a
May and June	16.29b	6.70a	0.17a	0.06b	0.23a
May and July	19.29b	7.83a	0.36a	0.21ab	0.57a
May and August	17.34b	7.96a	0.28a	0.12ab	0.40a
			After two growing seasons		
Control	28.20b	12.17b	0.62b	0.28b	0.91b
May	42.75a	13.92a	1.88a	1.29a	3.17a
May and June	28.00b	12.66ab	0.47b	0.28b	0.75b
May and July	28.56b	11.23b	0.74b	0.40b	1.14b
May and August	32.66b	13.31ab	1.12b	0.59b	1.71b

Table 3.4 Sea buckthorn mean height, main root length, lateral root length, stem diameter, shoot biomass, root biomass, and total biomass with different application timing. Means in columns with different letters are significantly different.

Application timing	Height	Main root length	Lateral root length	Stem diameter	Shoot biomass	Root biomass	Total biomass
	cm	cm	cm	mm	g/plant	g/plant	g/plant
After one growing season							
Control	54.92a	29.01a	8.05a	5.46a	10.68a	3.20a	13.88a
May	57.57a	28.56a	8.73a	5.46a	11.20a	3.57a	14.77a
May and June	45.41a	25.39a	7.62a	4.65a	6.59a	2.71a	9.31a
May and July	60.63a	27.42a	9.68a	5.85a	10.76a	4.10a	14.86a
May and August	56.57a	26.73a	7.10a	4.97a	9.73a	3.08a	12.81a
After two growing seasons							
Control	99.44c	105.57c	43.66c	14.41b	231.77b	106.03b	337.80b
May	136.34a	171.88a	91.96a	20.71a	611.91a	221.22a	833.14a
May and June	129.93ab	166.65ab	74.73b	20.90a	548.61a	200.57a	749.18a
May and July	110.99bc	132.58bc	65.63b	20.23a	458.98a	210.46a	669.43a
May and August	128.72ab	162.09ab	69.57b	20.69a	490.29a	277.71a	768.00a

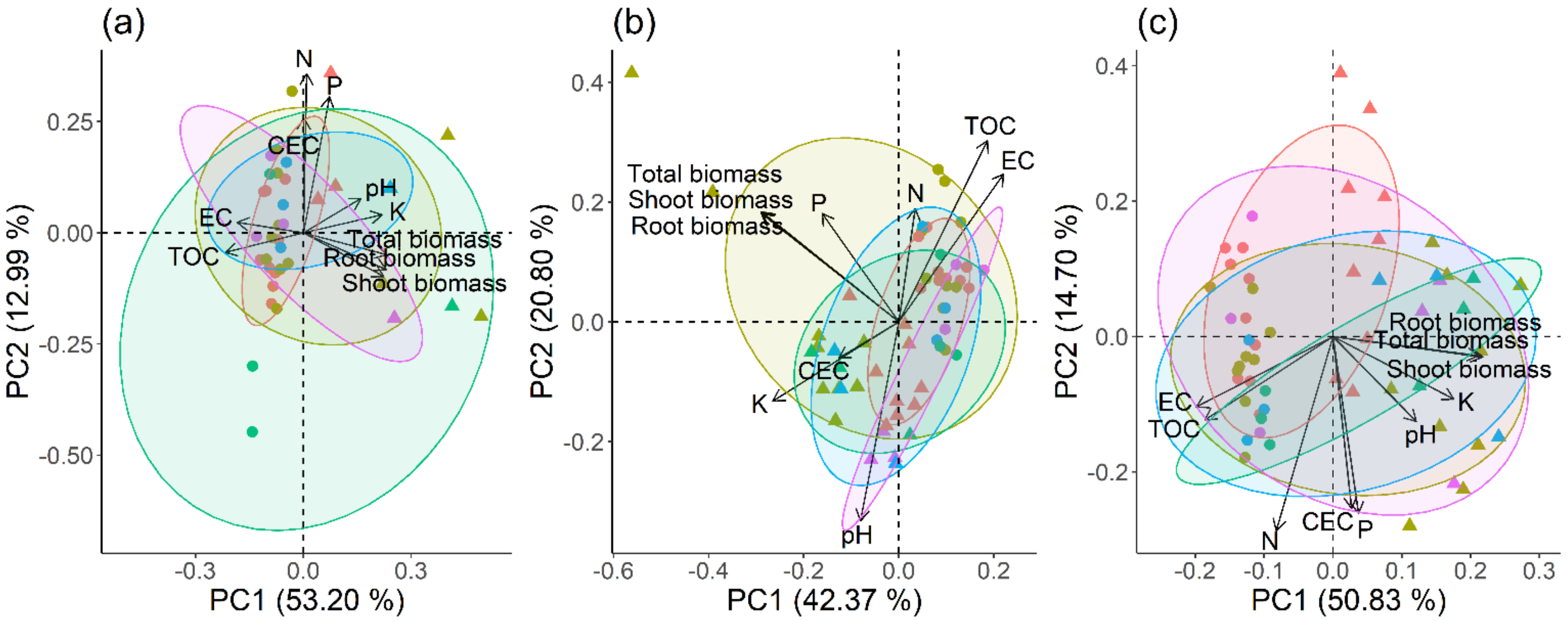

Figure 3.1 Principal component analysis of soil and plant parameters of (a) alfalfa, (b) barley, and (c) sea buckthorn. Soil parameters include pH, electrical conductivity (EC), total organic carbon (TOC), cation exchange capacity (CEC), and available nitrogen (N), phosphorus (P) and potassium (K). Plant parameters include root, shoot, and total dry biomass. Different colours represent application timing and point shapes represent experiment year. Data points of the same application timing are grouped together in an ellipse.

IV. Soil Amendment With A Humic Substance And Arbuscular Mycorrhizal Fungi Enhance Coal Mine Reclamation

1. Introduction

Heavy metal pollution is an increasing environmental concern worldwide due to high biotoxicity, mobility, and long persistence in the environment (Volesky & Holan, 1995; Qaiser et al., 2007). Coal mining is one of the major sources through which heavy metals enter the environment before being transmitted to the food chain (Musilova et al., 2016; Raj et al., 2019; Sidhu et al., 2019). Heavy metals are contained in coal refuse and fly ash, and they can be released into soils as a result of coal industry activities, such as production and accumulation of coal gangue, migration and sedimentation of wastewater, and coal transportation (Li et al., 2018). Agricultural lands are one of the most common end land uses after reclamation due to the shortage of agricultural lands worldwide (Palogos et al., 2017). The main difficulties for reclamation on contaminated sites may be attributed to lack of essential nutrients, together with high metal concentrations (Vangronsveld et al., 1996; Maiti, 2012). Sandy soils are particularly prone to nutrient losses due to their high infiltration capacity, low water holding capacity, and low nutrient retention capacity (Von Uexkull, 1986), making vegetation establishment difficult. Although inorganic fertilizers can provide essential mineral elements to obtain better growth, they easily leach in sandy soils.

Humic substances are the most stable component of soil organic matter and they are characterized as complex and supramolecular self-assembled mixtures of diverse heterogeneous hydrophobic, and hydrophilic compounds that survive microbial mineralization (Piccolo, 2002). They are known as distinctive heavy metal remediators due to high abundance of exchangeable functional groups (Havelcová et al., 2009), as soil conditioners to improve soil fertility by modifying soil physiochemical environments (Kalbitz et al., 2000; Bronick & Lal, 2005; Bezuglova et al., 2017), and as natural biostimulators to enhance plant growth (Chen et al., 2004; Tahir et al., 2011; Ciarkowska et al., 2017).

Arbuscular mycorrhizal fungi (AMF) are common soil microorganisms that build mutualism symbiotic relationships with the roots of most plant species, facilitating uptake of mineral nutrients from the soil in exchange for carbohydrates, and thus become an important plant root component (Smith & Read, 2008; Brundrett, 2009). They can improve soil properties by increasing aggregate stability (Rillig, 2004; Cozzolino et al., 2013), and improve plant-water relations through the AMF filament network which enables plants to search water and nutrients deeper and wider in the soil profile (Toro et al., 1998; Wang et al., 2009; Treseder, 2013). Only 24 % of AMF studies were conducted in the field (Berruti et al., 2016), and investigations on its effects in heavy metal contaminated soils are not abundant, and have inconsistent results (Khan, 2001; Vogel-Mikuš et al., 2006).

The combined use of humic substances and soil biota has generated great research interest. The use of microbial inoculants in combination with humic substances leads to better plant development and yield (Canellas et al., 2013; da Piedade Melo et al., 2017; da Silva et al., 2017). Humic substances are important to development of soil biota including AMF (Gryndler et al., 2005). Plant root biological activity (watercress), root cell proliferation, and length elongation (maize), and root branching (rockcress) were enhanced by humic substances (Piccolo et al., 1992; Canellas et al., 2002; Casimiro et al., 2003), which may increase contact between plants and AMF hyphae (Gryndler et al., 2009), stimulating mycorrhizal colonization and enhancing plant growth. However, studies on the synergistic effects of humic substances and AMF on plant growth and soil properties are very limited. Therefore, the objective of our study was to assess the effect of sole and combined applications of nano humus (a lignite derived humic substance product), arbuscular mycorrhizal fungi, and inorganic fertilizer, as soil amendments on soil properties, heavy metal remediation, and plant growth on a former coal mine under field conditions.

2. Materials and Methods

2.1 Study area

The study area was located in one of China's largest former underground coal mines, the Shendong mining area (39.31249°N, 110.2097°E), covering approximately 72.4 km^2 area in northern China. The mine area lies 1,200 m above sea level in Ordos plateau in Mu Us desert - Loess Plateau transitional zone and has a semi-arid climate. Approximately 60 to 70 % of precipitation in this region is concentrated in July, August, and September. Mean annual evaporation (2,300 mm) is almost six times greater than the annual precipitation. Mean annual temperature is 8.5 °C (-15.9 to 29.8 °C in a year). Soils were sand texture (90 % sand, 2 % silt, 8 % clay) with neutral pH, and generally lacking organic carbon (0.8 %) and essential nitrogen, phosphorus, and potassium (< 40 mg/kg). Soils were single grained structure and loose in consistency under both wet and dry conditions. They contained high concentrations of heavy metals, such as thallium (Tl), arsenic (As), cadmium (Cd), and zinc (Zn). The experimental location was procured from Shendong coal group, and three sites were established on a 500 m^2 unvegetated flat area, as slopes might cause soil and plant variability.

2.2 Plant materials and soil amendments

Alfalfa (*Medicago ruthenica* L.) and barley (*Hordeum vulgare* L.) were used for our study as they are common agricultural and reclamation species widely used around the world. They can form a symbiotic association with AMF (Goicoechea et al., 2014; Williams et al., 2017) and have high adaptability to a wide range of climate conditions (Michaud et al., 1988; Erice et al., 2010; Ligaba & Katsuhara, 2010; Gürel et al., 2016). Seeds were purchased from a local seed company.

2.3 Soil amendments

Humic substances, arbuscular mycorrhizal fungi, and fertilizer were used as soil amendments. A lignite derived humic product, nano humus, was obtained from a commercial source, Wuhan Shangyuan Environmental Protection Co, Ltd (Beijing, China). Nano humus is a black, fine grained, partially soluble substance, containing approximately 83.2 % organic matter, 50 % humic acid, and 1 % micronutrients (copper, iron, zinc, aluminum, manganese, boron); macronutrients include 0.86 % nitrogen, 1.11 % phosphorus (P_2O_5), 5 % potassium (K_2O), 0.29 % sulphur, 0.34 % magnesium; with 4 % silica sand and ash by weight. Functional groups of nano humus were determined by Fourier transform infrared spectroscopy (FTIR) (Table S4.), and surface elemental composition with energy dispersive X-ray analysis (Table S4.1). Nano humus had a pH of 9 and an electrical conductivity of 0.08 dS/m (Oakton 300 Series, IL, USA). Nano humus was dissolved in water (1:100 solid to water ratio) to produce an aqueous suspension, then sprayed on the soil surface at 150 g/m^2 (producer recommendations). AMF amendment was with spores of *Glomus mosseae* and *Glomus etunicatum* at a 1:1 mix provided by Gansu Research Academy of Forestry Science and Technology. There were 14 to 16 AMF infective propagules per gram of the material, approximately 3,500 to 4,000 propagules /m^2. The microbial inoculum was introduced using a targeted method widely used in field studies, in which AMF inocula and seeds were put in the same planting holes when seeding and covered with surface soils. AMF amendment was applied at 250 g/m^2 according to previous practices (Bi et al., 2007; Du et al., 2008; Wang et al., 2014). Inorganic fertilizer was purchased from a local supplier, containing 26 % nitrogen, 10 % phosphorus, and 9 % potassium. It was applied at 37 g/m^2 (producer recommendations).

2.4 Experimental design

A split plot experimental design was implemented in the field in two growing seasons (May to August) to determine amendment effects on soil properties, heavy metal remediation, and plant growth. There were seven amendments, including control (untreated soil); nano humus; AMF; inorganic fertilizer; nano humus with inorganic fertilizer; AMF with nano humus; and AMF, nano humus, and fertilizer together. Two plant species were randomly assigned in the main plot; 7 soil amendments were assigned in split plots nested within two plant species. The experiment was replicated 3 times (3 blocks). Experimental blocks were 5 m x 10 m, each divided into 2 main plots, 5 m x 5 m in size. Each main plot contained 7 split plots (1 m x 1 m) with a 0.5 m distance between split plots.

2.5 Vegetation measurements

Alfalfa and barley were seeded in May of the first study year, and barley at the same time in the second study year. A sprinkler system was installed on sites and managed by Shendong coal group. The seeding rate of alfalfa was 160 kg/ha (57 % pure live seed), equivalent to 2700 seeds per split plot. Barley was seeded at 30 kg/ha (76 % pure live seed), equivalent to 150 seeds per split plot. Plants were watered every day in the first two weeks, then every 2 days in June, and every week in July and August when precipitation more frequently occurred; this was repeated for two years. Emergence was estimated one month after seeding by dividing the number of emerged seedlings by the number of planted seeds. Plant height was measured with a ruler from the soil surface to the highest live leaf every month during the first and second growing seasons. At the end of each growing season, a final assessment was conducted including plant height, main and lateral root length, and dry biomass (shoot, root, total) for alfalfa; plant height, root length, leaf number, leaf width, seed production, and dry biomass (shoot, root, total) for barley. Root length was measured with a ruler. Leaf number was counted by hand, and leaf width was measured with a digital caliper. Each individual seed head was counted and the number of seeds in each head was averaged. Root and shoot samples were taken individually and washed gently before being oven dried for 48 hours at 80 °C. Dry biomass of root, shoot, and the total was then weighed. For each split plot, five plant samples were selected and measured along two intersecting diagonal lines to represent the mean value for each treatment. Biomass samples were delivered to a commercial laboratory for tissue analysis of total nitrogen, phosphorus, and potassium (LY/T 1269-1999). Due to a severe spring drought in April 2019 and damage of sprinklers in block 1 and 3, only alfalfa in block 2 remained in the second year for vegetation assessment.

2.6 Soil measurements

Soil was sampled from each block before planting to provide baseline information. At the end of each growing season (end of August), soil was sampled from each split plot. Soil pH and electrical conductivity (1:10 soil water suspension ratio) were determined electrometrically (Carter &

Gregorich, 2008). Cation exchange capacity was measured by exchange with calcium acetate buffered at pH 8.2 (Chapman, 1965). Total organic carbon was determined by combustion and available nitrogen by potassium chloride extraction (Carter & Gregorich, 2008). Available phosphorus and potassium were determined by modified Kelowna extraction (Ashworth & Mrazek, 1995). These soil variables were assumed to be the most influenced by addition of organic matter and mycorrhizae; and their levels represent soil productivity in agriculture. At the end of the experiment (in year two), soil thallium, arsenic, cadmium, and zinc concentrations were determined for each split plot. Thallium and cadmium were determined by graphite furnace atomic absorption spectrophotometry, arsenic by atomic fluorescence spectrometry, and zinc by flame atomic absorption spectrophotometry (Tüzen, 2003). All soil samples were taken with a hand shovel from the upper 15 cm of surface soil. Samples were kept cool until sending to a commercial laboratory for analysis.

2.7 Mycorrhizal measurements

To estimate root AMF colonization, root samples were collected from three randomly selected individual plants for each split plot at the end of August of each study year. Preparation procedure followed that of Vierheilig et al. (1998). For each individual sample, roots were washed to remove soil before cutting into 1 cm long segments and soaked in 70 % formalin-acetic acid-alcohol over 4 hours in a 50 ml plastic bottle for storage purposes. Root segments were clarified in 25 ml 10 % potassium hydroxide (KOH) in a 50 ml beaker and boiled at 90 ºC in a water bath for one hour. An extra fine sieve was used to collect root segments before transferring to another 50 ml clean beaker filled with tap water. Root segments were rinsed with water until water was clear. The rinsed samples were then acidified with dilute hydrochloric acid (2 %). The clarified root segments were stained with 5 % ink vinegar solution (95 ml 5 % acetic pure white household acid vinegar and 5 ml blue ink) for 3 minutes in a 50 ml beaker. Roots were then rinsed in tap water following the above rinse procedure. These steps removed the host cytoplasm and most nuclei which can make the vascular cylinder visible. AMF colonization was assessed according to Trouvelot et al. (1986). For each individual plant root sample, two slides were prepared. There were 15 root segments (1 cm) randomly selected from all prepared root segments and placed horizontally on one slide with a tweezer, 30 segments for two slides. Two to three drops of lactic acid (5 %) were placed on each slide. Root segments on slides were observed under the microscope and the presence or absence of colonization was recorded for calculation. In total, 210 root samples, 420 slides, 6,300 root segments were assessed for this two year study. Colonization % was calculated as:

(number of mycorrhizal segments / total number of segments) × 100.

2.8 Statistical analyses

Statistical analyses were performed with R software (version 3.6.1); significance was accepted at $p < 0.05$. Data were confirmed for normality (QQ plot and Shapiro-Wilk test) and equal variances assumptions (Levene's test). Three way analysis of variance (ANOVA) was performed separately to investigate the main effects and their interactions of three fixed factors (soil amendment, year, plant species) upon measured soil chemical variables and soil metals. Block and species nested in blocks were incorporated as random factors. One way ANOVA was used to test amendment effect (main effect) and block effect (random effect) on various barley growth parameters. Amendment effect on alfalfa growth parameters was conducted by one way ANOVA without taking random effects into account. Tukey's honest significant difference test was then performed for pairwise comparisons. Pearson product-moment correlation was used to determine relationships between mycorrhizal colonization rate and soil heavy metal concentrations, and between colonization rate and growth parameters. The correlation coefficient (r) is a measure of the dependence between two variables.

3. Results

3.1 Amendment effects on soil properties

There were no significant changes in measured soil chemical variables by amendment applications in the first year of the experiment, with differences becoming significant in the second year for most parameters (Figure 4.1). The combined application of AMF and humic substance was generally more effective in improving soil properties for plant growth than sole applications, although sole applications showed improvement for most measured soil parameters relative to the control.

Soil pH, a key variable that determines nutrient availability in soils, and electrical conductivity, a measure of salinity, did not vary significantly among amendments. The pH generally increased from 7.3 in the first growing season to 7.6 in the second; electrical conductivity decreased slightly from 0.33 to 0.29 dS/m over the experiment period.

Cation exchange capacity reflects the ability of soil to hold cation nutrients and it increased with all amendment combinations, except sole application of fertilizer. Nano humus with fertilizer showed the greatest enhancement (47 %) relative to the untreated control. Sole and combined applications of nano humus and AMF (with or without fertilizer) showed a significant improvement (37 to 38 %).

Total organic carbon is an important indicator of soil health. Relative to the control, it was 49 % greater with sole applications of nano humus, followed by 45 % with combined application of nano humus and fertilizer, 43 % with the three materials together, and 36% with AMF and nano humus. There were no obvious improvements with sole applications of AMF or fertilizer.

Soil available nitrogen, phosphorus, and potassium are essential macronutrients needed by all plants. Applying inorganic fertilizer released rapidly available forms of nutrients into sandy soils, increasing by 45 % nitrogen, 115 % phosphorus, and 67 % potassium relative to untreated soils. The combined application of AMF and nano humus resulted in a remarkably similar increase in soil available nitrogen (46 %) as fertilizer, a lower increase in phosphorus (20 %), and a higher increase in potassium (92 %). Sole applications of AMF and nano humus also increased soil nitrogen (51 and 29 %, respectively) and potassium (32 and 24 %, respectively).

3.2 Soil metals and mycorrhizal colonization

Applying amendments for two growing seasons reduced heavy metal concentrations, except for zinc, although the changes were not significant (Table 4.1). Nano humus reduced thallium by 25 %, cadmium by 18 %, and arsenic by 3 %. AMF showed a 20 % reduction in thallium, 17 % in cadmium, and 2 % in arsenic. Combining nano humus with AMF reduced cadmium 8 % and arsenic 7 % more than their sole applications.

The greatest difficulty for field experiments is that non inoculation controls usually contain natural AMF propagules. Coal mining sites with low nutrient sandy soils and large anthropogenic disturbance have reduced the abundance of natural soil AMF. There was no mycorrhizal colonization observed in non inoculated plants, confirming the effect of native AMF was eliminated with mining. In AMF inoculated plants, a typical structure of mycorrhizal colonization including arbuscules and highly branched hyphal structures inside cortical root cells after penetration were observed under the microscope (Figure 4.2).

AMF colonization was low in the first growing season when high concentrations of heavy metals were on sites. It was relatively higher in alfalfa (34 %) than barley (9 %). Mycorrhizal colonization was more successful in the second growing season when metal concentrations were reduced; 90 % on alfalfa roots and 81 % on barley roots. Colonization rate was significantly negatively correlated with thallium ($p < 0.001$, $r = -0.75$), arsenic ($p < 0.001$, $r = -0.87$), cadmium ($p < 0.001$, $r = -0.74$), and zinc ($p < 0.001$, $r = -0.75$) concentrations in soils. This indicates that the low mycorrhizal colonization in the first year can be attributed to the high soil heavy metal concentrations. Relative to sole application of AMF, AMF combined with nano humus significantly improved barley root mycorrhizal colonization rate from 76 to 86 %, while that of alfalfa was less significant (Table 4.2).

3.3 Amendment effect on alfalfa and barley growth

Alfalfa emergence was low in treated and untreated sandy soils at approximately 16 % with no significant difference among amendments. All amendments showed positive effects on alfalfa growth (Table 4.3). In the first growing season, nano humus with fertilizer most positively impacted alfalfa total biomass (10 times increase) and plant height (42 % increase) than other amendments. In the second growing season, mycorrhizal colonization was high with obvious beneficial effects on alfalfa growth. Relative to applying nano humus alone, combining it with AMF resulted in a more pronounced stimulation of alfalfa biomass production, with an additional increase of 8 times and 7 times in root and shoot biomass, respectively. The combination of nano humus and AMF enhanced 18 times in root biomass, 12 times in shoot biomass, 4 times in main root length, 1 time in height, and 0.5 times in lateral root length.

Barley emergence was 46 % higher than alfalfa, with slight variability among amendments. Barley responded positively to all amendments, although it was less obvious than alfalfa (Table 4.4). Barley might require greater soil nutrients and it responded most positively on most growth parameters to addition of fertilizer in the first growing season. Similarly, the synergistic effect of nano humus and AMF was greater than that of nano humus alone when AMF successfully colonized barley root tissues. The beneficial effect was particularly prominent with fertilizer added. The combination of nano humus, AMF, and fertilizer together enhanced 379 % in root biomass, 345 % in shoot biomass, 301 % in seed production, 71 % in leaf number, 69 % in plant height, 37 % in leaf width, and 29 % in root length relative to the control.

The relationship between mycorrhizal colonization rate and growth parameters was analyzed only in AMF treatment groups to avoid confounding effects from other materials. Both species showed a significant ($p < 0.05$) positive correlation between colonization rate and root development. Alfalfa colonization showed the strongest correlation with main root length ($r = 0.83$) and root biomass ($r = 0.72$). Barley colonization had the most significant correlation with root length ($r = 0.69$), followed by root biomass ($r = 0.57$). Enhanced growth was hypothesized to be associated with greater nutrient uptake by plants. Unexpectedly, there was no statistically significant difference between AMF inoculated and non inoculated plants at the final harvest (Table 4.5), and mycorrhizal colonization rate showed no significant correlation with total nitrogen, phosphorus, and potassium in root or shoot tissues ($p > 0.05$).

4. Discussion

Our findings provided insights on field assessment of the synergistic effect of AMF and nano humus, and added empirical support for greater benefits through combined applications than corresponding sole applications. Mechanisms of these effects (Figure 4.3) were multiple, each improving some facet of the soil environment for plant growth and development, that collectively had a major impact. Nano humus addition modified the soil physiochemical environment, providing better growth conditions to both host plants and AMF. The lower concentrations of soil metals and the enlarged plant roots induced by nano humus may collectively result in higher mycorrhizal colonization. The enhanced symbiotic relationship was likely to enable host plants to acquire more nutrients and water through the AMF filament network, which served as an extension of roots, leading to better growth of plants. In turn, more carbohydrates could be potentially allocated to AMF, which could improve AMF development and provide greater beneficial impacts.

In our experiment, addition of nano humus elevated soil nutrient availability, total organic carbon, and cation exchange capacity, consistent with previous findings on humic materials (Ibrahim & Goh, 2004; Turgay et al., 2011; Bezuglova et al., 2017; Ciarkowska et al., 2017). Humic substances are rich in soil available organic carbon and contain a small amount of nutrients that would be available to soil biota such as AMF and to plants (Mantoura et al., 1978; Kalbitz et al., 2000). Nano humus increased soil cation exchange capacity 38 % after two years, which means more cations could be held and there would be less nutrient leaching in sandy soils. Through cation exchange, more nutrient elements could be released from humic materials into the soil. With a 49 % increase in soil total organic carbon, nano humus served as a reservoir of organic carbon. In nutrient poor soils, carbon supplies from plants might not be the only limiting factor as addition of soil available nutrients increased AMF growth (Treseder & Allen, 2002). Consequently, changes in soil organic carbon and nutrient availability may have a direct impact on AMF development. Applying AMF with nano humus showed similar soil available nitrogen and greater available potassium relative to the sole application of inorganic fertilizer (26 % nitrogen, 10 % phosphorus, 9 % potassium). This implies that the amendment combination of AMF and nano humus may potentially substitute, or at least reduce, chemical fertilizer input in soils in reclamation.

Increased soil cation exchange capacity can be linked to high content of oxygen containing functional groups in humic substances, which leads to 500 to 1500 cmol (+) per kg cation exchange capacity (Tan, 2003). The higher the cation exchange capacity, the higher the negative charge, enabling humic materials to adsorb heavy metals in the soil (Uchimiya, Lima, Thomas Klasson, et al., 2010). Soil toxic metal concentrations, such as thallium, cadmium, and arsenic, were originally high in our soils, resulting in low mycorrhizal colonization for alfalfa and barley in the first growing season, exemplified by the significant negative correlation between colonization rate and metal concentrations. This finding was consistent with other studies showing inhibition of heavy metals on mycorrhizal colonization (Khan, 2001; Chen et al., 2005), although few studies reported the opposite results (Vogel-Mikuš et al., 2006; Dietterich et al., 2017).

Nano humus with fertilizer application acted as a nutrient carrier in both water soluble and complexed forms, alleviating nutrient deficiencies in sandy soils. They prominently increased root length and root biomass for two species, an effect also noted by Suman et al. (2017). Humic substances can chemically interact with plant cell membranes after penetration and trigger root development (Kulikova et al., 2013, 2016) due to the presence of auxin-like materials in humic substances (Canellas et al., 2002; Trevisan et al., 2010, 2011), thus affecting root development. The enlarged root system is likely to increase contact between plants and AMF hyphae (Gryndler et al., 2009), stimulating mycorrhizal colonization.

AMF colonization rate, an important measure of a symbiotic relationship, increased with humic substances directly, and indirectly in the second study year which eventually provided greater benefits. The filament network of AMF can bind particles and stabilize soil aggregates (Fulton, 2011) in which soil organic matter is the main agent (Rillig, 2004). It is likely to link increased soil available nitrogen and potassium with the interaction of AMF and solubilizing bacteria and other beneficial microorganisms (Mukhopadhyay & Maiti, 2011; Toro et al., 1998). AMF reduced heavy metals in soils by immobilization in their fungal structures (Gonzalez-Chavez et al., 2002; Andrade et al., 2010), and by production of glomalin protein that chelates or binds metals in the soil (Gonzalez-Chavez et al., 2002; Bothe et al., 2010). The interaction of humic substances and AMF thus likely improved AMF development by increasing spore quantity, glomalin contents (Pinos et al., 2019), and extraradical mycelium production (Gryndler et al., 2005). This could explain the greater improvement in soil properties with combined amendments, although published evidence of their combined effect on soil properties is rare.

Modifications in soil physiochemical environments and root contact collectively increased AMF colonization, suggesting a stronger mutualism relationship between mycorrhizae and host plants.

It was clear from our results that a higher colonization rate had greater enhancement on root length and biomass. Our results were in agreement with Lekberg & Koide (2005) who conducted a meta analyses of 290 published studies. A greater root system means a plant can better absorb and utilize water and nutrients from soils, maintaining better growth and development. This is especially important for plants growing in sandy soils with limited water and nutrient resources, as in our study. It was widely reported that AMF can improve plant growth by facilitating nutrient and water absorption from the soil (Smith & Read, 2008; Brundrett, 2009; Fulton, 2011). Therefore, we had hypothesized that biomass enhancement with combined AMF and nano humus amendment would be explained by enhanced nutrient contents in plant tissue. However, we did not observe significantly elevated tissue nutrients in alfalfa and barley at maturity. We thought this might be observed at an earlier growth stage rather than maturity as the differences can potentially disappear or be minimized over time (Gavito & Miller 1998); Singh & Tilak 1992). Whether this happens at early stages of plant development would be important to determine timing of amendment applications to most enhance reclamation success. The first growing season is critical to revegetation success. If plant roots do not develop well, the plant itself will be unable to move into later stages of development, including reproduction. Research will be needed to provide answers on the phenomenon observed in this study.

The combined application of AMF and nano humus showed distinct stimulation on root and shoot biomass for both species in our experiment. Several studies have proven the stimulating effect of sole applications of humic substances (Schmidt et al., 2007; Eyheraguibel et al., 2008; Ciarkowska et al., 2017) and AMF (Singh & Tilak, 1992; Bi et al., 2007; Treseder, 2013). However, only a few studies investigated the synergistic effect of AMF and humic substances and most were conducted in the greenhouse. Although greenhouse experiments are useful for testing specific hypotheses, studies with AMF and humic substances may not provide generalizable principles that affect plant growth under natural conditions. AMF inoculation might provide more benefits to plant root biomass under field than greenhouse conditions due to constraints imposed by growth containers (Berruti et al., 2016). Our field results were more significant than a greenhouse study by Wang et al. (2014) that used sandy soils from the same mining area. They observed a 73 % increase in corn biomass after applying AMF and humic substances. Biomass enhancement was evidenced by Pinos et al. (2019) who found an equivalent to 58 % and 33 % increase in root and shoot biomass of corn that grew in sandy soils with addition of humic substances and AMF in the greenhouse. Khaf et al. (2018) reported a 6 times increase in strawberry root development in nurseries (soil texture not mentioned). Field studies were very limited, making it difficult for direct comparisons due to different experiment conditions (field versus greenhouse), growth substrate (soil characteristics), and plant species.

5. Conclusions

Our study provided empirical evidence of the effect of sole and combined application of a lignite derived humic substance material (nano humus), AMF, and inorganic fertilizer on soil chemical properties, soil heavy metal concentrations, and alfalfa and barley growth during two field seasons. The combined use of humic substances and AMF positively impacted most measured plant and soil variables, particularly plant biomass, and soil cation exchange capacity, total organic carbon, nutrient contents, and heavy metals. The synergistic effects were quantitatively more pronounced on plant growth than corresponding sole applications. The addition of humic substances improved soil physiochemical environments, which increased mycorrhizal root colonization and strengthened symbiotic benefits to plant growth. These findings may have important implications for vegetation establishment in heavy metal contaminated reclamation sites.

Table 4.1 Changes of soil thallium, arsenic, cadmium, and zinc with different amendment applications after two growing seasons.

Amendment	Thallium	Arsenic	Cadmium	Zinc
	mg/kg			
Control	0.17	0.87	0.27	19.10
Nano humus	0.13	0.85	0.22	19.10
AMF	0.14	0.86	0.22	18.70
Fertilizer	0.11	0.90	0.16	19.60
Nano humus + fertilizer	0.13	0.81	0.20	17.70
AMF + nano humus	0.16	0.80	0.20	19.30
AMF + nano humus + fertilizer	0.15	0.86	0.20	19.10

Table 4.2 Mycorrhizal colonization rate (%) of AMF inoculated amendments. Means in columns with different letters are significantly different.

Amendment	First year		Second year	
	Alfalfa	Barley	Alfalfa	Barley
AMF	54.73a	7.42a	92.22a	75.56b
AMF + nano humus	21.03b	7.52a	86.67a	85.56a
AMF + nano humus + fertilizer	25.13b	7.43a	91.11a	82.22a

Table 4.3 Alfalfa mean total biomass, root biomass, shoot biomass, height, main root length, and lateral root length with soil amendments. Means in columns with different letters are significantly different.

Amendment	Total biomass	Root biomass	Shoot biomass	Height	Main root length	Lateral root length
	g/plant	g/plant	g/plant	cm	cm	cm
2018						
Control	0.23c	0.12c	0.11c	17.36a	23.14a	5.48a
Nano humus	0.30c	0.14c	0.17c	16.38a	23.78a	6.28a
AMF	0.94bc	0.33bc	0.61b	24.08a	30.40a	11.12a
Fertilizer	0.70bc	0.35bc	0.35bc	21.84a	30.32a	5.141a
Nano humus + fertilizer	2.56a	1.48a	1.08a	24.66a	37.82a	8.42a
AMF + nano humus	1.33b	0.71b	0.63b	18.62a	25.88a	6.38a
AMF + nano humus + fertilizer	0.54bc	0.23bc	0.31bc	18.80a	31.74a	5.50a
2019						
Control	6.18b	2.17b	4.01b	38.36c	43.96d	9.80c
Nano humus	50.29b	23.50ab	26.79b	76.10abc	108.86bc	17.38bc
AMF	233.59a	62.04a	171.55a	125.14a	170.80a	34.16a
Fertilizer	28.55b	10.57b	17.98b	45.44bc	72.22cd	18.72bc
Nano humus + fertilizer	98.70ab	41.14ab	57.56b	87.46abc	133.04ab	23.94ab
AMF + nano humus	93.53b	40.49ab	53.04b	94.74ab	118.06abc	15.04bc
AMF + nano humus + fertilizer	84.60b	34.31ab	50.29b	68.26bc	68.10cd	18.84bc

Table 4.4 Barley mean total biomass, shoot biomass, root biomass, height, leaf number, leaf width, and root length with soil amendments. Means in columns with different letters are significantly different.

Amendment	Total biomass g/plant	Shoot biomass g/plant	Root biomass g/plant	Height cm	Leaf number /	Leaf width mm	Root length cm
2018							
Control	0.32c	0.25b	0.06c	20.60cd	5.47b	2.60a	10.42ab
Nano humus	0.50bc	0.44b	0.06c	18.10d	5.53b	2.44a	13.10a
AMF	0.53bc	0.43b	0.10c	22.70cd	5.60b	2.64a	6.34c
Fertilizer	1.16a	0.93a	0.23ab	29.70ab	8.20a	4.07a	11.19ab
Nano humus + fertilizer	0.89ab	0.62ab	0.27ab	25.50abc	6.27ab	4.02a	8.20bc
AMF + nano humus	0.61bc	0.52ab	0.10c	20.60cd	5.67b	3.44a	11.10ab
AMF + nano humus + fertilizer	0.89ab	0.76ab	0.13bc	30.00a	4.53b	3.10a	10.77ab
2019							
Control	0.75b	0.53b	0.22b	26.20c	9.80c	3.95a	13.40c
Nano humus	0.86b	0.57b	0.29b	30.30bc	10.90bc	4.33a	14.30bc
AMF	2.23ab	1.52ab	0.71ab	37.20ab	15.10abc	5.38a	15.30ab
Fertilizer	2.92a	1.95a	0.97a	38.90ab	15.70ab	5.81a	16.20ab
Nano humus + fertilizer	1.88ab	1.30ab	0.58ab	33.20bc	14.70abc	5.03a	15.30ab
AMF + nano humus	0.96b	0.66b	0.30b	29.20c	12.20abc	4.73a	18.10a
AMF + nano humus + fertilizer	3.40a	2.37a	1.04a	44.20a	16.80a	5.42a	17.30ab

Table 4.5 Mean nitrogen, phosphorus, and potassium concentrations in plant tissues with soil amendments.

	Nitrogen		Phosphorus		Potassium	
Amendment	Root	Shoot	Root	Shoot	Root	Shoot
Alfalfa			g/kg			
Control	0.18	0.15	0.12	0.20	6.06	11.55
Nano humus	0.24	0.16	0.15	0.16	4.76	14.79
AMF	0.20	0.21	0.21	0.20	7.42	14.18
Fertilizer	0.24	0.21	0.12	0.21	5.35	13.22
Nano humus + fertilizer	0.17	0.19	0.17	0.18	6.80	14.20
AMF + nano humus	0.24	0.14	0.15	0.15	6.55	11.85
AMF + nano humus + fertilizer	0.23	0.22	0.24	0.19	6.85	13.22
Barley						
Control	0.12	0.10	0.15	0.20	5.00	6.76
Nano humus	0.14	0.22	0.20	0.21	7.82	6.74
AMF	0.11	0.25	0.11	0.15	5.63	6.59
Fertilizer	0.13	0.18	0.09	0.20	4.03	6.15
Nano humus + fertilizer	0.19	0.23	0.08	0.17	3.77	5.65
AMF + nano humus	0.13	0.19	0.07	0.26	5.77	8.23
AMF + nano humus + fertilizer	0.15	0.10	0.16	0.18	5.24	5.53

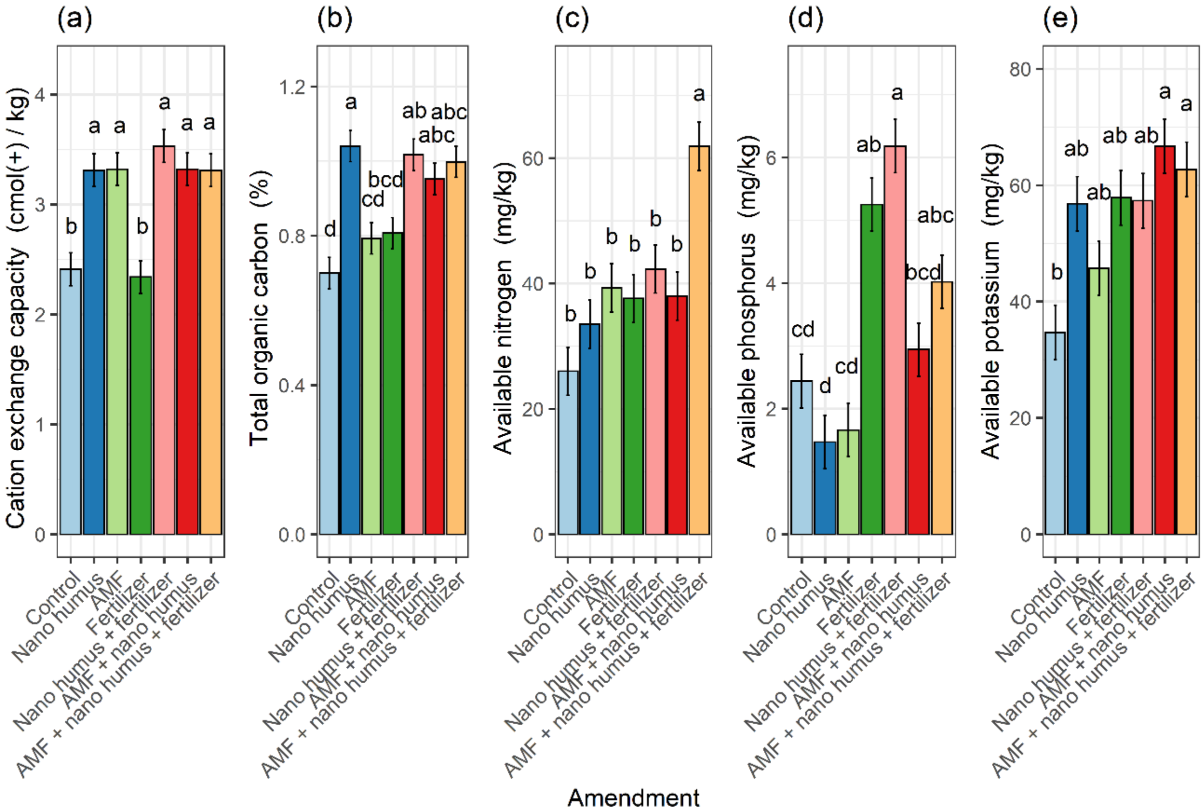

Figure 4.1 Mean and standard error of (a) cation exchange capacity, (b) total organic carbon, (c) available nitrogen, (d) available phosphorus, and (e) available potassium with different amendment applications after two growing seasons. Different letters indicate significant differences.

Figure 4.2 Photomicrograph of root after staining, showing (a) non AMF colonized plant root tissue at 20×, (b) colonized plant root tissue 10×, and (c) mycorrhizal colonization structure in root cortical cells at 40×.

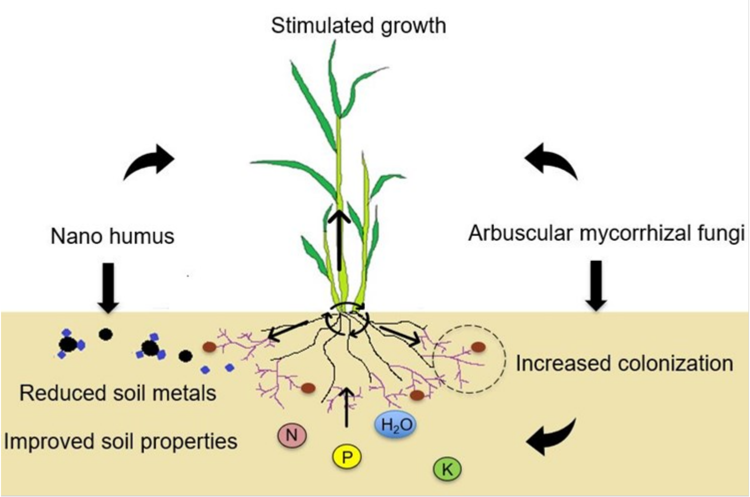

Figure 4.3 Possible mechanisms of combined effects of nano humus and arbuscular mycorrhizal fungi on plant growth.

Supplementary Materials

Table S4.1 Fourier transform infrared spectroscopy spectrum of nano humus. Spectra was determined with a 400 and 4000 cm-1 wavenumber spectrometer (Nicolet iS50, Thermo Scientific).

Functional groups/bands	Wavenumbers (cm^{-1})
Ambient water	> 3,600
O-H stretching	3,200 - 3,500
Carbon dioxide	2,324
CΞCH stretching	2,100
C-H bending	1981 and 700 - 900
C=C stretching	1,550 and 1,370

Table S4.1 Surface elemental composition of nano humus. Qualitative surface elemental information was identified with energy dispersive x-ray spectroscopy (Zeiss EVO MA10).

Element	Weight %
C	28.91
O	57.74
K	6.61
Al	1.59
Si	1.52
S	0.28
Ca	2.33
Fe	0.54
Mg	0.14

V. Lignite Derived Humic Products And Cattle Manure Biochar Are Effective Soil Amendments In Cadmium Contaminated And Uncontaminated Soils

1. Introduction

Cadmium (Cd) contamination in soils has raised serious environmental concern, and is recognized as a priority pollutant due to its high mobility and toxicity (Haider et al., 2021). Mining is one of the major sources through which Cd enters the environment before being transmitted to the food chain (Musilova et al., 2016; Sidhu et al., 2019). Cd is a non-essential element for plants with detrimental impacts on plant development and growth (Sandalio et al., 2001). Chlorotic spots on the leaves and leaf rolls are typical signs of Cd plant toxicity (Benavides et al., 2005). Cd contamination in soils is not biodegradable (Fu & Wang, 2011) and its accumulation in crops and animals poses severe health risks to humans due to dietary exposure (Tchounwou et al., 2012; Jaishankar et al., 2014). The global safety threshold of Cd in cereal crops is 0.1 mg/kg as suggested by Codex Alimentarius Commission (2004).

Several remediation technologies have been employed to mitigate Cd bioavailability in soils. Soil excavation obviously just transports contaminants elsewhere (Sidhu, 2016). Thermal desorption, conducted by heating contaminated soil to volatilize metals, is very expensive, labour intensive, and limited to small areas (Awa & Hadibarata, 2020). Electrokinetics, which involves application of high voltage to contaminated soil to remove metals is time consuming with low efficiency (Dhaliwal et al., 2020). Soil washing with water, chemicals, and other fluids, is very expensive and environmentally disruptive (Li et al., 2019). Solidification and stabilization is more economical, environmentally friendly, and suitable for large scale applications. It is applied by mixing or injecting chemically reactive soil amendments to contaminated soil (Dassekpo et al., 2018), such as animal manures, biosolids, and zeolites (Contin et al., 2019; Hamid et al., 2020; Saengwilai et al., 2020). Use of industrial and agricultural waste materials for heavy metal removal is usually cost effective and helps to reduce wastes accumulation.

Lignite waste derived humic substances and cattle manure derived biochar are capable of heavy metal immobilization due to their high abundance of exchangeable functional groups (Havelcová et al., 2009; Chen et al., 2011; Xu et al., 2013; Meng et al., 2017). They may also serve as natural biostimulators and soil conditioners with great ability to enhance plant growth and soil fertility (Chen et al., 2004; Ding et al., 2016; Ciarkowska et al., 2017). Both are highly porous and carbon rich, with substantial differences in raw materials, production, chemical structures, and density of functional groups (de Melo et al., 2016; Tomczyk et al., 2020). Humic substances are complex and supramolecular self-assembled mixtures of diverse heterogeneous compounds (Piccolo, 2002) from biochemical and chemical reactions during decomposition and transformation of plant and microbial residuals (humification) (Thorn et al., 1989). They naturally originate in soils, waters, sediments, and organic geological deposits (Schnitzer & Monreal, 2011). Relative to humic substances, biochar is produced over much shorter periods from pyrolysis of plant biomass or animal manures at temperatures less than 700 °C in an oxygen limited environment (Lehmann & Joseph, 2015). Therefore biochar may contain fewer oxygen containing functional groups than humic substances that form under aerobic conditions.

The majority of recent studies have focused on removing heavy metals from water systems with limited focus on soil and plant systems. To our knowledge, their direct comparison under experimental conditions is rare in the literature. Most previous humic and biochar studies were conducted on one soil texture, with clay (Beesley et al., 2010, 2014; Zhang et al., 2017), loam (Chan et al., 2008; Uchimiya et al., 2010), or sand soils (Khan et al., 2013; Ciarkowska et al., 2017; Suman et al., 2017). However, soil texture impacts remediation response (Duwiejuah et al., 2020). For example, Ciarkowska et al. (2017) found a lignite derived humic material had different effects in coarse and medium textured soils. The lack of available information on comparative effects of these products in different textured soils has impeded their applications in metal remediation and sustainable agriculture.

Our study assessed response of barley (*Hordeum vulgare* L.) to two lignite derived humic substances from different commercial sources, nano humus and humic powder, and a cattle manure biochar, as soil amendments in loamy sand and silt loam textured soils, with and without Cd contamination. Our results will contribute to improved knowledge on these waste derived materials, specifically to (i) phytotoxicity of Cd on barley growth, (ii) amendment effects on barley growth in Cd contaminated and uncontaminated soils with different soil textures, and (iii) amendment effects on Cd bioaccumulation in barley. Our results will thus provide important contributions to development of cost effective remediation strategies in heavy metal contaminated soils.

2. Material and Methods

2.1 Plant and soil materials

Seeds of barley, a widely cultivated crop species around the world, were obtained from a local commercial farm (Tribend Ranch Limited). Silty soils are prominent in river delta regions in Asia and North America (Assallay et al., 1998). Sandy soils are among the most widespread soils in the world, covering 7 % of the land surface (900 million hectares) (Driessen et al., 2001). Sandy soils are being cultivated more intensely as a result of rising population pressure. Thus, loamy sand (84 % sand, 9 % silt, 7 % clay) and silt loam (3 % sand, 86 % silt, 11 % clay) soils were used for this study. Loamy sand was procured near Devon (53°24'27.27"N, 113°45'34.94"W), silt loam was purchased commercially (Sungro®).

Each soil was thoroughly blended in a large plastic bin to ensure homogeneity. Total soil carbon and nitrogen were determined by combustion; total inorganic carbon (carbonate) by reaction with acid (Carter & Gregorich, 2008); and total organic carbon was calculated by the difference between total carbon and total inorganic carbon. Particle size (sand, silt, clay) was determined by hydrometer after treatment with calgon (Carter & Gregorich, 2008); pH and electrical conductivity (1:10 soil water suspension ratio) of soil samples using a pH and conductivity meter (Oakton 300 Series, USA), respectively. PH, electrical conductivity, total carbon, total inorganic carbon, and total nitrogen were considerably different for the two soils at 7.3, 0.70 dS/m, 4.5 %, 4.1 %, and 0.19 % for loamy sand soils, respectively; 5.8, 1.9 dS/m, 33 %, 33 %, and 0.84 % for silt loam soils, respectively.

Analytical grade Cd salt ($Cd(NO_3)_2 \cdot 4H_2O$) was obtained from Sigma-Aldrich (ON, Canada). The concentration of 50 mg/L divalent Cd was prepared by dissolving cadmium nitrate in deionized water at room temperature. 100 ml of prepared Cd solution was mixed into soils of the Cd treatment. This concentration was selected based on practical reference values resulting from various industrial activities in many regions and countries throughout the world (Kabir et al., 2012).

2.2 Soil amendments

Two coal mine lignite (humalite) derived humic products, nano humus and humic powder, were obtained from two commercial sources, Wuhan Shangyuan Environmental Protection Co, Ltd (Beijing, China) and Canadian Humalite International Ltd (Edmonton, Canada), respectively.

2.2.1 Nano humus

Nano humus is a black, fine grained, amorphous, mesoporous, and partially soluble substance with 7 μm particle diameters. Carbon, hydrogen, nitrogen, and oxygen are the primary elements. It contains approximately 83 % organic matter, 50 % humic acid, and 1 % micronutrients (copper, iron, zinc, aluminum, manganese, boron); macronutrients include 0.86 % nitrogen, 1.1 %

phosphorus (P_2O_5), 5 % potassium (K_2O), 0.29 % sulphur, and 0.34 % magnesium; with 4 % silica sand and ash by weight. Nano humus pH was 9.0 and electrical conductivity was 0.080 dS/m (Oakton 300 Series, IL, USA). Specific surface area was 2.1 m^2/g (Autosorb Quantachrome 1MP, Quantachrome Instruments, USA). Cd ion removal was 98.99 ± 0.05 % at 50 mg/L initial concentration (pH = 7). In each treatment pot, 5 g nano humus (producer recommendation) was dissolved in 500 ml water (1:100 powder to water) before application to the soil surface, providing the equivalent of 1.8 g carbon.

2.2.2 Humic powder

Humic powder is a fine grained, dark brownish, round shaped, and mesoporous solid material with high solubility. Particle diameter was approximately 10 times larger (74 μm) than nano humus. It has approximately 57 % organic matter, 67 % humic acid content, 1 % nitrogen, less than 0.05 % phosphorus (P_2O_5), 15 % potassium (K_2O), 0.40 % sulphur, 0.20 % magnesium, 35 % ash content, and micronutrients (copper, iron, zinc, aluminum, manganese, boron, barium). The values of pH and electrical conductivity were 8.8 and 3.4 dS/m, respectively (Oakton 300 Series, IL, USA). Specific surface area was 0.14 m^2/g (Autosorb Quantachrome 1MP, Quantachrome Instruments, USA). Cd ion removal was 10.87 ± 0.93 % at 50 mg/L initial concentration (pH = 7.0). Since there was no producer recommended rate, 4 g (~1.8 g carbon) was applied to provide the same amount of carbon in each treatment pot for all amendments. To investigate whether application rate would affect experimental results, a 200 times lower rate was also applied 0.02 g (~9 mg carbon) in each treatment pot. Powder was dissolved in water (1:100 powder to water) before application on the soil surface.

2.2.3 Biochar

Biochar was produced from cattle manure pellets provided by Paragon Soil and Environmental Consulting Inc (Edmonton, Alberta) by pyrolyzing them at 500 °C for 2 hours in nitrogen gas (Kwak et al. 2019). After pyrolysis, biochar was ground to pass through a 0.2 mm sieve. It has approximately 14 % carbon, 0.84 % nitrogen, and 80 % ash content (Kwak et al., 2019). The pH was 8.3 and electrical conductivity 0.66 dS/m (Oakton 300 Series, USA). Specific surface area was 3.9 m^2/g (Autosorb Quantachrome 1MP, Quantachrome Instruments, USA). Removal of the Cd ion was 37.20 ± 0.99 % at 50 mg/L initial concentration (pH = 7.0). In each treatment pot, 13 g biochar (~1.8 g carbon) was mixed in the top 15 cm of soil.

2.3 Experimental design

The experiment was conducted from February to May (100 days) under controlled greenhouse conditions set to 22 °C with 16 hours photoperiod. A complete randomized experimental design was implemented with 5 amendments (nano humus, high rate humic power, low rate humic powder, biochar, control), 2 soil textures (loamy sand, silt loam), and 2 Cd treatments (with, without), with 6 replications (n = 6). There were 120 experimental units in total (5 amendments x 2 soil textures x 2 Cd treatments x 6 replicates).

2.4 Greenhouse procedures and measurements

Greenhouse pots were 20 cm in diameter and 14 cm in height. To avoid metal leaching, a tray was placed under each pot to recycle drainage after each watering. Soil loaded pots and associated trays were randomly placed on a greenhouse bench. The Cd (~ 5 mg per pot) and amendments (nano humus 5 g, humic powder 4 g and 0.02 g, biochar 13 g) were added to pots before sowing. For each pot, 20 seeds were placed at 1 to 2 cm depth then covered with soil.

Emergence was estimated one week after seeding, by dividing the number of emerged seedlings by the number of planted seeds. Barley was then thinned to 5 plants per pot. Seedlings were watered when pots were lighter than the weight of moistened pots to maintain field capacity. Plant height was measured every week with a ruler for each plant in each pot from the soil surface to the highest live leaf. At the end of the experiment, a final assessment included root length, seed numbers produced per head, and dry biomass (shoot, root, total). Root and shoot samples were taken individually. Soil and non-root material were gently rubbed and shaken from the roots, then rinsed with tap water. Root length was determined with a ruler as a mean of longest and shortest root lengths for each individual plant. To measure dry biomass, roots and shoots were oven dried for 48 hours at 80 °C and weighed. Each individual seed head was counted and number of seeds in each head was averaged. Cd concentrations per unit of biomass in barley tissue was measured using inductively coupled plasma mass spectrometry (ICP-MS) at a local commercial laboratory. For quality control and assurance, relative percent difference was calculated for each pair of duplicates. Relative percent difference values were lower than 10 % in all cases, ensuring that analytical results were precise and accurate. However, the tested Cd concentrations (mg/kg) may not accurately reflect accumulated Cd contents (mg) in growing plants. Hence, we used the total amount of Cd per individual seedling (mg) for results interpretation, which was calculated by multiplying Cd concentrations (mg/kg) with the total biomass (kg) of each plant seedling.

2.5 Statistical analyses

Statistical analyses were performed with R software (version 3.6.1); significance was accepted at $p < 0.05$ for all tests. Two-way analysis of variance (ANOVA) was conducted separately to investigate treatment effects (amendment, soil texture) and their interactions on each of the measured growth parameters in soils with and without Cd addition. Before running the test, assumptions of normality (QQ plot and Shapiro-Wilk test) and equal variances (Levene's test) were conducted. Tukey's honest significant difference test was performed for pairwise comparisons. Principal component analysis (PCA) was performed to investigate the relationship between examined variables (measured growth parameters) and amendments.

3. Results

3.1 Effect of Cd on barley growth

The influence of Cd and soil texture on barley growth was statistically significant without interaction, suggesting the effect was consistent in both unamended soils. Seedling emergence, height, shoot biomass, and total biomass were significantly reduced in plants in soils with Cd (Figure 5.1). Relative to uncontaminated soil, Cd addition reduced emergence, height, shoot biomass, and total biomass by 18 % (from 93 to 75 %), 8 % (4.4 cm), 28 %, and 22 % in silt loam, respectively; 10 % (from 94 to 84 %), 17 % (8.5 cm), 37 %, and 9% in loamy sand, respectively. Cd toxicity on root length, root biomass, and seed production was not significant. Shoot growth tended to be more sensitive to Cd than roots as the greatest biomass reduction was on shoot biomass.

3.2 Growth response to soils with and without Cd

All measured growth parameters were considered in the projection of principal components (Figure 5.2). The first two, PC1 and PC2, explained 79 % of total variance among barley growth parameters and distinguished amendments according to soil texture. PC1 accounted for 63 % of the variation, with total biomass contributing greatly. PC2 represented 16 % of variation with major contributions from emergence. Close variables with small angles (less than 90 degrees) indicated high positive correlations between growth parameters, except emergence. Observations were grouped in ellipses according to soil amendment. Nano humus and biochar ellipses were separated from the control, implying their distinctive effect on barley growth. Humic powder ellipses at both concentrations partially overlapped that of the control, which means their beneficial effect might be less influential relative to nano humus and biochar. Ellipses of loamy sand and silt loam soils with and without Cd overlapped (not shown), suggesting a similar growth trend in different soils.

3.3 Barley response to amendments in soils without Cd

In soils without Cd, all growth parameters, except emergence, were significantly affected by amendment application and soil texture (Table 5.1). A significant interaction between two main effects occurred. In general, better growth occurred in silt loam than loamy sand. Overall, application of nano humus had the greatest effect on growth parameters; biochar best increased height; and the positive effect of humic powder became more significant with higher application rate.

In untreated soils (control), barley in silt loam was 4.5 cm taller than in loamy sand. Amendments significantly increased height in silt loam relative to loamy sand. Height enhancement was most significant with biochar, 8.3 cm taller than the control. It was followed by nano humus and high rate humic powder, showing increases of 5.7 and 4.8 cm, respectively. Low rate humic powder had no notable effect.

Mean root length was similar in untreated loamy sand and silt loam (control), approximately 15 cm. Nano humus and higher rate humic powder significantly increased root length, particularly in silt loam soils, with 12 and 8.2 cm enhancement relative to the control, respectively. Biochar had 4.9 cm longer roots in silt loam and 1.0 cm longer in loamy sand than that of the control, although the enhancement was not statistically significant.

Total biomass was 26 % greater in untreated silt loam than loamy sand. The beneficial effect of amendments was significant with stimulation in descending order of nano humus > biochar > high rate humic powder > low rate humic powder in loamy sand and nano humus > high rate humic powder > biochar > low rate humic powder in silt loam, respectively. Nano humus had the most prominent positive effect on total biomass production with a 97 % increase over the control. The stimulation was distinguished from other amendments where total biomass increase was 51 % with biochar and 34 % with high rate humic powder. Nano humus had a more pronounced effect on root biomass with a 230 % increase relative to the 40 to 100 % increase by other amendments. Enhancement was less significant on shoot biomass. Biochar increased shoot biomass by 53 % in loamy sand and 11 % in silt loam. Nano humus increased shoot biomass by 11 % in loamy sand and 62 % in silt loam.

Seed number significantly increased with nano humus, 14 % over the control. Effects of other amendments were less notable.

3.4 Effect of amendments on Cd bioaccumulation in barley

In all treatments, whether with amendments added or not, Cd concentrations in barley tissues exceeded the Codex safety threshold by more than 20 times. Bioavailability of Cd was significantly affected by soil texture, while the influence of amendment was not significant. A significant interaction effect occurred between amendment and soil texture, demonstrating that amendment effects are dependent on soil texture. Cd bioaccumulation was significantly higher for barley in silt loam than in loamy sand, with 0.005 to 0.01 mg higher tissue concentrations depending on amendments (Table 5.2). Most barley growth parameters differed significantly with soils, with small differences on root length elongation and seed production.

3.5 Barley response to amendments in soils with Cd

Although amendments did not reduce Cd uptake by barley from soils (Table 5.2), their influence on growth stimulation was significant in soils with Cd, except for low rate humic powder. High rate humic powder had noticeable stimulation effects on barley growth, low rate humic powder was relatively ineffective and not significantly different from the control.

Nano humus, high rate humic powder, and biochar had more pronounced stimulation effects on total biomass in soils with Cd than without, with 17, 25, and 142 % more enhancement in silt loam, respectively; 12, 32, and 11 % more enhancement in loamy sand, respectively. Effects were less for other growth parameters.

With Cd, nano humus, high rate humic powder, and biochar increased total biomass by 121, 49, and 88 %, respectively, relative to the control in loamy sand; it increased by 103, 167, and 76 % in silt loam, respectively. Height enhancement was less impacted by humic materials, with 5 to 19 % (2.3 to 8.2 cm) increase in two soils; biochar increased 25 % (11 cm) in two soils relative to the control. The positive effect on root length was more remarkable with humic materials, 55 % (8.4 cm) increase with nano humus and 31 % (4.7 cm) with high rate humic powder, relative to 17 % (2.5 cm) with biochar in two soils.

Seed production increased with biochar (18 %), high rate humic powder (15 %), and nano humus (14 %) in both soils although the promotion was mostly not statistically significant.

4. Discussion

4.1 Phytotoxicity of Cd on barley growth

Heavy metals can affect emergence as seeds are highly responsive and reflect their current living environment. Cd, a toxic, non-essential metal, is considered as a sole limiting factor influencing emergence in soils in our study since seeds were from the same batch. Decreased emergence could be attributed to altered physiological and metabolic activities with Cd addition. The inhibition effect can be attributed to reduced water absorption and transport (Li et al., 2005; El Rasafi et al., 2020) and embryonic damage through reduced hydrolyzing enzyme activities (Anwar et al., 2021). Similarly, Gubrelay et al. (2013) found significant inhibition of barley emergence, from 91 to 56 % under high Cd concentrations in sands (30 millimolar). Similar trends occurred with other cereal crops, such as wheat (*Triticum aestivum* L.), where emergence decreased from 88 to 58 % in Cd polluted sands relative to the control (50 mg/L) (Ahmad et al. 2012). Wheat emergence showed no negative response to Cd in sandy loam with Cd concentrations as high as 320 mg/kg (An, 2004). There was no inhibition of emergence in other common crops, including sweet corn (*Zea mays* L.), cucumber (*Cucumis sativus* L.), and sorghum (*Sorghum bicolor* L.). Cd toxicity effects on emergence is likely species dependent due to different degrees of sensitivity (McGrath et al., 2001).

There were no visible signs of metal toxicity in barley shoots throughout our study with Cd addition at approximately 5 mg per pot (~ 13 mg/kg). Vassilev et al. (2004) found barley showed no toxicity symptoms in Cd polluted sand at concentrations even as high as 28 mg per kg sand, although Hernández-Allica et al. (2008) found 81 % reduction of barley shoot biomass in hydroponic pots at 10 mg/kg of Cd. Wu et al. (2004) found barley shoot biomass decreased 32 % with Cd (5 μM, 0.028 mg/kg), and Vassilev et al. (1995, 2004) found adverse effects on barley biomass at various Cd concentrations. The growth inhibitory effect might be primarily due to the reduction of heavy metal induced photosynthetic rate (Sandalio et al., 2001).

4.2 Possible mechanism on growth stimulation

The distinct stimulation effects of lignite derived humic materials and cattle manure derived biochar on barley shoots and roots in both soil textures, with or without Cd are consistent with other studies (Schmidt et al., 2007; Chan et al., 2008; Mora et al., 2010; Uzoma et al., 2011; Revell et al., 2012; Ciarkowska et al., 2017). The morphological changes of barley, such as taller plant height and longer root length directly and/or indirectly induced by amendments may have greatly improved plant resource capture and utilization.

Interestingly, humic materials had greater impact on barley root length, while biochar had greater impact on barley height. The results were consistent with and without Cd on silt loam and loamy sand soils. The more significant root length elongation by humic substances could be explained by their direct impact on plant physiology. Humic substances, as a group of chemical compounds with a supramolecular structure, can chemically interact with plant cell membranes after penetration (Kulikova et al., 2013, 2016). Auxin-like materials in humic substances were evidenced in other studies (Canellas et al., 2002; Trevisan et al., 2010, 2011), which could contribute to root length elongation (Piccolo et al., 1992; Canellas et al., 2002; Casimiro et al., 2003). Humic materials could also slowly supply nutrients in the soil, indirectly promoting root development (Bronick & Lal, 2005; Bezuglova et al., 2017). The greater barley height with cattle manure biochar can be attributed to the improved soil environment, particularly enhanced nutrient availability. Other biochar studies are in agreement with this explanation (Gomez et al., 2014; Kim et al., 2016), as animal manure derived biochar normally contains more rapidly available macro and micro nutrients essential for crop production than plant derived biochar (Singh et al., 2010) and lignite derived humic materials.

4.3 Amendment application potential in soil metal remediation

In our study, the more pronounced stimulation in morphological characteristics of barley with humic materials and biochar with Cd than without may be due to increased nutrient availability from amendments via ion exchange. Nano humus, humic powder, and biochar are amendments with high oxygen containing functional groups and mineral components that are capable of exchanging cations with heavy metal ions (Uchimiya, Lima, Thomas Klasson, et al., 2010). Hence, mineral nutrients, such as calcium, magnesium, and iron ions, could have been released from amendments into the soil (Urrutia et al., 2014; Cieschi & Lucena, 2018), providing an additional nutrient source for barley use.

Several studies reported reduced metal uptake of plants grown in heavy metal contaminated soils amended with humic materials (Khan et al., 2017; Ondrasek et al., 2018) and biochar (Cui et al., 2011; Rizwan et al., 2018; Gonzaga et al., 2019). Contrary to expectations, nano humus, humic powder, and biochar did not decrease Cd uptake of barley from our contaminated silt loam and loamy sand soils. Some studies reported similar observations as ours with addition of humic materials (Park et al., 2011; Xu et al., 2018) and biochars (Lucchini et al., 2014), suggesting amendments may decrease readily soluble and exchangeable forms of Cd while increasing plant available forms. Future investigations on soil response to Cd addition will be needed to provide insights to understanding the inconsistent results in the literature.

4.4 Amendment effects in different textured soils

Our results confirmed the influence of soil texture on beneficial effects of amendments, as barley response was significantly different with soil texture. The better growth of barley in silt loam than in loamy sand can be attributed to leachability differences. Loamy sand is characterized by high water infiltration capacity, low water holding capacity, and low nutrient retention capacity (Von Uexkull, 1986), with 20 to 80 % of nutrient or chemical addition potentially lost through leaching (Manevski et al., 2015; Matichenkov et al., 2020). Silt loam contains relatively balanced sand, silt, and clay and better nutrient retention which might be less prone to leaching.

5. Conclusions

Cd significantly inhibited barley emergence and growth. Application of two lignite derived humic products, nano humus and humic powder, and a cattle manure biochar did not reduce cadmium uptake by barley significantly. Either with or without Cd, the beneficial effect of these amendments on barley growth stimulation was prominent in both silt loam and loamy sand soils. Application of nano humus had the greatest effect on plant growth parameters. Biochar best increased shoot growth, and the positive effect of humic powder became more significant on root growth with higher application rate (4 g per pot). Although the positive effects of humic materials and biochar were visible in both soils, they provided more marked enhancement of barley growth in silt loam than loamy sand. Our finding confirmed that lignite derived humic materials and cattle manure derived biochar are promising biostimulators for crop growth in silt loam and loamy sand soils that are contaminated with Cd.

Table 5.1 Mean (± standard deviation) emergence, height, root length, shoot biomass, root biomass, total biomass, and seed numbers (per head) with amendments in two soils without Cd (n=6). Within a column, different lower case letters indicate a significant difference between amendments.

Soil	Amendments	Emergence	Height	Root length	Shoot biomass	Root biomass	Total biomass	Seed number
		%	cm	cm	g/plant	g/plant	g/plant	number/head
Loamy sand	Nano humus	95.00±5.48 a	50.01±5.25 cd	20.90±6.41 abc	1.07±0.30 bcde	1.74±0.78 a	2.80±0.97 ab	21.39±1.41 a
	Humic powder (high rate)	92.50±7.58 a	41.63±2.59 e	9.94±0.73 f	0.92±0.15 de	0.65±0.11 bc	1.57±0.22 cd	17.50±0.89 cd
	Humic powder (low rate)	95.83±4.92 a	43.04±2.96 de	12.24±1.03 ef	0.83±0.10 e	0.57±0.11 bc	1.40±0.06 d	16.39±2.21 d
	Biochar	96.67±4.08 a	49.95±4.20 cd	16.01±2.46 cde	147±0.51 ab	0.91±0.17 bc	2.38±0.58 abc	20.11±1.49 abc
	Control	94.17±9.70 a	49.79±3.42 cd	15.03±2.17 def	0.96±0.07 cde	0.39±0.11 c	1.34±0.14 d	19.67±1.73 abc
Silt loam	Nano humus	90.00±8.37 a	60.03±3.47 ab	26.40±3.98 a	1.94±0.23 a	1.20±0.42 ab	3.14±0.28 a	20.78±1.66 ab
	Humic powder (high rate)	95.83±2.04 a	59.07±3.48 ab	23.00±1.82 ab	1.42±0.09 bc	1.13±0.39 ab	2.55±0.41 ab	19.94±1.79 abc
	Humic powder (low rate)	92.50±6.89 a	50.87±5.25 c	15.96±1.71 cde	0.84±0.29 e	0.64±0.30 bc	1.48±0.42 d	18.22±1.00 bcd
	Biochar	89.17±8.61 a	62.61±3.59 a	19.67±3.07 bcd	1.33±0.24 bcd	0.79±0.14 bc	2.12±0.26 bcd	19.89±1.42 abc
	Control	93.33±6.06 a	54.29±2.04 bc	14.80±1.40 def	1.20±0.18 bcde	0.49±0.05 c	1.69±0.21 cd	17.44±2.13 cd

Table 5.2 Mean (± standard deviation) emergence, height, root length, shoot biomass, root biomass, total biomass, and seed numbers (per head) with amendments in two soils with Cd addition (n=6). Within a column, different lower case letters indicate a significant difference between amendments.

Soil	Amendments	Emergence	Height	Root length	Shoot biomass	Root biomass	Total biomass	Seed number	Total tissue Cd
		%	cm	cm	g/plant	g/plant	g/plant	number/head	mg/plant
Loamy sand	Nano humus	81.67±14.72 a	49.09±2.39 cd	22.15±2.54 ab	1.07±0.19 cd	1.62±0.51 a	2.69±0.62 b	20.17±1.11 abc	5.95E-03±3.73E-03 cd
	Humic powder (high rate)	86.67±13.29 a	48.24±2.44 cd	20.28±3.12 bc	1.12±0.07 cd	0.69±0.10 c	1.81±0.09 cde	19.94±1.71 abc	6.40E-03±1.34E-03 cd
	Humic powder (low rate)	93.33±7.53 a	43.76±2.84 de	14.49±1.72 e	0.63±0.11 e	0.50±0.31 c	1.14±0.35 e	18.44±2.39 abc	2.52E-03±1.06E-03 d
	Biochar	93.33±8.76 a	52.09±1.94 bc	19.09±3.15 bcd	1.53±0.14 ab	0.76±0.26 c	2.29±0.24 bc	20.39±0.83 abc	7.38E-03±5.78E-03 cd
	Control	84.17±10.68 a	41.24±2.58 e	15.21±0.83 de	0.60±0.16 e	0.61±0.18 c	1.22±0.23 de	17.39±2.65 c	2.54E-03±7.54E-04 d
Silt loam	Nano humus	82.50±15.08 a	52.17±5.38 bc	25.31±2.44 a	1.28±0.16 bc	1.38±0.37 ab	2.66±0.42 b	19.78±1.00 abc	1.81E-02±6.16E-03 a
	Humic powder (high rate)	95.00±6.32 a	58.04±2.69 ab	19.74±1.25 bc	1.43±0.15 b	0.88±0.39 bc	2.31±0.49 bc	20.56±0.62 ab	1.18E-02±2.65E-03 abc
	Humic powder (low rate)	91.67±11.69 a	48.68±3.04 cd	15.16±0.95 de	1.10±0.11 cd	0.76±0.05 c	1.87±0.15 cd	18.56±1.22 abc	1.60E-02±5.75E-03 ab
	Biochar	80.00±15.17 a	62.02±4.02 a	16.66±2.09 cde	1.73±0.18 a	1.78±0.42 a	3.51±0.47 a	20.89±1.38 a	1.37E-02±7.57E-03 abc
	Control	75.00±13.42 a	49.87±3.40 cd	15.44±1.54 de	0.86±0.07 de	0.45±0.10 c	1.31±0.07 de	17.72±2.02 bc	8.31E-03±1.47E-03 bcd

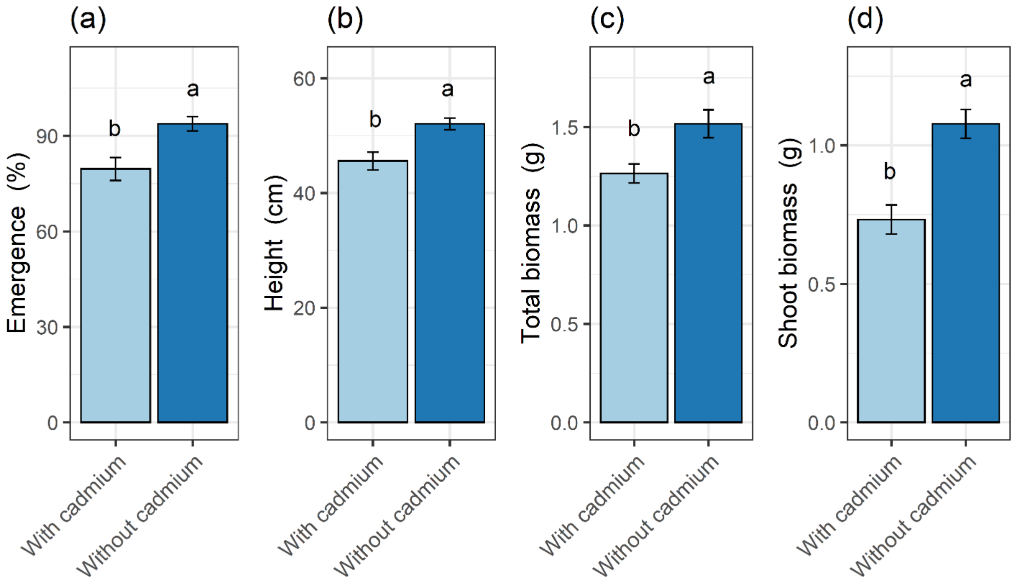

Figure 5.1 Mean and standard errors of (a) emergence, (b) height, (c) total biomass, and (d) shoot biomass in soils with and without Cd. Different letters indicate significant differences with and without Cd.

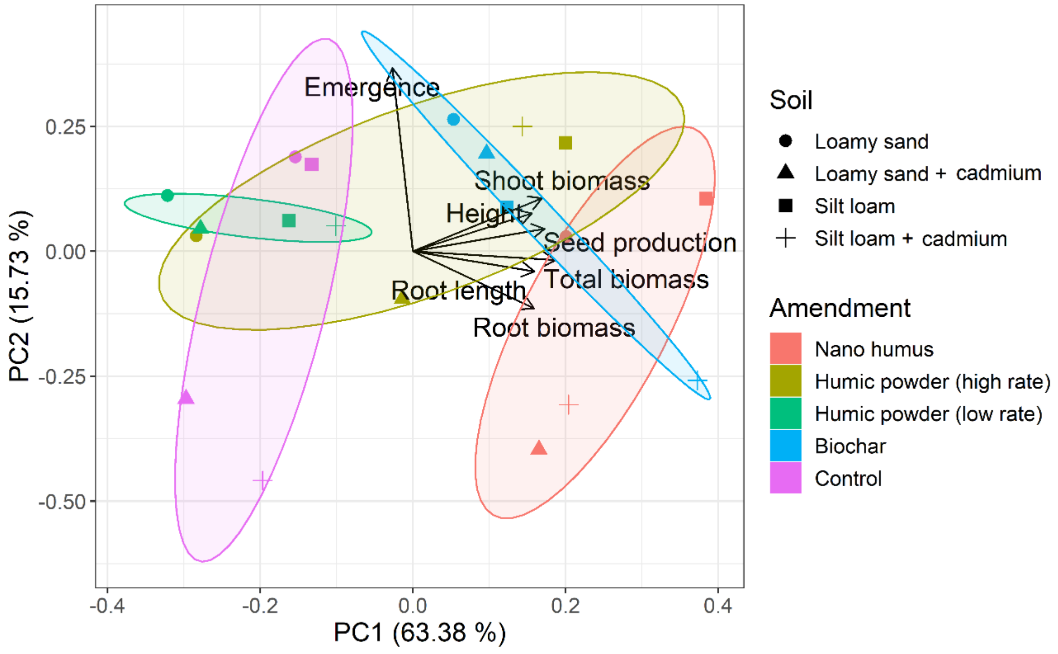

Figure 5.2 Principal component analysis of mean growth parameters treated with five amendments in four types of soil. Different colours represent amendments and different point shapes represent soils. Data points of the same amendment are grouped together in an ellipse.

VI. Cd(II) And Zn(II) Adsorption By Three Carbon Based Materials For Remediation Of Contaminated Water

1. Introduction

Excessive industrial release of heavy metals continues to pose great risk to the environment and public health. Heavy metals, unlike organic pollutants, are not biodegradable and can accumulate in living organisms (Fu & Wang, 2011). Due to their mobility and toxicity, heavy metals have been considered priority pollutants in many parts of the world (Volesky & Holan, 1995; Qaiser et al., 2007). Cadmium (Cd) and zinc (Zn) are among the heavy metals of greatest concern. Conventional methods of removing heavy metals from aqueous solutions such as chemical precipitation, ion exchange, membrane filtration, and electrochemical have considerable limitations (Nguyen et al., 2013), making less expensive remediation technologies an important research area. Adsorption is a mass transfer mechanism in which a material is moved from a liquid phase to a solid surface and becomes bound by physical and/or chemical interactions (Kurniawa & Babel, 2003). Adsorption is a fast, universal method, and has emerged as a favourable alternative for heavy metal remediation, especially from environmental and economic perspectives (Ali & Gupta, 2006; Demirbas, 2008).

Low-cost carbon based materials derived from agricultural waste, industrial by-products, natural materials, and/or modified biopolymers have been introduced as adsorbents for heavy metal remediation. Among various carbonaceous materials, lignite waste derived humic substances and cattle manure derived biochars are strong candidates for water remediation. Humic substances are complex colloidal organic materials with abundant functional groups (Stevenson, 1994); adsorption is one of their distinguishing characteristics (Ferro-García et al., 1998; Havelcová et al., 2009; Ghabbour & Davies, 2011). Commercially available humic materials are often derived from lignite and weathered coal (leonardite, oxihumolite) (Chen et al., 2004). Although humic substances and biochar are both highly porous carbon rich materials, they are physically, chemically, and functionally different (de Melo et al., 2016; Tomczyk et al., 2020). Compared to humic substances, biochar is produced over a much shorter time by low temperature pyrolysis (<700 °C) of plant biomass and animal manures under oxygen limited conditions (Lehmann & Joseph, 2015). Although less reported than plant residue based biochars, animal manure based biochars have shown a great affinity for heavy metals in aqueous solutions (Cao et al., 2009; Xu et al., 2013; Kwak et al., 2019; Nzediegwu et al., 2021).

Information is sparse on lignite derived humic materials and cattle manure derived biochars for heavy metal adsorption. Their direct comparison under the same experimental conditions is limited. It is not completely understood if these materials will work, and how they work, for cadmium and zinc ion removal from contaminated water. It remains unknown whether the chemical characterization of humic products with the same extraction origin are functionally the same. Such knowledge may facilitate use of a new, readily available, economical alternative technique for contaminant remediation in soil and water. Our study was undertaken to investigate use of two lignite derived humic products (different commercial sources) and cattle manure derived biochar as adsorbents in removal of two common contaminants, Cd(II) and Zn(II). Isotherm and kinetic experiments were used to determine chemical and physical properties, adsorption capacity, and adsorption isotherms and kinetics of three adsorbents, and effects of initial metal concentrations and contact time.

2. Materials and Methods

2.1 Adsorbent production

Two humic products, nano humus and humic powder, were produced from lignite deposits (humalite) from coal mines and procured from commercial sources. Nano humus is a black, fine grained, and partially soluble material, with 83 % organic matter and 50 % humic acids. Humic powder is a dark brownish, highly soluble, fine grained solid material, with 57 % organic matter and 67 % humic acids. Biochar was produced through 500 ℃ pyrolysis of cattle manure pellets for 2 hours in nitrogen gas conditions in a muffle furnace (Lindberg Blue M, Moldatherm, Thermo Scientific, Pittsburgh, PA, USA). Biochar was collected after cooling to room temperature, then ground and passed through a 0.2 mm sieve.

2.2 Adsorbent characterization and chemical preparation

Electrical conductivity and pH were determined by meter at 1:10 solid to water suspension ratio (Oakton 300 Series, Oakton Instruments). The Brunauer-Emmett-Teller surface area and pore size analysis (density function theory / Barrett-Joyner-Halenda) were measured with nitrogen gas adsorption at 77 Kelvin (Autosorb Quantachrome 1MP, Quantachrome Instruments).

To characterize functional groups on adsorbent surfaces, Fourier Transform Infrared Spectroscopy spectra was determined with a 400 and 4000 cm^{-1} wavenumber spectrometer (Nicolet iS50, Thermo Scientific). Functional groups were identified according to the spectra libraries of Socrates (2004) and Hesse et al. (2005). Surface morphology and elemental composition were obtained from scanning electron micrographs and qualitative elemental information on the sample surface (Zeiss EVO MA10), coupled with energy dispersive x-ray spectroscopy. Mean particle diameter was estimated from scanning electron microscope image analysis (ImageJ software) by measuring particle diameters on the morphological image. All chemicals were analytical grade, obtained from Sigma-Aldrich. Standard solutions with 25, 50, 75, 100 and, 125 mg/L concentrations were prepared by dissolving cadmium nitrate ($Cd(NO_3)_2{\cdot}4H_2O$) and zinc nitrate ($Zn(NO_3)_2 \cdot 6H_2O$) in deionized water. Nitric acid (HNO_3 10 %) and potassium hydroxide (KOH 10 %) were added dropwise to adjust pH to 7 before use.

2.3 Adsorption isotherm study

Adsorption experiments were performed by the batch technique at room temperature with three replicates per treatment. The mixture pH was measured at start and end of equilibration. Single metal ion concentrations were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) at a local commercial laboratory using collision reaction cell technology (APHA 3030B/6020A (mod)).

A 0.02 g of adsorbent was added to a 20 ml single metal standard solution in a capped test tube, then agitated 24 hours. After equilibrium, 10 ml of the mixtures were filtered with 0.45 µm membrane syringe filters. The filtrate was immediately acidified with the same volume of HNO_3 (2 drops) to pH less than 2 and stored in a plastic bottle for analysis.

For each sample at the end of the experiment, adsorption capacity (q_e), amount of metal adsorbed per unit mass (mg/g), and percent metal ion removal (%MR) were calculated by the equation: $q_e = \frac{(C_i - C_e)\,V}{m}$ and $MR = \frac{(C_i - C_e)100}{C_i}$

where C_i and C_e are initial and equilibrium concentrations (mg/L), respectively, m is adsorbent mass (g), V is solution volume (L).

Widely accepted isotherm models, Langmuir (1916) and Freundlich (1906), were regressed using experiment isotherm data. The linear Langmuir model is expressed as: $q_e = \frac{Q_{max} b C_e}{1 + bC_e}$

where q_e (mg/g) is amount of metal adsorbed per unit weight of adsorbent, C_e (mg/L) metal concentration in solution at equilibrium, Q_{max} (mg/g) maximum adsorption capacity, b (L/mg) constant related to bonding energy of metal ion adsorption (affinity).

The linear Freundlich model is expressed as: $q_e = K_f C_e^{1/n}$

where K_f is an indicator of adsorption capacity, *n* the Freundlich constant related to adsorption intensity.

The Redlich-Peterson isotherm model has features of Freundlich and Langmuir isotherms, incorporating three parameters (Redlich & Peterson, 1959). It is expressed as: $q_e = \frac{AC_e}{1+BC_e^g}$

where A is the isotherm constant (L/g), B the Redlich–Peterson isotherm constant (L/mg$^{1-1/A}$), g the exponent (0<g< 1). It reduces to the Langmuir isotherm when g = 1 and Freundlich isotherm when g = 0.

2.4 Adsorption kinetics study

Adsorption kinetics of Cd(II) onto three materials and effect of contact time (t) were investigated. A 0.02 g amount of adsorbent was weighed and added to 20 ml cadmium solutions (concentration = 100 mg/L, pH = 7) in a capped test tube. The mixture was agitated on a platform shaker (Excella E1 Orbital Shaker) at 180 rpm for 15 min, 30 min, 1 hour, 3 hours, 6 hours, 9 hours, and 24 hours. At specific times, a 10 ml sample was collected and filtered through 0.45 μm membrane syringe filters. The sample was immediately acidified for storage. The procedure was replicated three times.

Experimental data were studied using three kinetic models: pseudo first order model (Lagergren, 1898; Rudzinski & Plazinski, 2007), pseudo second order model (Ho & McKay, 1999), and intraparticle diffusion model (Weber & Morris, 1963).

The linear pseudo first order model is: $\log(q_e - q_t) = \log(q_e) - \frac{k_1 t}{2.303}$

where q_t (mg/g) is adsorbed cadmium ions at time t (min), q_e (mg/g) is adsorbed metal ions at equilibrium, and k_1 (L/min) is pseudo first order rate constant. Kinetic parameters were obtained by plotting log (q_e - q_t) against t.

The linear pseudo second order model is expressed as: $\frac{t}{q_t} = \frac{1}{k_2 q_c^2} + \frac{t}{q_e}$

The initial adsorption rate, h (mg/g per min), is: $h = k_2 q_e^2$

where k_2 (mg/g per min) is the pseudo second order rate constant. Values of t/q_t were plotted against t. Predicted adsorption capacity q_{cal} (mg g^{-1}) and k_2 are calculated from the linear regression slope and intercept, respectively.

The intraparticle diffusion model equation is: $q_t = k_{id} t^{1/2} + C$

where k_{id} (mg/g/min$^{0.5}$) is intraparticle diffusion rate constant (mg/g per min$^{1/2}$), C (mg/g) is intercept related to boundary layer thickness.

3. Results and Discussion

3.1 Characterization of adsorbents

The three adsorbents exhibited similar fourier transform infrared spectroscopy spectroscopic features (Figure 6.1), a broad and rounded band of H-bonded -OH stretching of carboxyl, phenol, and alcohol vibrations from 3,500 to 3,200 cm^{-1}. Alkynes (CΞCH stretching) appear at 2,100 cm^{-1}, C-H stretching and bending at 1,981 cm^{-1} (aliphatic) and 700 to 900 cm^{-1} (aromatic), and aromatic C=C stretching at 1,370 and 1,550 cm^{-1}. There were two bands at 2,919 and 2,850 cm^{-1} of humic powder, attributed to aliphatic C-H stretching. Ambient water (> 3,600 cm^{-1}) and carbon dioxide (2,324 cm^{-1}) were detected as background spectra in all adsorbents.

Peak absorbance (intensity) often reflects the relative amount of each functional group associated with the molecular bond. Biochar showed a relatively lower absorbance than nano humus and humic powder in all regions, indicating lower content of functional groups, especially phenolic hydroxyl groups in hydrogen bond regions (3,500 to 3,200 cm^{-1}). Identified phenolic hydroxyl groups are capable of ion exchange on the surface (Chojnacka et al., 2005), and considered one of the main functional groups for heavy metal adsorption (Dong et al., 2011) through -OH complexation (Xu et al., 2013). This implies phenolic groups may have less contribution in metal adsorption by biochar relative to nano humus and humic powder.

Porosity was similar between nano humus and biochar, and humic powder had an extremely low total pore volume (Table 6.1). Since the adsorbents had low specific surface areas (0.1 to 4 m^2/g), surface area dependent on physical adsorption may not be the dominant mechanism. All three adsorbents were highly heterogeneous, porous, and structurally complex (Figure 6.2). Nano humus had small, amorphous, and thin platy particles, which aggregated into larger and more isolated particles with mesopores distributed (2 to 50 nm). Humic powder particles were round shaped, smooth on the surface, and less porous. Biochar presented channels and longitudinal mesopores, together with a fibrous surface. Nano humus featured the smallest particle diameters, 1 to 20 µm (mean 7 µm) relative to humic powder (30 to 140 µm) and biochar (8 to 130 µm). All three were mesoporous materials with pore diameters from 14 to 37 nm.

Major elemental composition of the adsorbents varied (Table 6.2, Table S6.1). Nano humus was less carbonized, with significantly higher oxygen content than humic powder and biochar. Carbon content was greatest in biochar (53.1 %), followed by humic powder (45.32 %) and nano humus (28.91 %). Oxygen to carbon ratio is considered an indicator of content of carbohydrate derived polar functional groups and surface hydrophilicity (Chun et al., 2004). This ratio for nano humus (~1.5) was more than three times higher than that of biochar and humic powder (~ 0.4), indicating higher content of oxygen containing polar functional groups and more hydrophilic structure. Mineral components were released into solution from adsorbents via ion exchange (Uchimiya, Lima, Thomas Klasson, et al., 2010) and responsible for heavy metal adsorption via precipitation (Cao et al., 2009; Xu et al., 2013; Nzediegwu et al., 2021). Humic powder contains remarkably higher mineral components at approximately 26.5 %, double that of nano humus (~13 %), and almost four times that of biochar (~6.4 %). Higher electrical conductivity of humic powder also reflects its greater quantity of soluble salts (carbonates). The effect of heavy metals originating from adsorbents themselves was negligible.

With the high content of oxygen containing functional groups and hydrophilic nature of nano humus, abundant mineral contents of humic powder, and highly porous structure of biochar, all three materials have high potential as adsorbents in heavy metal removal from contaminated water. Although nano humus and humic powder are derived from lignite, they showed different morphology, specific surface area, porosity, and surface chemistry.

3.2 Influence of initial concentration

Adsorption reaction was greatly influenced by initial concentration of Cd(II) and Zn(II) metal ions (Figure 6.3). Adsorption capacity of metal ions on the adsorbents increased with initial concentration. Percentage removal of nano humus and biochar decreased gradually with increased Cd(II) and Zn(II) initial concentration, while humic powder showed an opposite trend. Adsorption capacity of nano humus increased gradually from 24.9 to 107.3 mg/g for Cd(II), and 24.5 to 79.6 mg/g for Zn(II) over all concentrations. This great adsorption capacity of nano humus contributed removal rates as high as 99.6 % for Cd(II) and 98.6 % for Zn(II), at relatively lower concentration (25 mg/L); 84.5 % for Cd(II) and 61.7 % for Zn(II) at highest concentration (125 mg/L). Adsorption capacity of humic powder increased rapidly from 4.1 to 69.9 mg/g for Cd(II) and 2.7 to 90.9 mg/g for Zn(II) with higher initial concentrations. Removal rate of humic powder increased at higher concentrations, from 16.3 % to 55.9 % for Cd(II) and 10.8 % to 72.7 % for Zn(II). Biochar adsorption capacity increased slowly with initial concentration and remained at 29.2 mg/g for Cd(II) and 30.2 mg/g for Zn(II). A smaller percentage of metal ions was removed by biochar, with an increase in concentration, dropping from 61.1 % to 23.3 % for Cd(II) and from 41.2 % to 24.2 % for Zn(II). Overall, nano humus was the most effective adsorbent among the three carbon rich materials for removing Cd(II) and Zn(II) in solutions for most concentration ranges.

Divalent metal ions, such as Cd(II) and Zn(II), share similar mechanisms (Li et al., 2017), of which ion exchange (Taha et al., 2011; Feng & Guo, 2012; Trakal et al., 2014; Zhang et al., 2015) and complexation with oxygenated functional groups (Panda et al., 2008; Sen et al., 2010; Vázquez et al., 2012) were most commonly considered key mechanisms on humic substances and biochar. Adsorption by presence of oxygen containing functional groups, such as carboxyls, phosphates, sulphates, aminos, amides, and hydroxyls (Neto et al., 2013) is preferred as they can enhance surface oxidation and metal complexation (Ambaye et al., 2020). The greater adsorption capacity of nano humus may be related to significantly higher content of oxygen containing functional groups indicated by the oxygen to carbon ratio and FTIR spectra discussed previously. Presence of mineral components may serve as additional adsorbing sites for ion exchange, meaning more metal ions can be adsorbed on the surface of nano humus. We found specific surface area had very limited influence on metal adsorption of the studied materials although a large surface area was considered a prominent determinant of adsorbent selection in most studies. Nano humus and humic powder showed very different adsorption capacities for both Cd(II) and Zn(II), which implied that humic materials could have different adsorption behaviours although derived from the same source. Their different performance can be attributed to the difference in their physical and chemical characteristics, particularly oxygen to carbon ratio which is directly linked to the content of oxygen containing functional groups. Higher oxygen to carbon ratio is suggested for selecting ideal humic materials working as adsorbents for metal removal from contaminated water.

3.3 Adsorption isotherm

To better understand adsorption mechanisms, the relationship between adsorbed metal ions on three adsorbents and their concentration in solutions under equilibrium conditions were analyzed by empirical models (R package PUPAIM), including 28 widely accepted physical and chemical isotherm models (Dąbrowski, 2001; Ayawei et al., 2017). Applying various isotherm models is necessary to completely describe equilibrium data obtained in adsorption experiments. The most commonly used models, Langmuir isotherm, Freundlich isotherm, and Redlich-Peterson isotherm provided the best fit in modeling and can be used to describe how Cd(II) and Zn(II) were adsorbed onto the three adsorbents (Table 6.2, Figure S6.2). The parameters of other models were not shown in this paper due to low regression.

Langmuir isotherm assumes adsorption was on a monolayer homogeneous surface containing a limited number of non-interacting identical sites (Langmuir, 1916). Freundlich isotherm assumes multilayer adsorption on the heterogeneous surface and gives an expression of exponential distribution of active sites and their energies (Freundlich, 1906). The Redlich-Peterson isotherm, incorporating three parameters, has a unique adsorption mechanism and does not follow ideal monolayer adsorption characteristics (Redlich & Peterson, 1959).

Maximum adsorption capacities (Q_{max}) from the Langmuir isotherm model for adsorption of Cd(II) on humic powder (389 mg/g) might be overestimated due to the very low fitness of the model, and thus it was not used for comparison. The Q_{max} of Cd(II) adsorption by nano humus and biochar were 99.9 and 32.7 mg/g, respectively. Adsorption capacities of Zn(II) on nano humus and biochar were lower than that of Cd(II) at 71.7 and 51.1 mg/g, respectively.

To find the most appropriate model for metal ions adsorption, coefficient of determination (R^2) were compared. With nano humus and biochar, the Freundlich isotherm ($R^2 = 0.91 - 0.98$) showed a higher coefficient of determination than the Langmuir isotherm ($R^2 = 0.82 - 0.96$) over the entire concentration ranges of Cd(II) and Zn(II) ions. Thus, the mechanism of the Freundlich isotherm more accurately described adsorption of metal ions. Parameters show adsorption of Cd(II) and Zn(II) onto adsorbents was favourable ($n>1$). The active energetic surface of nano humus and biochar are heterogeneous and formed multilayer coverage of metal ions instead of monolayer coverage. Heterogeneity of nano humus and biochar may be ascribed to its irregular shape and size of pores, surface functional groups, and impurities (Jaroniec & Madey, 1988). Fitting the Redlich-Peterson isotherm ($R^2 = 0.99$) further confirmed adsorption of metal ions on nano humus was not by monolayer coverage, which leads to a more thorough utilization of adsorption sites in the adsorbent.

None of the 28 empirical isotherm models showed acceptable R^2 values (< 0.60) to determine adsorption mechanism of Cd(II) and Zn(II) on humic powder. Although low R^2 seems rare in the literature, it was obtained in some studies (Elamin et al., 2019; Nzediegwu et al., 2021). Poor fitting can be linked with extremely small porosity of humic powder as a small pore size cannot hold large metal ions, regardless of charges or polarity (Ahmedna et al., 2004). Blockage might hinder and slow down adsorption. Increasing dosage of low porosity materials, such as humic powder, is suggested for future experiments as it could lead to increased adsorbent pore size, length, and availability of adsorption sites, which resulted in greater metal ion removal. Many studies mentioned that percent removals positively correlated with adsorbent dosage (Meena et al., 2008; Azouaou et al., 2010; Jung et al., 2013).

Our materials were compared with various carbon rich adsorbents with relatively closer experimental pH conditions reported in the literature (Table 6.3). Adsorption capacities of different carbon based materials varied significantly. Although a direct comparison is difficult due to differences in experimental conditions, nano humus and other similar humic substance materials had relatively higher adsorption capacity (Q_{max}) than compared materials.

3.4 Contact time

Effect of contact time on Cd(II) adsorption efficiency varied (Figure S6.3a), with 89.1 % Cd(II) quickly removed by nano humus at a metal concentration of 100 mg/L (pH = 7) within15 minutes, and with a very steep slope. Removal gradually reached equilibrium with increased contact time and eventually removed 92.8 % at 24 hours. Removal of Cd(II) by humic powder followed a gently increasing trend in the initial 3 hours, increasing slowly to 30.1 % at 24 hours. Removal by biochar was lowest in the first 15 minutes at 7.1 %, increasing gradually with increased contact time until equilibrium after 24 hours at 28.7 %. Results highlighted that nano humus adsorbed cadmium best, wherein most adsorption was rapidly achieved in 15 minutes.

Adsorption of metal ions onto solid adsorbents is a complex and dynamic process in which metal ions bind to the adsorbent's surface before equilibrium is reached. The variation with contact time can be attributed to the initial large number of vacant surface sites available for adsorbing metal ions that consequently become saturated. This explains why initial adsorption was rapid and slowed with time.

3.5 Adsorption kinetics

Adsorption kinetics analysis was conducted to gain insight into rate controlling steps affecting adsorption kinetics. Adsorption kinetics of Cd(II) by three adsorbents were analyzed by pseudo first order, pseudo second order, and intraparticle diffusion kinetic models (Table 6.4, Figure S6.3bcd). The pseudo first order model assumes adsorption rate is controlled by diffusion steps (Lagergren, 1898; Rudzinski & Plazinski, 2007). The pseudo second order model assumes rate is dominated by chemisorption (Ho & McKay, 1999). The intraparticle diffusion assumes adsorption is diffusion controlled, and rate is dependent upon rate of adsorbate diffuse towards adsorbent particles (Weber & Morris, 1963). The intercept C reflects boundary layer thickness, the larger the value the greater the effect (McKay et al., 1980; Wu et al., 2009). When C equals zero, adsorption is entirely governed by intraparticle diffusion; a non zero C usually indicates a multistep mechanism (An, 2020; Kuang et al., 2020).

The pseudo second order model provided a better correlation of experimental data of nano humus (R^2 = 0.99). Equilibrium adsorption capacity of nano humus calculated from the model (q_{cal}) showed good agreement with the corresponding experimental value (q_e). With humic powder data, the pseudo second order model provided a relatively higher R^2 and more accurate q_{cal}, regardless of low R^2 for both models. Neither mono nor multilinear fitting of the intraparticle diffusion model was appropriate for modeling nano humus and humic powder data which confirmed adsorption was not diffusion controlled. Thus the adsorption rate determining step of Cd(II) on humic substance, nano humus, and humic powder systems is assumed to be chemisorption. It might involve valency forces through sharing or exchange of electrons between Cd(II) and functional groups on humic substance surface (Ho & McKay, 1999). Comparing initial adsorption rate (h), nano humus was significantly largest. This supported our experimental results in the previous section of contact time where rapid adsorption of Cd(II) onto nano humus was observed.

With biochar, pseudo first order models fit better than pseudo second order models with slightly higher R^2 and theoretically q_{cal} predicted from both models were in accordance with the experimental value q_e. A high R^2 was also given by the intraparticle diffusion model in mono linearity. Herein, diffusion, including boundary layer diffusion and intraparticle diffusion, might be the rate controlling step for cadmium-biochar systems. The small value of C tended to limit the effect of boundary layer diffusion. Therefore, intraparticle diffusion played the most important role as the rate determining step for the Cd(II)-biochar system. Diffusion resistance (external and internal) to mass transport was greatly influenced by particle size; smaller particles have a shorter distance of diffusion, higher mass transfer rate, and consequently better adsorption utilization of internal surface (Xu et al., 2018). Thus, particle size as an important adsorbent evaluation parameter for cattle manure biochars in cadmium removal should be considered.

4. Conclusions

Cadmium adsorption of humic materials tended to be by chemisorption dominated multilayer adsorption, while diffusion dominated for cattle manure biochar. These materials have shown their ability to adsorb metal ions with various efficiencies. Due to the large number of available sites (oxygen containing functional groups), nano humus was the most efficient adsorbent for both cadmium and zinc, followed by humic powder and biochar with lower capacity. Although nano humus and humic powder were derived from lignite, they showed notable differences in physical and chemical characteristics (morphology, particle size, specific surface area, porosity, and surface chemistry) and adsorption behaviours. Humic materials with higher oxygen to carbon ratio and porosity were more effective in metal adsorption. Particle size could be one of the important determinants of cattle manure biochar since a smaller particle size could result in a higher diffusion rate. Specific surface area had very limited influence on adsorption effectiveness of the three materials studied. In general, the great adsorption capacity of nano humus suggested its high suitability for heavy metal removal from contaminated water. The capacity of humic powder and biochar was highly comparable with reported carbon based materials. We recommend using lignite derived humic materials and cattle manure derived biochar in future heavy metal remediation.

Table 6.1 Properties of adsorbents.

Adsorbent	pH	Electrical conductivity	Specific surface area	Total pore volume	Mean pore diameter
		µS/cm	m^2/g	cm^3/g	nm
Nano humus	8.98	80.67	2.05	0.02	37.43
Humic powder	8.82	341.33	0.14	4.99E-04	14.18
Biochar	8.33	66	3.91	0.03	27.94

Table 6.2 Isotherm parameters for Cd(II) and Zn(II) adsorption on adsorbents.

Adsorbent	Metal	Langmuir isotherm			Freundlich isotherm			Redlich Peterson isotherm			
		Q_{max}	b	R^2	k_f	n	R^2	A	B	g	R^2
		mg/g	L/mg		mg/g			L/g		L/mg	
Nano humus	Cd(II)	99.85	1.92	0.94	55.31	4.30	0.97	557.75	8.34	0.84	0.99
	Zn(II)	71.70	0.70	0.87	32.16	4.39	0.98	503.30	14.56	0.79	0.98
Humic powder	Cd(II)	389.00	0.00	0.13	0.37	0.93	0.12	0.46	0.05	0.45	0.04
	Zn(II)	43.71	0.08	0.00	16.39	5.32	0.00	1.36	0.00	2.66	0.11
Biochar	Cd(II)	32.71	0.07	0.82	6.81	3.10	0.91	4.85E+05	6.66E+04	0.81	0.60
	Zn(II)	51.11	0.02	0.96	2.22	1.73	0.96	-1.30E+04	-5.08E+03	0.56	0.74

Table 6.3 Comparison of adsorption capacity of nano humus, humic powder, and biochar with selected carbon based adsorbents.

Adsorbent	Best fit isotherm	Maximum adsorption capacity (Qmax) (mg/g)	pH	Reference
Cadmium				
Nano humus	Freundlich	99.85	7.5	Our study
Humic powder	Poor fitting		7.9	Our study
Cattle manure biochar	Freundlich	32.71	7.2	Our study
Dairy manure biochar	Freundlich	54.4 (486 mmol/kg)	5.85-6.98	Xu et al., 2013
Bagasse fly ash	Langmuir	1.24	6	Gupta et al., 2003
Oak wood char	Freundlich	0.37	5	Mohan et al., 2007
Pine bark char	Freundlich	0.34	5	Mohan et al., 2007
Oak bark char	Freundlich	5.4	5	Mohan et al., 2007
Chemically modified maize straw	Langmuir	196.1	5.8	Guo et al., 2015
Castor seed hull	Langmuir	11.90	6	Sen et al., 2010
Oxidized granular activated carbon	Langmuir	5.74	6	Huang et al., 2007
Humic materials	Langmuir-Freundlich	433.4 (3.87 mmol/g)	5	Havelcová et al., 2009
Oxyhumolite humic acids	Langmuir	62.7 (0.56 mmol/g)	/	Čežíková et al., 2001
Soil extracted humic acids	/	415.5 (3.71 mmol/g)	4	Coles & Yong, 2006
Leonardite humic substances	Langmuir	129	4	Meng et al., 2017
Zinc				
Nano humus	Freundlich	71.7	7.2	Our study
Humic powder	/	/	7.6	Our study
Cattle manure biochar	Freundlich	51.11	7.1	Our study
Dairy manure biochar (DM350)	Langmuir	31.66 (487 mmol/kg)	5.85-6.98	Xu et al., 2013
Fir tree sawdust	Langmuir	13.4	6	Šćiban et al., 2006
Hardwood biochar	Langmuir	4.54	5	Chen et al., 2011
Corn straw biochar	Langmuir	11	5	Chen et al., 2011
Castor seed hull	Langmuir	15.13	6	Sen et al., 2010
Humic materials	Langmuir-Freundlich	64.55 (0.993 mmol/g)	5	Havelcová et al., 2009
Oxyhumolite humic acids	Langmuir	27.95 (0.43 mmol/g)	/	Čežíková et al., 2001

Table 6.4 Kinetic parameters for Lagergren and intraparticle diffusion models.

Adsorbent	Pseudo first order				Pseudo second order				Intraparticle diffusion		
	q_e	k_1	q_{cal}	R^2	k_2	q_{cal}	h	R^2	k_{id}	C	R^2
	mg/g	h^{-1}	mg/g		g/mg/h	mg/g	mg/g/min		mg/g/$h^{0.5}$	mg/g	
Nano humus	93.10	2.53E-06	5.50	0.53	1.30E-03	92.59	11.12	0.99	0.09	87.82	0.18
Humic powder	36.80	9.25E-07	24.13	0.18	1.37E-04	27.96	0.11	0.52	0.48	8.10	0.30
Biochar	30.00	2.88E-06	27.37	0.86	1.13E-04	31.08	0.11	0.84	0.63	2.70	0.90

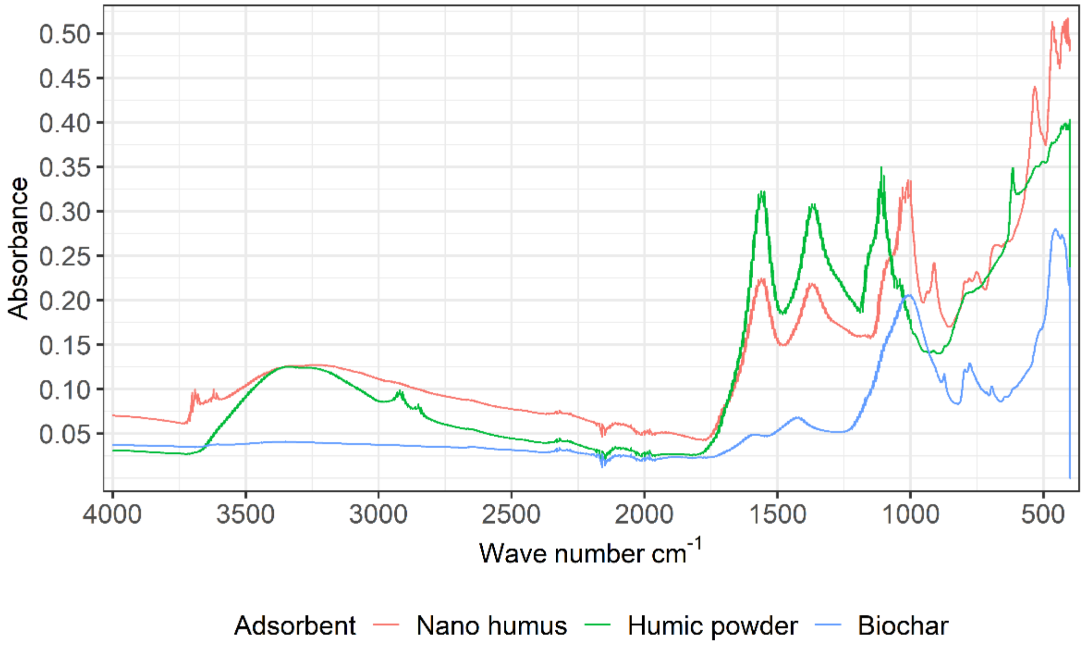

Figure 6.1 Fourier transform infrared spectroscopy spectrum of nano humus, humic powder, and biochar.

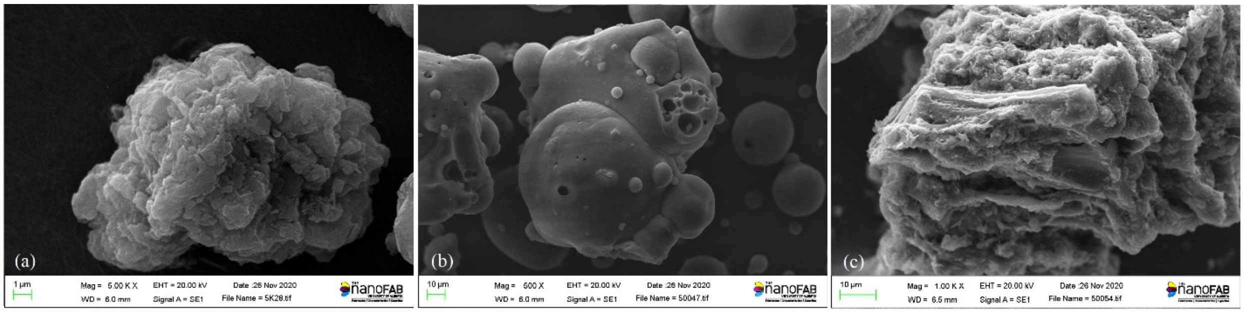

Figure 6.2 Scanning electron microscope images of (a) nano humus at magnification 5K ×, (b) humic powder at magnification 100 ×, and (c) biochar at magnification 100 ×.

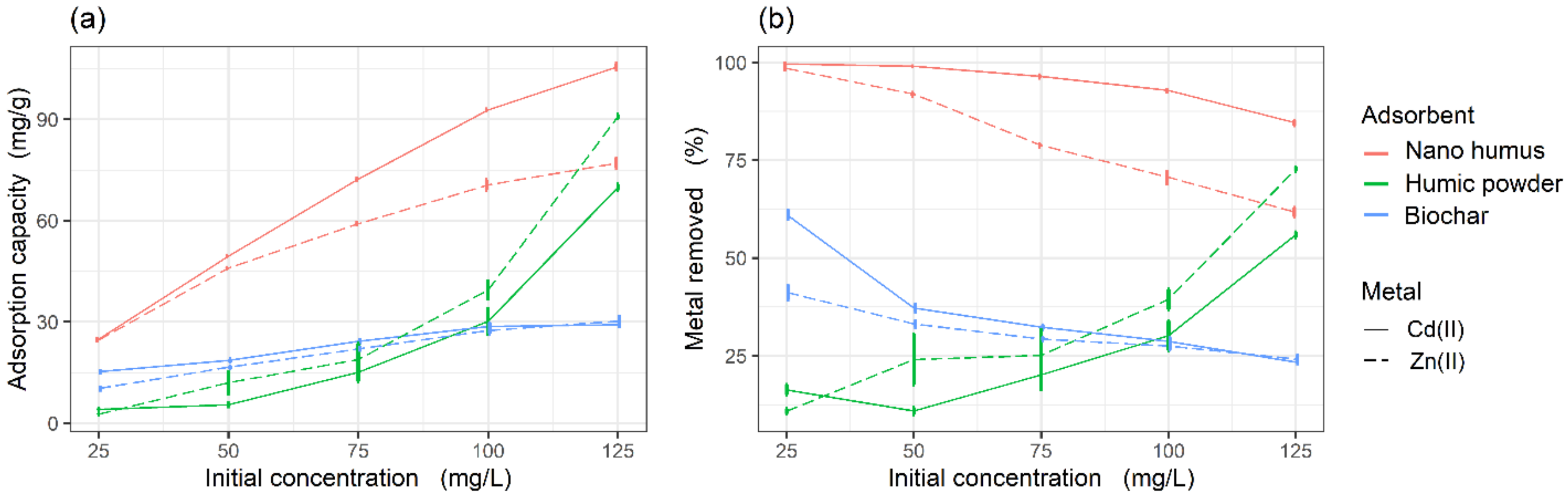

Figure 6.3 Effect of initial concentration on (a) percentage of removal, and (b) adsorption capacity of three adsorbents.

Supplementary Materials

Table S6.1 Elemental composition of adsorbents from energy dispersive X-ray analysis.

Adsorbent	Element (Wt %)												Atomic ratio
	C	O	K	Al	Si	S	Ca	Fe	Na	Mg	P	Cl	O:C
Nano humus	28.91	57.74	6.61	1.59	1.52	0.28	2.33	0.54	/	0.14	/	/	1.50
Humic powder	48.05	25.49	18.37	0.90	0.40	2.66	2.60	0.89	0.41	0.23	/	/	0.40
Biochar	59.65	33.94	0.19	0.46	0.21	0.63	0.60	1.41	0.20	0.72	0.72	1.27	0.43

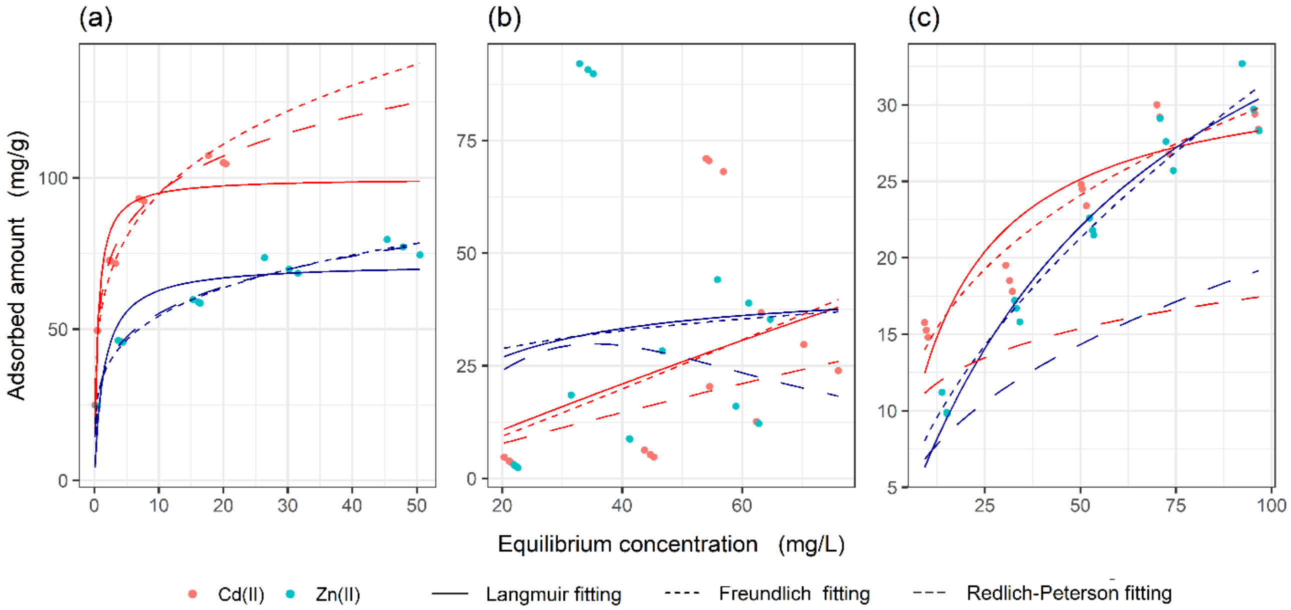

Figure S6.2 Langmuir, Freundlic, and Redlich–Peterson model fitting for (a) nano humus, (b) humic powder, and (c) biochar.

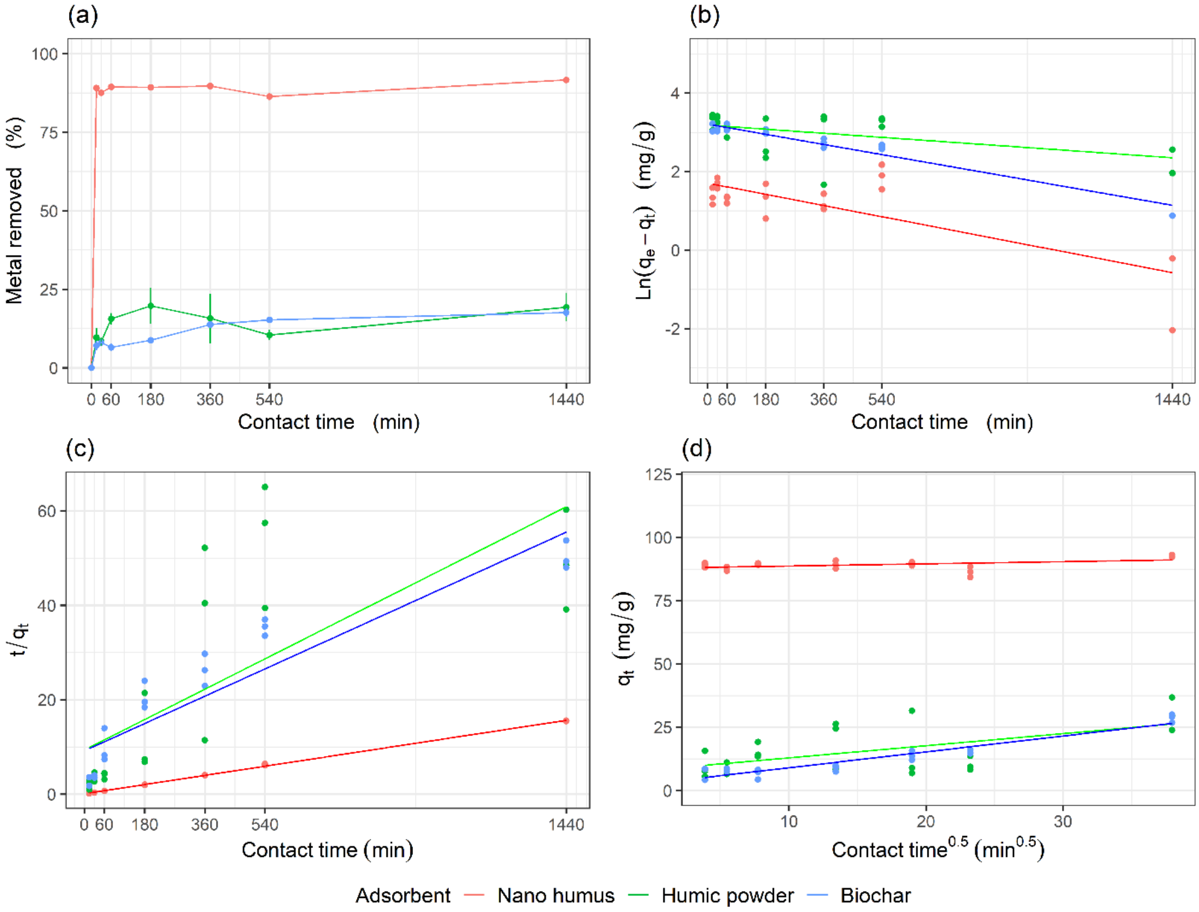

Figure S6.3 Regression plot for (a) percentage of removal, (b) first order kinetics, (c) second order kinetics, and (d) the intraparticle diffusion model.

VII. Synthesis Of Research

1. Research Summary, Contributions, and Recommendations

1.1 Introduction

Our research assessed the potential of an industrial waste derived humic material, called nano humus, as a soil amendment in coal mine reclamation and remediation. This work was conducted in the greenhouse and laboratory at the University of Alberta, and at a field site in northern China (Shendong coal mine). Our research results contribute to a better understanding of existing knowledge on whether, how, and why humic substances will affect plant growth, soil properties, and heavy metal removal in coal mine reclamation in sandy soil regions.

We gave constructive and practical suggestions on nano humus applications that can be applied in other regions of the world with similar reclamation challenges. Some of our work was implemented under actual field conditions on a coal mine site for two consecutive growing seasons. We included the entire growth cycle of three agricultural plant species; whereas the majority of published studies were conducted in greenhouses and/or other controlled growing chambers or cells with a single phase of plant growth. To our knowledge, we are the first to investigate the biological effect of application timing of lignite derived humic materials in coal mine reclamation. Our work contributes significantly to its value and provides generalizable principles that affect soil properties and crop growth under natural conditions. Our findings on humic substance characterizations and adsorption models demonstrated a sound knowledge of heavy metal removal mechanisms which are not well studied. We provided valuable evidence of the positive effect of humic substances where uncertainty remains in previous observations and has impeded their use in land reclamation and sustainable agriculture.

1.2 Application rate and timing

We determined the most appropriate rate of nano humus in the greenhouse which was then applied in all of our following experiments, and we investigated the combined use of nano humus with inorganic fertilizer as soil amendments in sandy soils (Chapter II). We evaluated nano humus application timings with different numbers of applications and frequencies in the field and identified the best timing for land reclamation in sandy soil regions (Chapter III). A single time application would be a more suitable reclamation strategy in sandy soil regions from a practical perspective. We showed it could provide conditions for better growth of alfalfa, barley, and sea buckthorn, and healthier mined soils, than by splitting the application amount into multiple times. We recommended an early application of humic materials for future practice in sandy soil regions as the change in soil nutrient status takes time. Early stage plant developments can also benefit, which is a major determinant of later biological developments and reclamation success. We recommended direct use of nano humus at a rate of 150 g/m^2 as an aqueous suspension (1 % concentration) on the surface of the soil at the beginning of the growing season (May).

1.3 Sole application of nano humus

We assessed the sole application of nano humus as a soil conditioner and a plant growth biostimulator (Chapters III and IV), and a heavy metal ameliorant in soils of two textures (Chapter V). We studied the chemical characterization of nano humus and provided insights into the underlying heavy metal removal mechanisms in contaminated water (Chapter VI). The impact of a sole application of nano humus on soil available nitrogen, phosphorus, and potassium, and plant biomass was remarkable. As a soil conditioner, nano humus positively changed most soil variables after two consecutive years of application, including soil cation exchange capacity (38 %), total organic carbon (49 %), and soil available nutrients (29 to 64 %). As a biostimulator, its key benefits on plant growth were root elongation and greater total biomass production (alfalfa by 729 %, barley by 250 %, sea buckthorn by 147 %). As a heavy metal adsorbent, the great adsorption capacity for divalent cadmium (Q_{max} = 99.9 mg/g) and zinc (Q_{max} = 71.7 mg/g) suggested a high suitability for heavy metal removal from contaminated water where the process tended to be chemisorption dominated multilayer adsorption. However, alleviation of heavy metal bioaccumulation in crop tissues was not obvious. We recommended using nano humus in reclaiming mined sandy soils in coal mine reclamation due to its significant effects on enhancing soil properties and crop growth. We recommended using nano humus in heavy metal remediation of contaminated water.

1.4 Combined application of nano humus

We investigated effects of nano humus combined with inorganic fertilizer (Chapter II), and/or arbuscular mycorrhizal fungi (Chapter IV) under greenhouse and field conditions. The synergistic effects of nano humus and arbuscular mycorrhizal fungi on soil chemical properties, soil metal remediation, and plant biomass production were pronounced, demonstrating the great possibility of these combinations as effective soil amendments in reclamation. Application of arbuscular mycorrhizal fungi with nano humus increased soil cation exchange capacity (38 %), total organic carbon (36 %), and available nutrients (20 to 92 %); they reduced soil concentrations of cadmium

(25 %), thallium (11 %), and arsenic (9 %); they enhanced total biomass of alfalfa (1413 %) and barley (28 %). These findings contribute to a better understanding of humic substances and mycorrhizae interactions in the rhizosphere and provide important implications in development of cost effective remediation strategies in contaminated soils. The pronounced performance of the combined application of humic substances with fertilizer or (and) arbuscular mycorrhizal fungi provided insights for future land reclamation strategies. We recommended these amendment combinations, and the synergistic application could be quantitatively more pronounced on altering soil physiochemical environments and plant growth than corresponding sole applications. We provided important information to assist in filling existing knowledge gaps where little information is available. We recommended using these combinations for future applications in sandy soil regions.

1.5 Comparison with other materials

We made a direct comparison of nano humus with humic powder (another lignite derived humic product) and cattle manure biochar for promoting plant growth, reducing heavy metal bioaccumulation, and removing heavy metal from contaminated water (Chapters V and VI). Relative to humic powder and biochar, nano humus had the greatest stimulation effect on plant growth, and both humic products exhibited the most visible enhancement on root development in soil of two textures. The direct comparison in our research suggested nano humus is a highly comparable soil amendment. Nano humus showed outstanding heavy metal removal efficiency from contaminated water, which was more distinct than cattle manure biochar and the other humic product, most likely due to the presence of oxygen containing functional groups in nano humus. It should be noted that lignite originated humic materials can be functionally different in heavy metal adsorption, which can be attributed to differences in physical and chemical properties (morphology, particle size, specific surface area, porosity, surface chemistry). When selecting lignite derived humic materials for metal removal, we recommended considering oxygen to carbon ratio and total pore volume instead of specific surface area, since humic materials with higher oxygen contents and porosity were more effective in metal adsorption. Lignite derived humic materials, nano humus, and humic powder were less effective in soil than water, and they did not reduce heavy metal uptake by plants significantly.

2. Limitations of the Research

As with every research project, there are things that could have been done differently if we had more time and money. These aspects may be addressed in future research on these topics.

In the greenhouse experiment we focused on metal accumulation in plant biomass in two cadmium contaminated soils, we were unable to analyze the exact soil samples due to budget constraints. Although total cadmium concentration was known at approximately 13 mg per kg soil by calculation, we did not have data for the available cadmium content in the two research soils after cadmium solution addition. The fate of cadmium in soils were unknown, and variabilities in cadmium chemical forms may have different effects on plants (Fu et al., 2011; Lai et al., 2021).

We were unable to determine the translocation coefficient due to insufficient biomass materials. We could not measure cadmium content in barley roots, stems, leaves, and seed heads separately as many roots and seed head samples did not reach the minimum sample volume required for analysis (dry weight > 1 g), particularly those grown in loamy sand. Since metals can be accumulated by plants and the metals within plants are dynamic with metal ion distribution changing in plant organs (Álvarez-Fernández et al., 2014), it would have been good to understand these dynamics in our plants.

In the field experiment, we observed an interesting phenomenon that plant biomass enhancement at maturity with the combined application of nano humus and AMF was not associated with a change of plant tissue nutrient contents. We questioned if this could be observed at an earlier growth stage. This is because plant demand for nutrients will shift as plants grow and mature, which regulates nutrient uptake by roots and distribution in plant tissues (Hawkesford et al., 2018). Unfortunately, our data were not able to explain this phenomenon as more frequent sampling would have been needed. A monthly sample collection for nutrient content analysis was not possible due to budget and logistical constraints. Plant sampling was very challenging for deep rooted species in the field; alfalfa had an active root zone at 2 m depth in only two years. Although 420 individual plants were excavated and sampled in total, and despite considerable efforts, we were only able to conduct nutrient content analysis on an annual basis.

3. Future Research Directions

Future research can build on our results and move forward to ascertain more detailed applications of humic materials for land reclamation, agriculture, and other potential uses.

- The efficacy and mechanism of lignite derived humic substances to treat industrial process water with multiple organic and inorganic toxic elements.

- Joint use of lignite derived humic substances adsorption and phytoremediation in constructed wetlands.
- Biological functions of humic materials derived from different origins, such as lignite, compost, and sewage sludge in reclamation and remediation.
- Application of lignite derived humic materials as soil amendments in the reclamation of mined saline soils.

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