Examining biochar as a carrier for *Rhizobium spp.* on pea crop

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ABSTRACT

The symbiotic relationship formed between legumes and rhizobia plays an integral role in the agriculture industry as the bacteria fix atmospheric dinitrogen (N_2) to plant available nitrogen (N). Inoculating legume crops with rhizobia is a common agricultural practice with peat and clay being the preferred inoculant carriers. Both peat and clay are slowly renewable, natural resources with limited availability. This leaves room to explore alternative, more sustainable, carriers that can compete biologically and economically with current carriers. A potential alternative carrier is biochar which is the product of thermal degradation of organic materials in the absence of air (pyrolysis). Feedstock, pyrolysis temperature, and degree of oxidation during the production of biochar affects the resulting biochar characteristics. The aim of this research was to (i) characterize the physical and chemical properties of a variety of biochars and examine their abilities to support rhizobia, (ii) manipulate a subset of biochars to achieve increased surface area, (iii) assess the potential phytoxicity of each biochar, and (iv) evaluate the ability of each biochar to deliver nodulating rhizobia to pea seed. Nine biochars produced from different feedstocks and sources were examined. The biochars displayed a wide range of physical and chemical properties resultant from the varying feedstocks and production conditions. Six of the nine biochars were able to support rhizobia while three showed an inability to support rhizobia. The % carbon (C) and C:N ratio of the biochars was found to positively correlate with *Rhizobium* survival. The manipulated biochars had exponentially larger surface areas than the original biochars but failed to support rhizobia immediately following inoculation. It was observed that the manipulated biochars were very alkaline most likely causing rhizobia to become stressed upon inoculation and subsequently unable to survive in the high pH conditions. There were some phytotoxic effects on garden cress seed with undiluted biochar extracts where, conversely, the diluted biochar treatments resulted in the biostimulation of garden cress. There were no conclusive results assessing the biochars ability to deliver rhizobia to pea seed as the uninoculated and sterile treatments were successfully nodulated via native rhizobia. These findings suggest that biochar has the ability to support rhizobia but due to biochar's complex nature, further research is needed in developing it as an inoculant carrier.

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1.0 General Introduction

1.1 Introduction

The inoculation of legume seeds with *Rhizobium* and *Bradyrhizobium* bacteria has been a common practice in the agriculture industry for more than 90 years (Brockwell and Bottomley, 1995). The symbiotic relationship formed between legumes and rhizobia bacteria plays an integral role in the agriculture industry as the bacteria fix atmospheric dinitrogen (N_2) to plant available nitrogen (N). Currently in North America there are three formulations offered to consumers: powdered, granular, and liquid. Limited research has been conducted to examine new carriers for *Rhizobium* and *Bradyrhizobium* inoculants with *Rhizobium* spp. research largely focusing on the genetics of rhizobia (Xavier et al., 2004). Several studies have been conducted examining new potential inoculant carriers including compost (Wall, 2003), charcoal (Beck, 1991; Crawford and Berryhill, 1992), biochar (Hale et al., 2015) and, agro-industrial waste (Rebah et al., 2007).

Powdered peat, the most common formulation, maintains favorable nutrient and environmental conditions that encourage rhizobial survival and growth (Xavier et al., 2004). Concerns associated with using inoculant grade peat include its vulnerability to weather conditions (CSPMA, 2008), and its valuable environmental services (IPS and IMCS, 2012). Clay is commonly used in granular formulations because of its large surface area and desirable surface area and moisture properties (Malusa et al., 2012). Peat and clay are both slowly renewable, natural resources that are subject to availability in certain geographic regions. Additionally, they both have associated environmental concerns with their obtainment. The extraction of peat can release nutrients and minerals into water downstream potentially causing water quality issues (Swystun et al., 2013). Clay is obtained via mining which causes disturbances to the landscape and can have adverse environmental effects. Liquid formulations can be easily applied however they require refrigeration during transport and storage which makes them unfavorable when compared to more easily stored powdered or granular inoculants. The current state of microbial inoculants leaves room for research examining alternative, more sustainable, carriers that can compete biologically and economically with existing commercial formulations. Biochar (Hale et al., 2015) and charcoal (Beck, 1991; Crawford and Berryhill, 1992) are both capable of supporting microorganisms. Further research is needed to provide a

better understanding of how these materials interact with the inoculant microorganism and the soil environment.

Biochar is the product of thermal degradation of organic materials in the absence of air (pyrolysis). Studies examining biochar as a soil amendment for agricultural (Lehmann et al., 2011; Stefankiw, 2012) and reclamation purposes (Strobbe, 2013) have been conducted. Factors such as feedstock and pyrolysis temperature during production have been identified as affecting the resulting biochar's properties and its effectiveness as an amendment. Physical properties such as surface area, pore volume and pore diameter have been linked to production temperature (Day et al., 2005; Sohi et al., 2010; Downie et al., 2011) whereas chemical properties tend to be influenced by both feedstock and production conditions (Amonette and Joseph, 2009; Enders et al., 2012). Due to the charred nature of biochar, it has been inherently linked to harmful environmental compounds including volatile organic compounds (VOCs) and polycyclic aromatic hydrocarbons (PAHs). Both VOCs and PAHs are known to persist in the environment and can cause adverse effects to organisms and human health. It is apparent that biochar is a complex material to study and can display a wide range of characteristics. The characteristics of biochar and their relationship with microorganism survival is important to understand when considering biochar as an inoculant carrier.

The aim of this study was to (i) characterize the physical and chemical properties of a variety of biochars and examine their abilities to support rhizobia bacteria, (ii) manipulate a subset of biochars to achieve increased surface area and pore volume, (iii) assess the potential phytoxicity of each biochar, and (iv) evaluate the ability of each biochar to deliver nodulating rhizobia to pea (*Pisum sativum*) seed. This thesis was prepared in traditional format and consists of seven chapters. It begins with the introduction (Chapter 1) and is followed by the literature review (Chapter 2). The materials and methods are outlined in Chapter 3. The results (Chapter 4) include the biochar physical and chemical characterization, *Rhizobium* survival studies, biochar phytotoxicity bioassay, and the biochars ability to deliver *Rhizobium* to pea crop. These results are discussed in Chapter 5 where relationships between biochar properties and Rhizobium survival are examined, and biochars overall potential as an inoculant carrier is discussed. Chapter 6 concludes this research and outlines future research directions for exploring biochar as an inoculant carrier. Literature cited (references) are listed in Chapter 7.

2.0 LITERATURE REVIEW

2.1 Legume crops and *Rhizobium:* A symbiotic relationship

Globally, it is estimated that legumes are grown on approximately 250 million hectares (Mha) and fix approximately 90 Tg of atmospheric dinitrogen (N_2) a year (Xavier et al., 2004). Agriculture and Agri-food Canada (2013) reported that the total area in Canada seeded to the four major leguminous pulse crops (dry pea (*Pisum sativum*), lentil (*Lens culinaris*), dry bean (*Phaseolus vulgaris*) and chickpea (*Cicer arietinum*) increased from 1.26 Mha in 1995 to 3.02 Mha in 2010. Domestically, these pulse crops are predominantly used for livestock feed with the remainder being used for human consumption and seed. Canada exports the majority of its pulse crops for human consumption earning an estimated $2.1 billion in 2009-2010 (Agriculture and Agri-Food Canada, 2013).

Legumes are unique among agricultural crops in that they form symbiotic relationships with *Rhizobium* bacteria in which the bacteria fix atmospheric N_2 to ammonia (NH_3) where it is quickly protonated to plant available ammonium (NH_4^+). When in close proximity to legume roots, the bacteria release nod factors that induce the root hairs to curl allowing the bacteria to penetrate the root system. The root cells begin to rapidly multiply resulting in nodules being formed at these infection sites. Inside these nodules the *Rhizobium* bacteria differentiate into a cell-type called a bacteroid that facilitates the N_2 fixation (Oke and Long, 1999). In return, the host plant provides nutrients and energy, in the form of photosynthates, to the rhizobia. Nitrogen additions to the soil can decrease the need for plants to form symbiotic relationships with rhizobia when compared to N limited environments (Lehmann et al., 2011). Quantifying the amount of nitrogen (N) fixed by leguminous crops is a complex task with some global estimates ranging from 15 Tg of N yr^{-1} (Smil, 1999) to 21.45 Tg of N yr^{-1} (Herridge et al., 2008). Lindstrom et al. (2010) reports that legumes fix on average 66% of the N recovered in the crop, equaling 23 to 176 kg N ha^{-1}, depending on plant species and the rhizobia present in the nodules. The N that is biologically fixed from the atmosphere reduces the need for additional N inputs including both organic and inorganic sources.

Legumes are recognized as having positive effects on soil quality when properly managed by increasing the N supply in the soil, increasing soil organic matter, and stimulating soil biological activity (Saskatchewan Agriculture and Food, 2005). Legume crops have been

reported to increase soil N concentrations through above- and below-ground biomass inputs. These inputs can be beneficial for subsequent crops making legumes advantageous to include in crop rotations. Legumes contribute to below-ground carbon (C) and N inputs via rhizodeposition although high variability has been reported based on plant species and field conditions (Wichern et al., 2008). Studies examining the N economy of pulse crops in the Northern Great Plains were subject to variability but when studied over a long-term period it has been found that faba bean, field pea, and lentil are likely to contribute positively to soil N while other crops such as desi or kabuli chickpea are more likely to be neutral or cause a N deficit in the soil (Walley et al., 2007). Field pea have been reported to release 129 kg N ha^{-1} in the soil, with rhizodeposition accounting for 56 kg N ha^{-1} (Wichern et al., 2008). Furthermore, it is reported that for a pea crop the amount of total crop residue N (including above-and below-ground inputs) remaining in the soil system following harvest was 35.6% of total plant N with 61% of that being derived from belowground inputs including root N and N rhizodeposits (Arcand et al., 2013). This residual N is beneficial to future crops and to overall soil health.

Some of the first recorded evidence of the legume and *Rhizobium* symbiotic relationship dates back to the 17^{th} century when Malpighi published a diagram with "bump" being observed on legume roots (Deaker et al., 2004). Although the mechanisms of N_2 fixation were unknown at the point, further studies in 1887 by Hermann Hellriegel and Hermann Wilfarth recognized that it was in these "bumps" (nodules) that the conversion of N_2 to NH_3 was occurring (Deaker et al., 2004). One year later, *Rhizobium* were first isolated and cultured by Martinus Beijerinck (Deaker et al., 2004). Following these discoveries, much has been learned about *Rhizobium* bacteria and the processes by which they infect legume roots.

The symbiotic relationship formed between rhizobia and legumes is a highly specified interaction that requires compatibly of the bacteria and host at all stages of nodulation including nodule infection, nodule invasion and nodule development (Sharma et al., 1993). Initially, flavonoids are secreted by the plant subsequently inducing the rhizobia to produce host specific *nod* factors involved in nodulation (Sharma et al., 1993; Spaink, 1994). The structure of the *nod* factor allows the legume host to distinguish between species and biovars of rhizobia (Sharma et al., 1993). Each rhizobial strain is able to interact with a limited number of host plant species (Spaink, 1994). *Rhizobium leguminosarum* biovar *viciae* can inoculate several legume species

including peas, vetches, lentils and sweet peas (Sharma et al., 1993; Spaink, 1994). Other rhizobia are more host specific. For example, *Rhizobium leguminosarum* biovar *trifoli* is specific to clover (Spaink, 1994). Optimal N_2 fixation can occur when plant genotypes are adapted to local rhizobial populations and when the soil is inoculated with strains adapted to local environmental conditions (Lindstrom et al., 2010). A challenge associated with inoculant production is presented in identifying which *Rhizobium* strain is the most efficient N_2 fixer with a particular legume species as several strains could be compatible.

Rhizobia are a free-living bacteria that are naturally occurring in many soil environments. Within the soil matrix, they are commonly found in legume plants' rhizosphere with their population decreasing in non-rhizosphere soil. Rhizobia are medium-sized, rod-shaped cells ranging from 0.5-0.9 µm in width and 1.2-3.0 µm in length (Somasegaran and Hoben, 1994). They are gram-negative bacteria that do not form endospores and move by a single polar flagellum or two to six peritrichous flagella (Somasegaran and Hoben, 1994). Rhizobia are classified as aerobic chemoorganotrophs and grow well in the presence of oxygen. They do not fix N_2 in the free-living form except under special conditions (Somasegaran and Hoben, 1994). Optimal growth of most rhizobia strains occurs at temperatures ranging between 25-30^{o}C and a pH range of 6.0-7.0 (Somasegaran and Hoben, 1994). It is important to have a carrier material that can support rhizobia and effectively deliver the bacteria to legume seed.

2.2 Current *Rhizobium* inoculant production and carriers

Inoculants are important in the agricultural industry as they deliver beneficial bacteria, including rhizobia, to crops. Various inoculant carriers have been studied over the years including powdered, granular and liquid formulations. Characteristics of a suitable carrier include, but are not limited to, a pH readily adjustable to 6.5 – 7.0 (buffering capacity), a good moisture-holding capacity, a readily sterilizable material, and free of toxic materials (Thompson, 1983). Important characteristics of the inoculant carrier formulation include a stable formulation during production, distribution, storage and transportation as well as being easy to handle and apply to ensure effective application (Xavier et al., 2004).

Limited research has been conducted to examine new carriers for *Rhizobium* and *Bradyrhizobium* inoculants (Wall, 2003; Rebah et al., 2006; Albareda, 2008; Hale et al., 2015) with *Rhizobium* research largely focusing on the genetics of rhizobia (Xavier et al, 2004).

Powdered peat, the most common formulation, maintains favorable nutrient and environmental conditions that encourage rhizobial survival and growth (Deaker et al., 2004; Xavier et al., 2004; Albareda, 2008). The shelf-life of a powdered peat inoculant is dependent on the manufacturers indicated expiry date although it is typically produced for use within one growing season. Liquid and granular formulations are also currently available on the inoculant market. Other materials have been examined as alternative carriers including sterile and non-sterile carriers. Albareda et al. (2008) studied six alternative carriers to peat including bagasse, cork compost, attapulgite, sepiolite, perlite and amorphous silica with cork compost and perlite showing superior results in maintaining the survival of different rhizospheric bacteria. Compost has been examined as a granular bacterial inoculant with results showing selected sterile composts were able to support large populations of rhizobia which were comparable to those of commercial inoculants. The author recommended future studies to manipulate the water content to enhance the survival of *Rhizobium* (Wall, 2003). Biochar (Hale et al., 2015) and charcoal (Beck, 1991; Crawford and Berryhill, 1992) also supported inoculant microorganisms but further research is needed to understand the effects of each material on microorganisms.

2.2.1 Peat

In Canada, it is estimated that 70 million tonnes of peat accumulates each year over 113.4 x 10^6 ha with approximately 1 million tonnes of peat being harvested (Daigle and Gautreau-Daigle, 2005). Canadian peat is harvested largely for horticultural and agricultural uses, used domestically and exported internationally. There is no indication of how much is consumed by the inoculant production industry. Peat extraction activities steadily increased in 1990 from 10 kha to 18.5 kha in 2005, almost doubling the land area under extraction (Environment Canada, 2007). In Canada, peat harvesting companies typically have a policy for the preservation of environmentally sensitive peatlands and for site restoration or reclamation of harvested sites (Daigle and Gautreau-Daigle, 2005). Concerns associated with utilizing inoculant-grade peat include its expense because of its difficulty in excavating (Brockwell, 1985) and its vulnerability to weather conditions. At best, peat can be considered a slowly renewable resource. Some estimates report peatlands naturally recovering in 15 to 20 years (Daigle and Gautreau-Daigle, 2005). Furthermore, peat is not readily available in many parts of the world due to the lack of natural peat deposits (Graham-Weiss et al., 1987) and as such can be difficult and/or expensive

to obtain (Brockwell, 1985; Beck, 1991). Additionally, peat is vulnerable to weather conditions. In the 2008 growing season, persistently wet conditions led to only 43% of typical volumes being harvested resulting in a peat shortage (CSPMA, 2008). Peat provides essential environmental services that includes habitat for rare or unusual species, carbon storage, and water quality protection (IPS and IMCS, 2012).

The abiotic conditions associated with peatlands typically include high water levels, and acidic conditions. This often results in special adaptations for species that live there. This has led to a variety of mosses, carnivorous plants, shrubs and orchids adapted to peatlands that are not commonly found in other locations (CSPMA, 2016). Peatlands also provide habitat for several species of mammals, birds and insects (CSPMA, 2016).

Peatlands act as natural filters in the hydrological cycle by accumulating nutrients, minerals, sediments and pollutants. This can prevent these constituents from moving downstream potentially affecting water quality in freshwater bodies via processes such as eutrophication. This accumulated matter is relevant to peat extraction as it can be released during harvesting. A common practice in peat extraction is draining of the peatland to induce aerobic conditions within the peat column to increase decomposition which subsequently releases the nutrients and minerals contained within the peatland water (Swystun et al., 2013). This can lead to higher nutrient and suspended solid concentrations in downstream water potentially causing water quality issues (Swystun et al., 2013).

Peatlands are inherently linked to carbon storage as a large quantity of organic matter accumulates in them. The harvesting of peatlands can contribute to greenhouse gas emissions chiefly through carbon dioxide (CO_2) outputs. Environment Canada (2007) reports that peatlands managed for peat extraction, in 2005, contributed 0.6 Mt of CO_2 emissions. Peat extraction is an anthropogenic carbon source and to neutralize the CO_2 emissions additional sinks must be created without relying on natural, pre-existing sinks (ie. natural and restored peatlands) (Schilstra, 2001). The ecological importance of peatlands and subsequent environmental effects of peat harvesting leave room for examining more sustainable inoculant carriers.

2.2.2 Liquids

Liquid inoculants are typically produced via a peat culture mixed in water, or a mineral- or organic-based liquid that is inoculated with the desired bacteria (Deaker et al., 2004). These inoculants may be directly applied to the seed or, alternatively, applied directly in the seed furrow (Deaker et al., 2004). However, this formulation does leave the bacteria readily exposed to extreme environmental stresses after inoculation and seed planting which can adversely affect bacterial survival thus, making it less favored then powdered or granule inoculant carrier forms (Tittabutr et al., 2007; Albareda et al., 2008). Additionally, the inoculant needs to be refrigerated during shipping and storage, a condition not required for powdered or granular formulations. Liquid inoculants offer a long shelf life with observed rhizobial populations being sufficient after a 2-year period (Black et al., 2006). Some adverse physiological changes have been reported in on-seed stability and rhizobia's ability to form nodules by storing commercial liquid formulations for several years (Xavier et al., 2004).

2.2.3 Clay

Granular inoculant formulations are typically produced from inert clay or peat, with the granules containing inoculum being sown with seed in the seedbed. The direct inoculation of granular carriers into the seed furrow or slightly below the seed furrow reduces the possibility of detrimental effects on rhizobia caused by pesticides and fungicides that are applied directly to the seed (Deaker et al., 2004). Legume crop nodulation and yields associated with granular inoculants have been observed to be equivalent to those obtained with powdered peat and liquid products (Stephens and Rask, 2000).

Clay is commonly used in granular inoculant formulations. It is a naturally occurring material that possesses desirable properties as an inoculant carrier including its large surface area and the ability to absorb microorganisms in its matrix (Malusa et al., 2012). Its availability is subject to location with the United States, Brazil and South Africa typically leading clay mining and subsequent processing (USGS, 2014). Two clays commonly used in the agricultural industry are Kaolin and Vermiculite. These aforementioned clay materials are natural, soil-based materials that require mining and processing before they can be used agriculturally or industrially. Kaolin clay is used as a component in media formulations that are applied as a sorbent coating to seed or seedling roots for protection, and to supply plant growth promoting

rhizobacteria (PGPR) (Allen et al., 2010). Vermiculite is commonly used as a granular inoculant carrier in North America. It provides a good buffering zone to organisms introduced into a foreign environment due to its ion-exchange capacity (Paau, 1998). Vermiculite and kaolin can be obtained via open-pit or underground mining with underground clay deposits largely being associated with coal deposits (EPA, 1995). Although clay is a suitable inoculant carrier that is currently used in agriculture, it is subject to availability depending on location and is collected via mining which has negatively associated environmental impacts. Additionally, it can be considered a non-renewable resource making room for other, more renewable and readily available, materials to be explored as inoculant carriers.

The vulnerability, availability, cost, and environmental impacts of acquiring current inoculant carrier's leaves opportunity for research examining alternative carriers that can compete biologically and economically with the leading existing commercial formulations.

2.2 Biochar: A potential *Rhizobium* inoculant carrier

Biochar is the product of thermal degradation of organic materials in the absence of air (pyrolysis). Various organic materials have been used as feedstocks including animal litter and manures, plant residues, food waste, and sewage sludge. During production, the feedstock is placed in an oxygen-depleted kiln and heated to high temperatures (200-750^{o}C) for various durations of time (Novak et al., 2009). Factors such as feedstock, temperature, duration of pyrolysation, and amount of oxygen present during pyrolysis are known to affect the resulting biochars properties. Okimori et al. (2003) developed a preliminary set of seven core properties to examine in the evaluation of biochars including pH, volatile compound content, ash content, moisture-holding capacity, bulk density, pore volume, and specific surface area. The variability of these properties, particularly surface area and pore volume, make it a desirable candidate to explore as an inoculant carrier.

Biochar use has largely been investigated in agricultural and environmental applications. Biochar additions to the soil can increase crop yields, reduce nutrient leaching and increase biological N_2 fixation in leguminous plants (Quilliam et al., 2013a). Biochar can be of environmental benefit as its application to the soil sequesters C subsequently increasing soil carbon storage (Day et al., 2005; Lehmann, 2007; Sohi et al., 2010). During pyrolysis of organic matter, the C in plant biomass is rapidly converted into a more resistant, stable, form allowing C

to persist in the char form (Lehmann, 2007). Additionally, biochar can alter greenhouse gas emissions. The additions of biochar to soil in otherwise normally managed agricultural systems suppressed nitrous oxide (N_2O) and methane (CH_4) emissions, both of which have high global warming potentials (Sohi et al., 2010). A considerable amount of research with biochar has been focused on its function in contaminated soils and its remedial properties. Biochar has a high sorption capacity which increases nutrient retention (Fellet et al., 2011) and affects the bioavailability and mobility of inorganic pollutants (Fellet et al., 2011; Beesley et al., 2010; Beesley and Marmiroli, 2011). Although studies show promise for biochar in environmental applications, its use and cost of use at a large scale still needs to be further examined.

Limited research has been conducted examining biochar as a carrier for bacterial inoculants, including *Rhizobium* (Crawford and Berryhill, 1982; Beck, 1991; Hale et al., 2015). Mineral soil, with and without wood charcoal amendment, was studied as an inoculant carrier with the amended soil supporting higher populations of rhizobia while being equally as effective as peat in maintaining a viable population of *Rhizobium* (Beck, 1991). In a recent study, biochar was examined as a carrier for a plant growth promoting rhizobacteria (PGPR). The bacteria's survival on several biochars was examined (Hale et al., 2015). Biochar chemical characteristics, particularly N and pH, had the greatest effect on PGPR survival. Although once incorporated into soil physical properties such as surface area, pore opening diameter and water filled-pore spaces had the greatest effect on PGPR survival (Hale et al., 2015).

Biochars have been reported to support microbial communities with pH, pore structure, surface matter and mineral matter being important factors in determining how a biochar effects soil microbes (Pietikainen et al., 1994; Lehmann et al., 2011). The stability of the biochar material dictates how much C is available as an energy source. Furthermore, the mineral-rich ash proportion of biochar includes several macro-and micro-nutrients for uptake by the microorganisms (Lehmann et al., 2011). The beneficial mechanisms by which abundance of rhizobia is increased includes improved hydration, greater P, Ca, Mg and K availability, greater micronutrient availability, and increased biofilm formation (Lehmann et al., 2011). Biofilm is a thick layer of polysaccharides excreted by microorganisms that facilitates attachment to surfaces, and in the case of rhizobia to legume roots (Fujishige et al., 2006). The sorption of signaling compounds (important to the rhizobia/legume symbiotic relationship), sorption of inhibitory

compounds, and sorption of dissolved organic matter to biochars can have varying effects on rhizobia populations (Lehmann et al., 2011). Biochar's high variability in properties has a prominent effect, in both favorable and unfavorable capacities, on microbial communities.

Important chemical properties of biochar include volatile matter content, functional group composition, ash content, pH, elemental composition and biochar toxicity. Volatile matter, or the labile fraction, can indicate the quantity of nutrients readily available for microorganisms (Lehmann et al., 2011). Volatile matter ranges from 13.2% to 70.0% with content largely depending on pyrolysis temperature rather than feedstock (Enders et al., 2012). Higher temperatures typically result in lower volatile matter contents due to greater degradation of the materials (Enders et al., 2012). The organic functional groups present in biochars are mainly dependent on feedstock, with effects on microorganisms being positive, neutral and/or negative depending on the functional group (Downie et al., 2009). Biochar surface chemistry is typically heterogeneous in nature with N, H, O, K, and S being incorporated in the aromatic C rings as heteroatoms (Downie et al., 2009). The composition of these aromatic rings can create diverse micro-environments within biochars with acidic and basic sites coexisting micrometers from each other (Downie et al., 2009). Aqueous acid solutions used in common soil tests have been effective in extracting portions of Si, Fe, S, P, K, Mg, and Ca from biochar indicating the quantity of nutrients that may be available to organisms (Bourke et al., 2007; Major et al., 2010). Feedstock is known to be a precursor to biochar elemental composition with high concentrations of total P, N, Ca, Mg, K and Na in original feedstock material translating into high concentrations of the same elements in biochars (Enders et al., 2012).

The ash content of biochar is directly related to pH as the ash portion accumulates base cations such as Mg and Ca. Therefore, biochars with a high ash content generally have higher pH values than those with lower ash contents (Enders et al., 2009; Lehmann, 2011). Ash content of biochars can range from 0.4% to 88.2% with greater variations occurring between feedstocks than with pyrolysis temperature, although ash content did tend to increase with temperature (Enders et al., 2012). Biochar pH can range from below pH 4 to above pH 12 although biochar tends to be more alkaline by nature (Lehmann, 2011).

Notable physical properties of biochar include surface area, pore volume, pore width, and moisture properties. Surface area is directly related to the temperature at which the biochar is

produced (Sohi et al., 2010). Day et al. (2005) reported temperatures of 400°C and 900°C resulting in surface areas of 120 $m^2 g^{-1}$ and 460 $m^2 g^{-1}$, respectively, although this can vary greatly with feedstock. Pore volume displays a linear relationship with surface area and often surface areas in micropores are significantly greater than those of macropores (Downie et al., 2009). The surface area of biochar can be measured by gas adsorption although it should be noted that this measurement is influenced by microporosity (nm) which does not pertain to plant roots, microbes, or the mobile soil solution (Sohi et al., 2010). The average pore diameter of a batch of biochar is indicative of the porosity of the material being micro- (< 2 nm), meso-(2–50 nm), or macro-porous (>50 nm) (Downie et al., 2009) which relates to microorganisms being able to physically fit inside the pore spaces. It is important to recognize that biochar is a heterogeneous material and a sample analyzed from a batch of biochar may not be an accurate representation of the batch.

Biochar hydrological properties are important to consider when examining it as a carrier for microorganisms. A biochar's water holding capacity, the maximum amount of water biochar can hold, is inherently linked to porosity as greater pore space offers more area for adhesion and cohesion of water within the biochar. The inherent moisture content of a biochar is the moisture held within the biochar itself with no additional moisture being added. Water activity (A_w) is the partial vapor pressure of water in a substance divided by the standard state partial vapor pressure of water. It is desirable for an inoculant carrier to have low A_w to limit microbial growth while sufficiently supporting acceptable levels of rhizobia during the products shelf-life (Stephens and Rask, 2000). When interacting with moisture, biochar can display hydrophobic or hydrophilic tendencies. Research has shown that hydrophobicity can be controlled by choice of pyrolysis temperature with mid-range temperatures (400°C – 600°C) yielding desirable hydrophobicity ranges (Kinney et al., 2012).

There is the potential for biochar to contain toxic compounds whose presence can affect plants, soil, and microorganisms. The presence of these toxic compounds is strongly linked to biochar production conditions and subsequent handling, with biochar feedstock being seemingly unrelated (Spokas et al., 2011). The production process has the ability to contaminate biochar via the re-condensation of pyrolysis vapors containing volatile organic compounds (VOCs) and can result in biochar containing highly mobile, phytotoxic compounds (Buss and Masek, 2014).

Processing, handling, and storage of biochar can result in the reduction of VOCs (Buss and Masek, 2014). Volatile organic compounds are of concern in the environment as they are a primary precursor in the formation of ground-level ozone and particulate matter which lead to smog formation which negatively affect human health and the environment (Government of Canada, 2015). Some biochars have been reported to have over 140 individual VOCs desorbed from them (Spokas et al., 2011) which illustrates the complex and diverse nature of biochar chemistry.

Another group of compounds known as polycyclic aromatic hydrocarbons (PAH) have long been associated with biochars as they result from the incomplete combustion of organic materials. Polycyclic aromatic hydrocarbons are a concern in the environment as they can persist for long periods of time and can affect organisms through various toxic activities therefore, it is important to consider PAH content when introducing biochars into the environment. Analytical methods that efficiently extract PAHs from biochars are lacking which results in limitations in quantifying their PAH content (Hilber et al., 2012) especially quantification of bioavailable PAHs (Hale et al., 2012). While quantitative measurements are lacking, PAH association with biochars has been fairly well studied. When biochar is applied as an amendment to soil, PAH mineralization is repressed due to increased sorption and reduced bioavailability (Quilliam, 2013b). A study examining the phytotoxicity of biochar on garden cress seed (*Lepidium sativum*) concluded that PAHs are rather immobile in biochars making them unlikely to be the cause of toxicity in germination tests (Buss and Masek, 2014). Further research on the mobile compounds associated with biochar and their associated effects on plants, soils and micoorganisms would be beneficial in providing a link to biochar toxicity.

As previously discussed, biochar properties are often resultant of feedstock source and pyrolysis conditions. Biochars can be further processed to achieve desirable properties with the activation process typically aiming to enhance the pore diameters of the initial biochar and to create new porosity (Alaya et al., 2000). In addition to the initial biochar pyrolysation, which occurs in an inert atmosphere at moderate temperatures (400^{o}C to 800^{o}C), a second stage of processing can be added. Physical activation involves exposing biochars to partial gasification using oxidizing gases such as steam, CO_2, air or a mixture of these gases at higher temperatures (usually >900^{o}C) resulting in well-developed and accessible internal pores (Downie et al., 2009).

The different oxidizing gases increase surface area and porosity via different reactions. The steam manipulation of biochars can increase surface area and porosity by removing constituents in the effluent gasses from the reactor (Azargohar and Dalai, 2008). Carbon dioxide activation results in the removal of C atoms (or burn-off) which facilitates the development of a more porous structure (Downie et al., 2009) while steam activation leads to the release of volatiles with partial devolatilization and enhanced crystalline C formation (Alaya et al., 2000). Following physical activation, biochar's have been shown to exhibit basic properties with negligible acidic groups being present (Azargohar and Dalai, 2007). The ability to manipulate biochar properties can be of benefit when examining biochar as an inoculant carrier as it can potentially be designed with properties suitable to microorganism survival.

Biochar has the potential to be a suitable carrier for bacterial inoculants based on previous research documenting positive effects of biochar on microorganisms (Beck, 1991; Lehmann et al., 2011; Hale et al., 2015). Biochar has properties, including surface area and pore volume, which can be manipulated to levels that may more effectively support a target microorganism. The varying properties of biochar can produce differing living conditions in the biochar pore spaces favorable to microorganism's survival. However, the high variability of biochar properties, relating to feedstock and production conditions, produces a challenge when considering biochar as a carrier for inoculants. Research is needed to examine the mechanisms by which biochar properties influence inoculant efficiency and survival (Lehmann et al., 2011).

3.0 MATERIALS AND METHODS

3.1 Biochar physical and chemical characterization

Procedures outlined by Somasagaren and Hoben (1994) were followed for preparing each biochar (Table 3.1) as an inoculant carrier. The biochars were ground in a hammer mill and sieved to 200 mesh (75 µm) and finer particle size. This preparation took place prior to further physical and chemical analysis as grinding the sample had the potential to affect subsequent biochar properties. It was decided to characterize the biochar following grinding because the 200 mesh and finer particles are the standard size for industry inoculants (Somasagaren and Hoben, 1994).

Biochars were prepared in a 1:10 biochar:water dilution, shaken by hand, and left to sit for 30 min. Following this, they were shaken again, left to sit for 1 h, and then the pH and EC of the solution were measured using an Accumet™ AP85 pH/conductivity Meter (Fisher Scientific™, Pittsburgh, PA, USA). The percent water holding capacity (WHC) was calculated by placing approximately 2 g of biochar in a column made from a 50 mL syringe barrel that was fitted with a cheesecloth filter at the bottom. The biochar was then saturated with water and allowed to drain for 24 hrs. Following this, the WHC was calculated using the mass of water retained in the biochar per g of dry biochar x 100%. A subset of air-dried biochars (BMB, FB, FFB2, WB and SPF2) were weighed (1.35 +/- 0.1 g) into disposable dishes and analysed for available water (A_W) (n=3) using a Model Series 3TE water activity meter (Aqua Lab, Decagon Devices, Pullman, WA, USA). Air-dried biochars were analyzed for elemental composition

Table 3.1. Biochar feedstock and source.

Biochar ID	Feedstock	Source
BMB	Bone meal	Titan Clean Energy, Saskatoon, SK
FB	Fish meal	Titan Clean Energy, Saskatoon, SK
FFB1 (Flin Flon 1)	bone meal or creosote/greenwood	Titan Clean Energy, Saskatoon, SK
FFB2 (Flin Flon 2)	bone meal or creosote/greenwood	Titan Clean Energy, Saskatoon, SK
OHB	Oat hull	Titan Clean Energy, Saskatoon, SK
FHB	Flax	Saskatchewan Research Council, Saskatoon, SK
WB	Wheat	Saskatchewan Research Council, Saskatoon, SK
SPF1	Spruce/pine/fir	Out of Ashes BioEnergy Inc., Prince George, BC
SPF2	Spruce/pine/fir	DiaCarbon Energy Inc., Burnaby, BC

using HF-HNO_3 Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Geology Department, University of Saskatchewan). Biochars were analyzed for the quantity of each of the following elements: Li, Sc, V, Rb, Sr, Y, Zr, Nb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Hf, Ta, Tl, Pb, Th, U, P , Ti, Cr, Co, Ni, Cu, Zn, As, Ge, Mo, Ag, Cd, Sn, Sb, W, Na, K, Al, Fe, Mg, Ca, and Mn. Nitrogen and C content in the biochars were analyzed using a isotope mass spectrometer coupled to the elemental analyzer (Soil Science Department, University of Saskatchewan).

Biochar surface area, total pore volume and average pore width were analyzed using the BET (Brunauer-Emmett-Teller) gas adsorption method via the BET ASAP 2020 instrument (Department of Engineering, University of Saskatchewan, Saskatoon, SK). The nine original biochars and three manipulated biochars (section 3.2) were measured for meso-pore (2-50 nm) surface area and pore volume. The Barret, Joyner, and Halenda (BJH) method used to calculate surface area and pore volume is based on a desorption model. All samples (0.2 g) were degassed for 6-8 h with N_2 prior to conducting the analyses (n=1).

Procedures outlined by ASTM method D1762 – 84 (2013) Chemical Analysis for Wood Charcoal were followed to analyze moisture content, volatile matter and ash content (n=3). Each biochar sample (1 g) was air-dried and then place in muffle furnace heated to 105^{o}C for 2 h. The dried samples were placed in desiccator for 1 h and weighed. The calculation for inherent moisture (%) is as follows:

$$Moisture\ (\%) = \frac{A-B}{A}\ x\ 100 \qquad \text{(Eqn. 3.1)}$$

Where A = grams of air-dry sample and B = grams of sample after drying at 105^{o}C. Following moisture analysis, the samples were analyzed for volatile matter. The same samples were placed in a preheated 950^{o}C muffle furnace. Samples were initially placed on the outer ledge of the furnace (300^{o}C) for 2 min and then placed on the edge of the furnace (500^{o}C) for 3 min. The samples were then moved to the back of the furnace (950^{o}C) for 6 min with the muffle door closed. The samples were cooled in a desiccator for 1 h and weighed. Volatile matter (%) was calculated using the following equation:

$$\text{Volatile matter (\%)} = \frac{B-C}{B}\ x\ 100 \qquad \text{(Eqn. 3.2)}$$

Where C = grams of sample after drying at 950°C. Following volatile matter analysis, the same sample was used to determine ash content. The samples were placed in a muffle furnace for 6 h at 750°C. The samples were cooled for 1 h in a desiccator and then weighed. Following this, samples were repeatedly burned for 1 hr periods until heating resulted in a loss of less than 0.0005 g. Ash content (%) was calculated by the following equation where D = grams of residue:

$$Ash\ (\%) = \frac{D}{B}\ x\ 100 \qquad \text{(Eqn. 3.3)}$$

Biochar surface functional groups were characterized using Attenuated Total Reflectance Fourier-Transform Infrared (ATR-FTIR) spectroscopy via a Bruker FRA 1061A spectrometer equipped with a liquid N_2-cooled MCT detector. All spectra were collected in attenuated total reflectance (ATR) mode using a Bruker PlatinumIR accessory with a single bounce diamond coated ZnSe crystal for optics. Samples were pressed onto the ATR-FTIR window as a fine powder. Measurement of air was collected and subtracted from all spectra for background correction. All samples were measured with 512 scans from wavenumbers 500-4000 cm^{-1} with 4 cm^{-1} resolution. The resulting FTIR-ATR spectra for the biochars were matched to known literature spectra for functional groups associated with biochars.

The surface morphology of each biochar was studied using scanning electron micrographs which were obtained via a Phenom G_2 Pure scanning electron microscope (SEM) (Biology Department, University of Saskatchewan). To prepare the samples, sieved (200 mesh) biochar was placed on an adhesive disc holder and coated with gold using a Gold Sputter Coater (Edwards S150B). Images at varying magnifications were captured using the SEM (Appendix I).

The 'Molarity of an Ethanol Droplet' (MED) test was completed following methods outlined by Doerr (1998). The MED test is an indirect measure of the surface tension of a soil surface and indicates how strongly a water drop is repelled by a soil at the time of application (Doerr, 1998). For the study, 6-8 g of biochar was weighed into a Petri dish, and levelled and

packed down. Solutions of increasing ethanol concentration were prepared: 0, 3, 8.5, 13, 24 and 36 % by volume. In increasing order of concentration, a drop (0.05 mL) of each solution was placed on the biochar surface until drop penetration occurred within 3 s. The MED test was performed on three subsamples within the same petri dish of each biochar, except for biochars FBM, SPFM1, and WBM which only had 1 subsample (to conserve biochar material). To facilitate statistical analysis, the MED values are categorized into a simple numerative and descriptive scale ranging from 1 (very hydrophilic) to 6 (extremely hydrophobic).

3.2 Biochar manipulation

Three biochars were selected for manipulation to increase surface area. Biochars SPF1, FB and WB were selected as they were representative of the strongest, mid-, and weakest performing biochars in survival study 1 (refer to section 4.1) thus representing the spectrum of biochar performances. For each biochar, two samples of 20.0 g each (total of 40 g of each biochar) were manipulated using physical (steam) activation with the parameters indicated in Table 3.2. The two samples for each respective biochar were thoroughly mixed together prior to any physical and chemical analysis, and prior to using in the *Rhizobium* survival study.

Table 3.2. Parameters for pressurized steam activation of biochars.

Parameter	Description
Set temperature:	704ºC
Actual temperature:	795 ºC
Boiler set temperature	250 ºC
Set flowrate:	26 % at 90 psi
Actual N_2 flowrate:	140 mL min^{-1}
Mass:	20 g
Actual steam flowrate:	15.25 g h^{-1}
Injection time:	1.4 h

3.3 *Rhizobium* survival study

3.3.1 Study 1

Biochars were inoculated with *Rhizobium leguminosarum* biovar *viciae* and *Rhizobium* survival on each of the biochars was examined over an 84 d period. The *R. leguminosarum* bv. *viciae* strain was isolated from the commercial peat inoculant (Nodulator XL® by Becker Underwood), used as a control in the study. The *Rhizobium* was isolated by thoroughly mixing 1

g of the peat inoculant in 9 mL of sterile deionized water. A sample was streaked onto Yeast-Mannitol Agar with Congo Red indicator dye (YMA + CR) (Somasagaren and Hoben, 1994) and incubated (28^{o}C) in the dark for 7 d. YMA + CR is a selective medium for growing *Rhizobium* (Somasagaren and Hoben, 1994) and was the enumeration medium used in this study. Following the initial isolation, *Rhizobium* colonies were identified and restreaked onto YMA + CR and incubated (28^{o}C) in the dark for 7 d. Typically, *Rhizobium* produce white colonies, or weakly absorb the CR dye, while other bacteria strongly absorb the CR dye (Kneen and LaRue, 1982). This was repeated once more to ensure a pure *Rhizobium* colony was obtained. The isolated *R. leguminosarum* bv. *viciae* was grown in Yeast Mannitol broth for 7 d on a rotary shaker (160 rpm, 25-30^{o}C) (Somasagaren and Hoben, 1994) and used to inoculate the biochars.

To prepare biochar as a carrier, each biochar was weighed (40 g) and transferred into a sterile polyethylene bag. The bags were partially sealed and sterilized via autoclaving using the G15 cycle (15 min at 121^{o}C) and allowed to cool overnight in a biosafety cabinet. The following day, the bags were sealed in aseptic conditions using an electric heat sealer. *R. leguminosarum* bv. *viciae* was aseptically injected into the polyethylene bag using a syringe. The *Rhizobium* concentration in the nutrient broth at the time of application was 6.86 x 10^{8} cells mL^{-1} broth. The *Rhizobium* was applied at a rate suitable to each biochars' respective WHC while ensuring that each biochar remained friable (Table 3.3). The theoretical concentration of rhizobia applied (Table 3.3) is an estimate based on the enumeration of inoculum and the volume of inoculum applied to the biochar. The broth was worked into the biochar by kneading the bag until the liquid inoculum was uniformly absorbed into the biochar. On Day 0, inoculation day, a sample from each biochar was obtained to enumerate an initial *Rhizobium* population for each biochar (Table 3.3); enumeration method is described below. To sample the biochar, the bag containing the biochar was cut open with sterile scissors and a sterile scoopula was used to collect 1 g of biochar. The bag was sealed using laboratory tape following sample collection. Subsequent biochar samples were obtained from the original opening and the bag was resealed following sampling. The inoculated biochars were incubated in the dark at 25-30^{o}C for 4 wk to facilitate *Rhizobium* growth and stabilization (Somasagaren and Hoben, 1994; Albareda et al., 2008). Following incubation, the inoculated biochars were stored at the recommended temperature of 4^{o}C (Somasagaren and Hoben, 1994). The biochars were enumerated weekly, starting at day 0, using traditional spread plate methods until the lower limit of detection (10^{6} rhizobia g^{-1} biochar)

was reached. A low limit of detection (10^6 rhizobia g^{-1} biochar) was established indicating when *Rhizobium* populations were no longer acceptable for industry standards (Somasagaren and Hoben, 1994).

Table 3.3. Study 1: Rate of *R. leguminosarum* bv. viciae applied to each biochar at inoculation time (D 0) and enumeration of *R. leguminosarum* bv. *viciae* rhizobia on day of inoculation†.

Biochar‡	Theoretical rhizobia applied (Log CFU g^{-1} biochar)	Enumerated Rhizobia (Log CFU g^{-1} biochar)
Bone Meal Biochar; **BMB**	7.84	8.14 (7.97)§
Fish Biochar; **FB**	7.74	8.31 (8.01)
Flin Flon 1; **FFB1**	7.74	8.53 (7.91)
Flin Flon 2; **FFB2**	7.84	8.56 (8.20)
Oat Hull Biochar; **OHB**	7.74	8.31 (6.95)
Flax Biochar; **FHB**	7.84	7.35 (7.79)
Wheat Biochar; **WB**	7.74	8.09 (7.84)
Spruce/Pine/Fir 1; **SPF1**	8.05	8.31 (8.12)
Spruce/Pine/Fir 2; **SPF2**	7.84	8.36 (7.81)

† Enumeration was performed the same day as initial inoculation
‡ The source for each biochar is listed in Table 3.1.
§ Number in brackets is the standard deviation of the mean of 3 replicates.

To enumerate rhizobia on the biochars, 1 g (n=3) of each inoculated biochar was placed in 99 mL of sterile distilled water in a dilution flask, shaken by hand for 1 min and left to sit for 30 min. The solution was shaken for 1 min prior to sampling to ensure a representative sample was collected. A 1 mL aliquot was placed in 9 mL of sterile distilled water in a dilution tube and mixed using a vortex mixer. A dilution series was created by pipetting 1 mL of the biochar solution into subsequent dilution tubes containing 9 mL of sterile distilled water to achieve dilutions in the range of 10^{-4}-10^{-7} (Somasegaran and Hoben, 1994). A 0.1 mL sample was plated on an YMA + CR and spread via a sterile plate spreader. Plates were inverted and incubated (28^{o}C for 5 d). *Rhizobium* colony forming units (CFU) were identified and counted. The CFU were used to estimate the *Rhizobium* population per g of biochar (Table 3.3).

3.3.2 Study 2

Biochars SPF1, FB and WB were selected for steam-activated manipulation as they were representative of the strongest, mid-, and weakest performing biochars in survival study 1 (refer to section 3.2) thus representing the spectrum of biochar performances. For each biochar the respective pre- and post-manipulated biochars were used (3 original biochars + 3 manipulated biochars = 6 biochars) to examine rhizobia survival as a function of surface area. The procedures followed in Survival study 1 (section 3.3.1) were used to inoculate, store and sample the biochars. The *Rhizobium* concentration in the nutrient broth at the time of application was 4.75×10^8 cells mL^{-1} broth with the biochars being sampled on inoculation day (Table 3.4).

Table 3.4. Study 2: Rate of *R. leguminosarum* bv. *Viciae* applied to each biochar at inoculation time (D 0) and enumeration of *R. leguminosarum* bv. *Viciae* rhizobia on day of inoculation†.

Biochar‡	Rhizobia applied (Log CFU g^{-1} biochar)	Rhizobia enumerated (Log CFU g^{-1} biochar)
Original biochars		
Fish Biochar; **FB**	7.71	6.74(6.46)§
Wheat Biochar; **WB**	7.62	7.18(6.67)
Spruce/Pine/Fir 1; **SPF1**	7.83	7.50(6.99)
Manipulated biochars		
Fish Biochar; **FBM**	7.71	0
Wheat Biochar; **WBM**	7.71	0
Spruce/Pine/Fir; **SPFM1**	7.83	0

† Enumeration was performed the same day as initial inoculation
‡ The source for each biochar is listed in Table 3.1.
§ Number in brackets is the standard deviation of the mean of 3 replicates.

3.4 Biochar phytotoxicity bioassay

A garden cress (*Lepidium sativum*) phytotoxicity bioassay was conducted following the protocol outlined by Leege and Thompson (1997). The tests consisted of (i) obtaining an aqueous extract from each biochar; (ii) incubating cress seeds in contact with the biochar water extracts; and (iii) quantifying seed germination and root elongation.

The biochars were grouped into two groups based on the date they were analyzed. Group 1 included BMB, FB, FFB1, FFB2 and OHB, and Group 2 included FHB, WB, SPF1 and SPF2. Separate water controls were included in each group. An aqueous extract of each biochar was collected by adding 50 mL of deionized distilled water (DI) to 40 g of biochar. The solution was incubated at ambient temperature (25°C) for 30 min. Water extract was collected using vacuum

filtration (Whatman #2 filters) and then centrifuged at 4800 G for 15 min. The supernatant was gently decanted to avoid resuspending the pellet. Dilutions (undiluted, 3x and 10x) of the supernatant were prepared. Filter paper (Whatman #2) was placed in sterile Petri dishes (n=5) and 1 mL of test solution was added to each Petri dish. Control dishes (n=5) were prepared with 1 mL of deionized (DI) water. Eight garden cress seeds were placed, evenly spaced, in each Petri dish. The dishes were incubated (25^{o}C) in the dark for 24 h. The plates were removed and the number of seeds germinated and radicle length were recorded. Group 1 and Group 2 biochar calculations were done using the respective control from that time. The percent germination (PG), percent radicle length (PRL) and germination index (GI) were calculated using the following equations:

$$\text{PG} = \frac{MTG}{MCG}\ x\ 100 \qquad \text{(Eqn. 3.4)}$$

$$\text{PRL} = \frac{MTRL}{MCRL}\ x\ 100 \qquad \text{(Eqn. 3.5)}$$

$$\text{GI} = \frac{PG\ x\ PRL}{10000} \qquad \text{(Eqn. 3.6)}$$

Where MTG is the mean treatment germination (count), MCG is the mean control germination (count), MTRL is the mean treatment radicle length (mm) and MCRL is the mean control radicle length (mm). The GI is on overall index for the bioassay as it encompasses both PG and PRL into one measure (Leege and Thompson, 1997).

3.5 Examining biochar's ability to deliver *Rhizobium* to pea seed

3.5.1 Study 1

The six biochars (BMB, FB, FFB1, FFB2, SPF1, SPF2) used in the growth chamber study were selected based on the survival study results. The biochars were used to inoculate pea seed with *Rhizobium leguminosarum* bacteria. Other treatments included two sterile biochar treatments (SPF1 and FFB2), an uninoculated control, an inoculant grade peat (Nodulator XL® by Becker Underwood) as an industry standard, and a reference wheat crop to measure biological

N_2 fixation using the ^{15}N enriched isotope dilution method. Each carrier treatment was replicated 6 times (6 formulations x 6 replicates + 2 sterile biochars x 6 replicates + 1 commercial inoculant x 6 replicates + 1 uninoculated control x 6 replicates + 1 wheat reference x 6 replicates = 66 pots).

Soil for this experiment was collected from a field near Bruno, SK which had no history of pulse crops. The soil has a clay loam texture. The soil was air-dried (7 days, 25°C) and screened to pass a 2 mm sieve. Soil was screened for the presence of native rhizobia by growing pea in a sample of the soil prior to the start of the growth chamber study. Soil was weighed into a 20-cm dia pot and distilled water was added to bring the soil to 70% field capacity. Five holes, 2.5 cm deep, were made and 1 surface sterilized pea seed was placed in each soil and covered remaining soil. Pea seed was surface sterilized by placing seed in 10% ethanol solution for 5-10 s. Ethanol was decanted off and the seed was covered with hydrogen peroxide for 5 min. Seed was rinsed a minimum of five times with sterile DI water. The pot was placed in a growth chamber with a cycle of 16 h day/22°C and 8 h night/18°C for 7 wk. Following germination, plants were thinned to three. Roots were collected and washed, and nodules were removed and counted. There were minimal nodules (11 per pot) observed. It was decided that native rhizobia presence was minimal and the soil would be used for the growth chamber study. The macronutrient content (kg ha^{-1}) of the soil was determined by ALS Laboratory Group, Saskatoon, SK.: NO_3-N, 14.6; P, 68.6; K, >1215; SO_4-S, 30.4. The pH of the soil was 8.1 and the electrical conductivity was 0.2 mS cm^{-1}.

Prior to seeding, germination tests were performed on the pea seed by placing seeds on moistened filter paper in Petri plates. Plates were incubated at ambient temperature (approx. 25°C) in the dark for 5 d. There was 100% germination. To set-up the growth chamber study soil (2 kg) was weighed into 20-cm dia pots, adjusted to approximately 70% field capacity, and allowed to equilibriate at room temperature for 1 wk prior to seeding. Soil moisture was maintained at approximately 70% field capacity with distilled water for the duration of the study by watering every two days. Five holes, 2.5 cm deep, were made in each pot. The inoculated biochars were applied at a rate of 1.0×10^6 rhizobia per seed. At the time of seeding, there was variation among the biochars in respect to *Rhizobium* populations so different weights of biochar were applied for each of the treatments to achieve an equal application of rhizobia per seed.

Additional sterile biochar was applied as necessary to achieve equal weights of biochar applied per seed for all treatments. Biochar was placed in the seed hole to ensure rhizobia-seed contact. One sterilized seed was placed in each hole. The seeds were covered up with remaining soil and placed in the growth chamber. There were two sterile biochar treatments (FFB2 and SPF1). For this, sterile biochar was placed in the seed hole at a weight equivalent to that of the inoculated biochar.

On the day of seeding, all biochar treatments were sampled and enumerated on YMA + CR following the protocols outlined for the survival study (section 3.3.1). After germination, pots were thinned to three plants per pot based on recommended seeding rates (Saskatchewan Pulse Growers, 2011). Pots were randomized weekly in a growth chamber with a cycle of 16/8 h day/night cycle and day/night temperatures of 22 ^{o}C/16^{o}C. Relative humidity was maintained at 50%.

For the N_2 fixation protocol a small amount (5.6 kg N ha^{-1}) of ^{15}N-urea (10 atom %) solution was surface applied to each pot. The wheat reference provides an estimate of plant uptake in the absence of biological fixation. The higher the amount of N_2 fixed the more the ^{15}N in the pea tissue is diluted. By comparing ^{15}N in the pea tissue to ^{15}N in the wheat tissue, an estimate of % N derived from atmosphere (%Ndfa) can be calculated (Australian Center for International Agricultural Research, 2013) as follows:

$$\%\text{Ndfa} = \frac{^{15}\text{N of reference plant} - {}^{15}\text{N of N2 fixing legume}}{^{15}\text{N of reference plant} - {}^{15}\text{N of } N_2} \times 100 \qquad \text{(Eqn. 3.7)}$$

Pea were grown to maturity (64 d) and harvested. Roots, shoots and seed were separated. The roots were washed, and the nodules removed and counted. Roots, shoots and seed were dried for 7 days at 60^{o}C and dry weight determined. Nodules were oven dried (60^{o}C) for 3 days, removed and dry weight determined. Following harvest, the uninoculated control was observed to have nodule counts similar to those of the *Rhizobium* inoculated treatments so the biomass samples from this study were not analyzed for N or ^{15}N content.

3.5.2 Study 2

The original objective of this study was to compare the original and manipulated biochars as carriers for *Rhizobium*. The manipulated biochars did not support *Rhizobium*. However, original biochars SPF1 and FB were the only biochars observed to support *Rhizobium* following *Rhizobium* survival study 2 (section 3.3.2) and therefore were the selected biochars for the growth chamber study. In addition to the inoculated SPF1 and FB biochars, a sterile biochar treatment for each respective biochar was used along with an uninoculated control, an inoculant grade peat (Tagteam® by Novozymes) as an industry standard and a wheat reference to measure biological N_2 fixation using the ^{15}N enriched isotope dilution method (Warembourg, 1993). Each carrier treatment was replicated 6 times (2 formulations x 6 replicates + 2 sterile biochars x 6 replicates + 1 commercial inoculant x 6 replicates + 1 uninoculated control x 6 replicates + 1 wheat reference x 6 replicates = 42 pots).

For this experiment a soil-less potting mix (LG3 Blend by Sungro Horticulture, Agawam, MA) was used to minimize potential interference of *Rhizobium* bacteria naturally occurring in the growth medium. Additionally, samples of the potting mix were prepared in a dilution series and spread plated on YMA + CR to further screen for rhizobia presence. For this, 1 g (n=3) of potting mix was thoroughly mixed in 9 mL of sterile deionized water. Refer to section 3.3.1 for protocol followed for preparing dilution series, preparing spread plates and incubation details. No distinguishable rhizobia colonies were observed on the plates although it should be noted that a suite of other bacteria were observed and could have hindered any rhizobia colony growth. To prepare for the growth chamber study, potting mix (1 kg) was weighed out into 20-cm dia pots, adjusted to approximately 70% field capacity, and allowed to equilibriate at room temperature for 1 wk prior to seeding. Soil moisture was maintained at approximately 70% field capacity with distilled water for the duration of the study. Germination tests were performed on the pea seed prior to seeding by placing seeds on moistened filter paper in petri plates. Germination was 100%. Pots were randomized weekly in a growth chamber with a cycle of 16/8 h day/night cycle and day/night temperatures of 21 ^{o}C/16^{o}C. The relative humidity was maintained at 50%.

Pea were grown to maturity (61 d) and harvested. Refer to section 3.5.1 for seeding protocol, harvest protocol and biological nitrogen fixation protocol. Shoot biomass was finely ground on a ball mill and analyzed for N concentration and atom% ^{15}N using a Delta V mass

spectrometer (Thermo Scientific®, Bremen, Germany) coupled to an ECS4010 elemental analyzer (Costech Analytical Technologies Inc., Valencia, California). The average N content per g dried biomass and the average N content derived from the atmosphere per pot were calculated based on N content and biomass values.

(Eqn. 3.8)

$$\text{N content (N mg g}^{-1}\text{ shoot biomass)} = Total\ shoot\ biomass\ per\ treatment\ x \frac{Percent\ N}{100}$$

(Eqn. 3.9)

$$\text{N content derived from atmosphere per pot (mg pot}^{-1}\text{)} = \frac{\%Ndfa}{100}\ x\ Average\ N\ content\ x\ total\ shoot\ biomass$$

3.6 Statistics

SAS 9.4 was used to perform all statistical procedures. Data from day 21 and 84 of the *Rhizobium* survival studies were used to examine correlations between biochar physical and chemical properties and rhizobia survival. Day 21 of the study was when the biochars capable of supporting rhizobia and the biochars incapable of supporting rhizobia became evident. Day 84 represented the end of the study where *Rhizobium* populations on biochars had declined but were observed to be relatively stable. The original biochars and their corresponding properties (physical, chemical and elemental composition properties) were for analysed. Biochar properties were examined for interacting effects correlating to *Rhizobium* survival using logistic multiple-regression using the forward stepwise method in SAS 9.4 (SAS Institute Inc., 2015). It was noted that this method has a moderate risk of making a Type II error and is most appropriately used when trying to fit a model to data and are uninterested in causality (Fields, 2005).

The log transformed *Rhizobium* survival data was analyzed using linear regression in SAS 9.4 (SAS Institute Inc., 2015) to assess the rate at which *Rhizobium* population declined on the biochars over time. Only the biochars the supported *Rhizobium* passed day 21 of survival study 1 were examined (BMB, FB, FFB1, FFB2, SPF1, SPF2). Following this, the regression slopes

expressing *Rhizobium* survival on the biochars were compared by calculating a z-value using the following equation:

$$z = \frac{\text{slope a} - \text{slope b}}{\text{SQRT}((\text{standard error of slope a})^2 + (\text{standard error of slope b})^2)} \quad \text{(Eqn. 3.10)}$$

Where a and b are the respective regression slopes being analyzed.

Univariate ANOVA and Tukey's honestly significant difference (HSD) tests ($p < 0.05$ level) were used to assess differences between treatments in both Growth Chamber Study 1 and Study 2. Normality was assessed using Shapiro-Wilk's test. Because of the cost of analyses biochar characterization analyses were typically run as single samples (n=1) so treatments were not analyzed for significant differences.

4.0 RESULTS

4.1 Biochar characterization

4.1.1 Physical characterization

The physical properties of the biochars showed a high level of variability among biochars within a parameter (Table 4.1). The original biochars yielded high ranges in BET surface area (0.11 to 153.25 $m^2\ g^{-1}$), pore volume (0.003 to 0.097 $cm^3\ g^{-1}$), average pore width (9.58 to 95.32 nm) and WHC (45 to 214%) (Table 4.1). Inherent moisture content had a smaller range from 1 to 4%.

Manipulation of the biochars resulted in physical changes to all three of the biochars processed (FB/FBM, WB/WBM, SPF1/SPFM1) (Table 4.1). The manipulation of biochars resulted in pores with a smaller average width contributing to larger surface areas and pore volumes. Following manipulation, the surface area for biochars FB, SPF1 and WB increased 4454%, 13544% and 20294%, respectively. This increase in surface area coincided with an increase in pore volume ($cm^3\ g^{-1}$) and a decrease in average pore width (nm) with biochars FB, SPF1 and WB changing by 420%, 4580% and 1880% and -71%, -88% and -87%, respectively. The inherent moisture content of FB did not change with manipulation while manipulation of SPF1 increased inherent moisture by 33%. Due to a limited amount of biochar materials and

several of the tests being cost prohibitive, only one sample for each biochar was typically analyzed per parameter.

4.1.2 Chemical characterization

The original set of biochars had highly variable chemical properties including EC (1.28 to 18.61 µS cm^{-1}), % C (40.7 to 82.7 %), C:N ratio (12:1 to 497:1), % volatile matter (15 to 55 %) and % ash (6 to 64 %) (Table 4.2). All biochars exhibited alkaline pH ranging from 8.59 to 10.01. The hydrophobicity of the original biochars ranged from very hydrophilic (MED index = 1) to very hydrophobic (MED index = 6). Biochars FHB and WB were very hydrophobic (MED index =6) with the other seven biochars having an MED index ranging from 1-3. The results for biochar elemental composition (excluding C and N) are reported in Appendix I.

The manipulation of the biochars resulted in chemical changes to all three of the biochars processed (Table 4.2). Collectively, there were increases in pH, C:N ratio and ash content and decreases in %N and volatile matter content. Changes in %C, EC and hydrophobicity varied between the three biochars. Carbon (%) decreased between FB/FBM and WB/WBM while it marginally increased between SPF1/SPFM1. The manipulation of the biochars produced a substantial increase in EC between SPF1/SPFM1 (+2978 %), a relatively small increase between FB/FBM (+25 %) and a relatively small decrease between WB/WBM (-43 %). There was an increase in hydrophobicity between FB/FBM and SPF1/SPFM1 while biochars WB/WBM were observed to have the highest rating of 6 corresponding to extreme hydrophobicity.

The biochar spectra from FTIR-ATR were matched with spectra of known functional groups of biochars found in the published literature (Table 4.1; Table 4.3). In all biochars, the shift from the pre- to post-manipulated biochars involved a relative increase in normalized reflectance from wavenumber 600 to 1000 cm^{-1} (Fig. 4.1). Following manipulation, the spectra from 600 to 1000 cm^{-1} for FB shifted upwards with a decrease in noticeable peaks. FB exhibited a peak at 1575 cm^{-1}, associated with lignin, where FBM did not (Table 4.3). WB also shifted to a higher normalized reflectance although distinguishable peaks could be identified. At 665 and 870 cm^{-1} WBM had peaks distinguishable from WB whereas WB had peaks distinguishable from WBM at 1695, 1575, 1413 and 1080 cm^{-1}. SPF1 had peaks at 1575 and 870 cm^{-1} where SFPM1 did not. Similar to the other two manipulated biochars, SPFM1 displayed increased normalized reflectance relative to SPF1 in the range of 600 to 1000 cm^{-1}.

Table 4.1. Physical characteristics of original and manipulated biochars. Manipulated biochars were treated with pressurized steam to increase surface area for *Rhizobium* attachment.

Biochar	BET surface area (m^2 g^{-1})	Pore volume (cm^3 g^{-1})	Average pore width (nm)	Water holding capacity (% by weight)	Inherent moisture content (% by weight)	Available Water	Source†
			Original biochars				
	(*n*=1)	(*n*=1)	(*n*=1)	(*n*=1)	(*n*=3)	(*n*=3)	
Bone Meal Biochar; **BMB**	113.35	0.097	9.58	140	1	0.19¤	TCE
Fish Biochar; **FB**	9.22	0.030	26.87	96	4	0.23	TCE
Flin Flon 1; **FFB1**	77.60	0.071	13.87	138	3	ND§	TCE
Flin Flon 2; **FFB2**	12.35	0.037	16.62	131	1	0.22	TCE
Oat Hull Biochar; **OHB**	0.11	0.003	95.32	195	2	ND	TCE
Flax Biochar; **FHB**	2.99	0.004	37.22	96	1	ND	SRC
Wheat Biochar; **WB**	2.92	0.005	44.34	154	1.5	0.21	SRC
Spruce/Pine/Fir 1; **SPF1**	4.93	0.005	40.66	214	1.5	ND	OAB
Spruce/Pine/Fir 2; **SPF2**	153.25	0.016	9.85	45	2.5	0.19	DCE
			Manipulated biochars				
	(*n*=1)	(*n*=1)	(*n*=1)		(*n*=3)	(*n*=3)	
Fish Biochar; **FBM**	419.96(+4454)‡	0.156(+420)	7.89(-71)	ND§	4.0(0)	ND	TCE
Wheat Biochar; **WBM**	595.52(+20294)	0.099(+1880)	5.63(-87)	ND	ND	ND	SRC
Spruce/Pine/Fir 1; **SPFM1**	672.66(+13544)	0.234(+4580)	4.91(-88)	ND	2.0(+33)	ND	OAB

† The source for each biochar is listed in Table 3.1.

‡ Value in parentheses indicated percent change from original biochar value

§ Value not determined (ND) for sample due to an inadequate amount of material to perform analysis.

Table 4.2. Chemical characteristics of original and manipulated biochars. Manipulated biochars were treated with pressurized steam to increase surface area for *Rhizobium* attachment.

Biochar	pH	EC (µS cm^{-1})	%N	%C	C:N Ratio	Volatile matter (%)	Ash (%)	Hydrophobicity (MED index)†	Source‡
				Original biochars					
	(*n*=1)	(*n*=1)	(*n*=3)	(*n*=3)	(*n*=3)	(*n*=3)	(*n*=3)	(*n*=3)	
Bone Meal Biochar; **BMB**	9.05	12.36	1.07	40.71	38	15	63	1	TCE
Fish Biochar; **FB**	9.65	10.44	4.34	51.33	12	28	36	2	TCE
Flin Flon 1; **FFB1**	9.15	18.61	1.27	40.60	32	20	64	1	TCE
Flin Flon 2; **FFB2**	9.86	17.65	3.19	40.63	13	29	52	2	TCE
Oat Hull Biochar; **OHB**	9.88	8.30	1.34	69.44	52	25	15	3	TCE
Flax Biochar; **FHB**	8.58	8.63	1.24	64.94	53	55	7	6	SRC
Wheat Biochar; **WB**	8.88	12.03	0.74	63.44	86	50	14	6	SRC
Spruce/Pine/Fir 1; **SPF1**	8.75	1.28	0.24	82.57	345	33	6	3	OAB
Spruce/Pine/Fir 2; **SPF2**	10.01	2.26	0.17	82.68	497	28	8	1	DCE
				Manipulated biochars					
	(*n*=1)	(*n*=1)	(*n*=3)	(*n*=3)	(*n*=3)	(*n*=3)	(*n*=3)	(*n*=1)	
Fish Biochar; **FBM**	11.14(+15)§	6.74(-43)	1.21(-72)	43.75(-15)	39(+225)	26(-7)	49(+36)	4	TCE
Wheat Biochar; **WBM**	11.17(+26)	13.05(+25)	0.24(-68)	58.65(-8)	205(+138)	ND¶	ND	6	SRC
Spruce/Pine/Fir; **SPFM1**	12.30(+41)	39.40(+2978)	0.05(-79)	84.50(+2)	585(+70)	23(-30)	8(+33)	4	OAB

† MED index of 1 = very hydrophilic and 6 = extremely hydrophobic

‡ The source for each biochar is listed in Table 3.1.

§ Value in parentheses indicated percent change from original biochar value

¶ Value not determined (ND) for sample due to an inadequate amount of material to perform analysis.

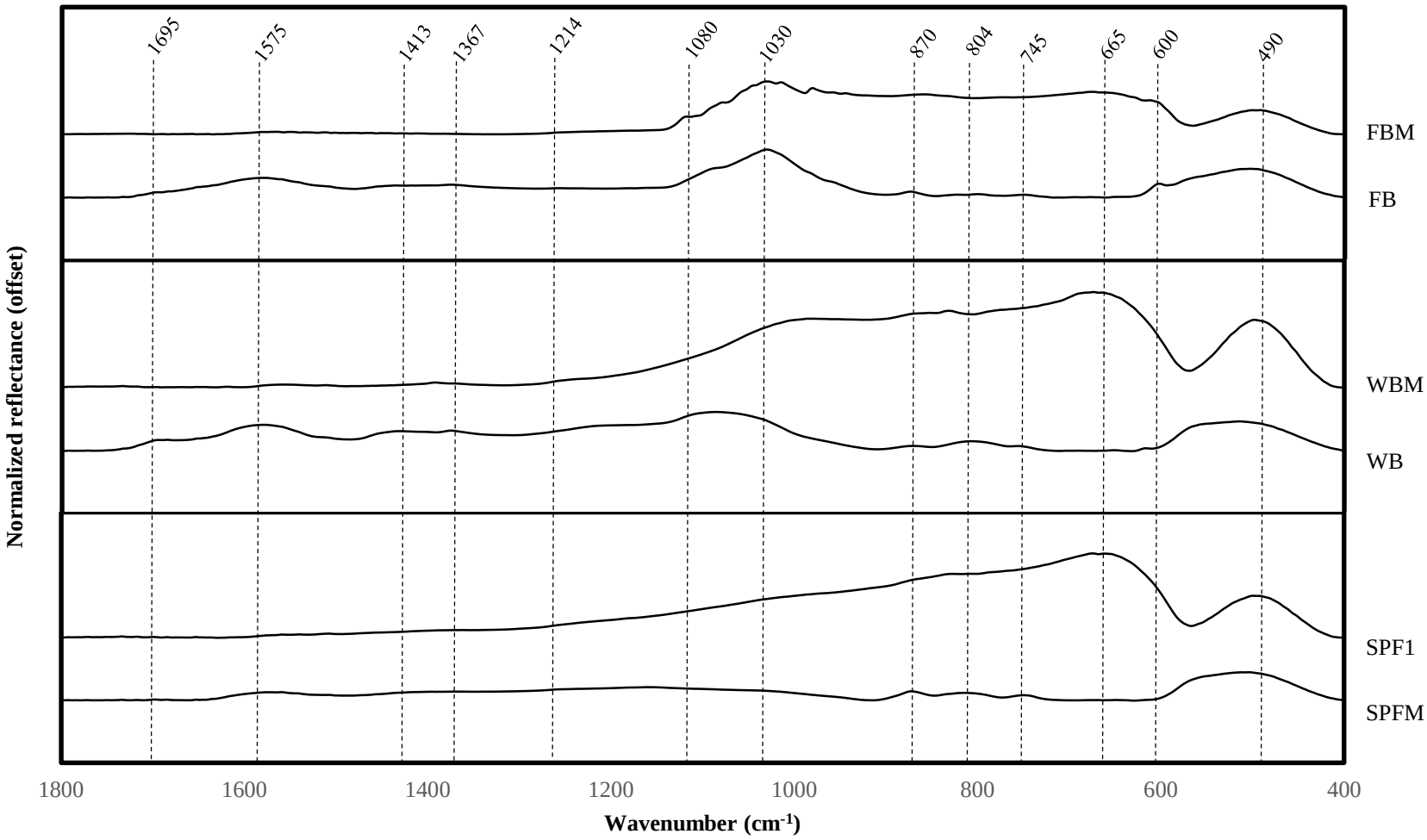

Fig. 4.1. FTIR-ATR spectra for biochar treatments of fish biochar (FB), wheat biochar (WB), and spruce/pine/fir biochar (SPF1) of Turtleback Biochar ®. Each of the biochars was altered using steam manipulation (FBM, WBM, and SPFM1). Refer to Table 4.1.3 for functional groups corresponding to the identified wavenumbers.

Table 4.3. Absorption frequencies of functional groups in the original and manipulated biochars.

Wavenumber (cm^{-1})	Functional groups	Components	Reference
1689	C=C in aromatic structure and C=O vibrations aromatic carbonyl/carboxyl C=O stretching	hemicellulose	Mukome et al., 2013; Hsu et al., 1999; Keiluweit et al., 2010.
1575	Skeletal C=C vibration, aromatic C=C ring stretching	lignin	Keiluweit et al., 2010; Sharma et al., 2003; Ozcimen and Ersoy-Mericboyu, 2010; Mukome et al., 2013.
1413	Skeletal C=C vibration, C=C, C-O, C-H, Aromatic C-C ring stretching	lignin	Mukome et al., 2013; Hsu et al., 1999; Ozcimen and Ersoy-Mericboyu, 2010; Sharma et al., 2003.
1367	Aliphatic CH_3 deformation and O-H bending	ND†	Ozcimen and Ersoy-Mericboyu, 2010; Keiluweit et al., 2010; Sharma et al., 2003.
1214	Aromatic CO – stretching	ND	Ozcimen and Ersoy-Mericboyu, 2010.
1080	C-O stretching	cellulose and hemicellulose	Mukome et al., 2013; Hsu et al., 1999; Ozcimen and Ersoy-Mericboyu, 2010.
1030	C-O stretching	cellulose and hemicellulose	Ozcimen and Ersoy-Mericboyu, 2010; Mukome et al., 2013.
870	1 adjacent H deformation	ND	Ozcimen and Ersoy-Mericboyu, 2010
804	2 adjacent H deformations	ND	Mukome et al., 2013.
788	2 adjacent H deformations	ND	Ozcimen and Ersoy-Mericboyu, 2010.
770	2 adjacent H deformations	ND	Ozcimen and Ersoy-Mericboyu, 2010.
745	C – H bending	ND	Keiluweit et al., 2009.
665	O – H bend	ND	Mukome et al., 2013; Sharma et al., 2003.
600	ND	ND	
490	ND	ND	
400-700	C-C stretching	ND	Mukome et al., 2013.

† ND indicates components of functional group not determined

4.2 *Rhizobium* survival on biochar

4.2.1 Study 1

The capacity to support *Rhizobium* varied between the nine biochar's. There were six biochars (SPF1, SPF2, FFB1, FFB2, FB, BMB) observed to support *Rhizobium leguminosarum* bv. *viciae* over the 84 day study (Figure 4.2). All six of these biochars exhibited a decline in rhizobia populations over time (Table 4.6). SPF1 consistently supported the highest rhizobia

population for the duration of the study and had the lowest rate of decline in *Rhizobium* population. SPF1 rate of *Rhizobium* population decline was significantly lower than that of biochars FB and BMB. Three biochars (WB, OHB, and FHB) were terminated from the study after 28 days there were no detectable *Rhizobium* in the samples.

4.2.2 Study 2

The capacity to support *Rhizobium* varied between the six biochars, particularly between the original and manipulated biochars (Fig. 3.2). The manipulated biochars (FBM, WBM, SPFM1) were sampled on inoculation day had no detectable *Rhizobium* indicating that the biochars were immediately unable to support *Rhizobium* (Fig. 3.2). Biochars FB and SPF1 supported *Rhizobium* over the 28 day and displayed results similar to those in survival study 1 (section 4.2.1).

4.3 Correlation between *Rhizobium* survival and biochar

The day 21 dataset showed a significant correlation between the C:N ratio and *Rhizobium* survival ($p < 0.05$) (Table 4.4). Using day 84 data, there was a significant relationship between both %C and the C:N ration and *Rhizobium* survival ($p < 0.05$) (Table 4.5). Percent C was weakly correlated with *Rhizobium* survival at day 21 ($p = 0.077$). Similarly, %N was weakly correlated with survival on day 84 ($p = 0.075$). Multiple regression analyses failed to identify combinations of physical/chemical properties that affected *Rhizobium* survival (data not shown).

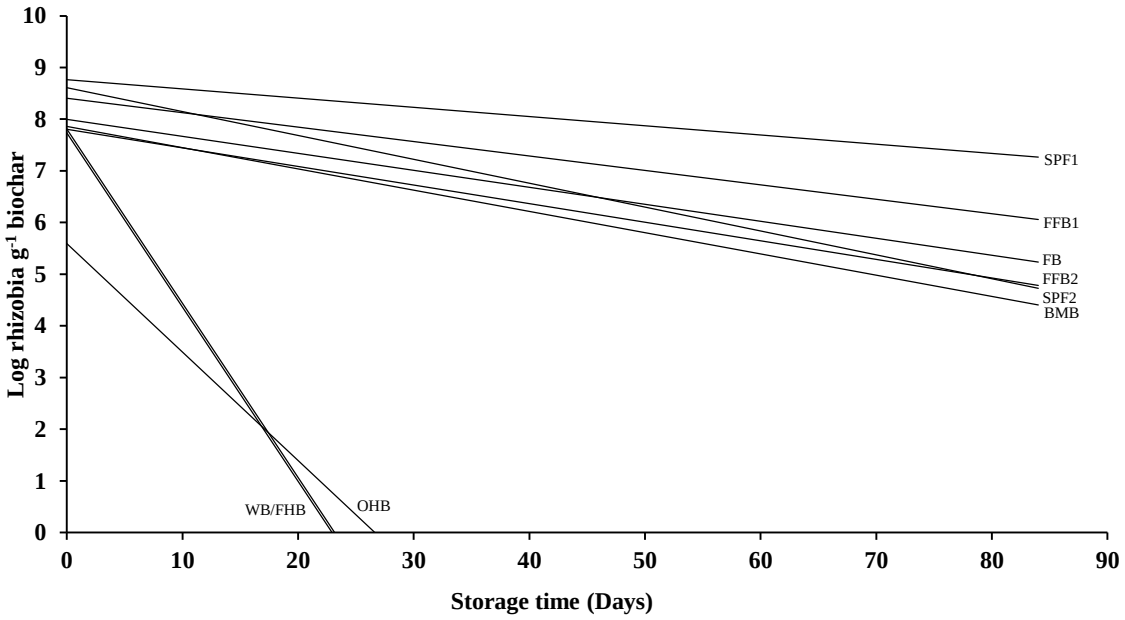

Fig. 4.2. Regression of rhizobia survival on 9 biochars over an 84 day period. Biochar treatments of bone meal biochar (BMB), fish biochar (FB), Flin Flon biochar 1 (FFB1), Flin Flon biochar (FFB2), oat hull biochar (OHB), flax hull biochar (FHB), wheat biochar (WB), spruce/pine/fir biochar (SPF1) of Out of Ashes BioEnergy Inc. and spruce/pine/fir biochar (SPF2) of DiaCarbon Energy Inc. Each point is the mean of 3 measurements (Appendix 4).

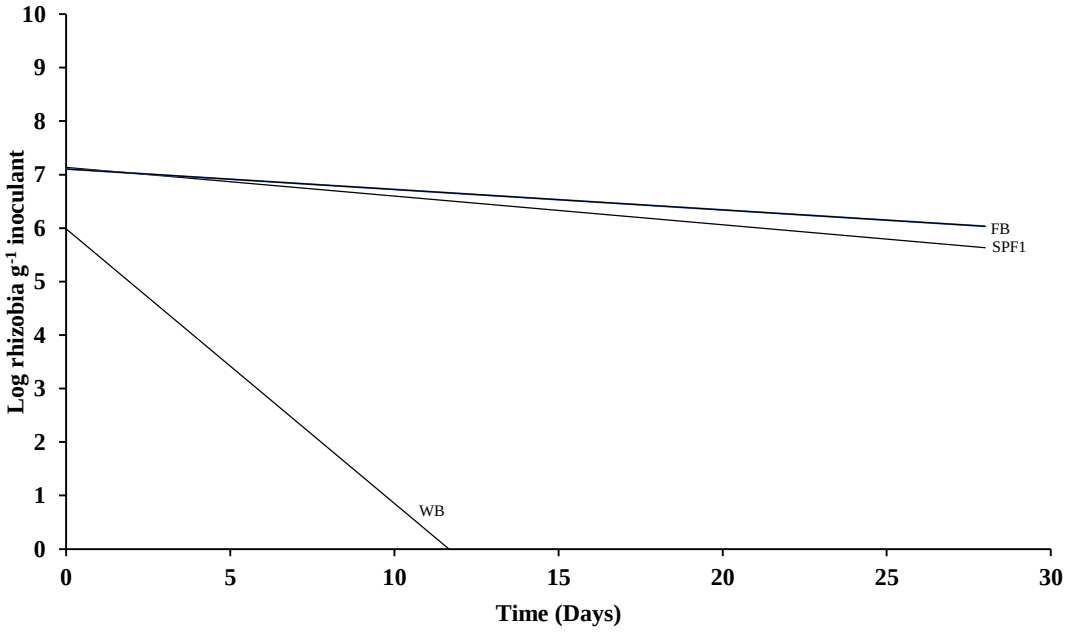

Fig. 4.3. Regression of rhizobia survival on 3 biochars (pre- and post-steam manipulation) over a 28 day period. Biochar treatments of fish biochar (FB), wheat biochar (WB), spruce/pine/fir biochar (SPF1) and the manipulated biochar for each of the previous three respective biochars (FBM, WBM, SPFM1). Each point is the mean of 3 measurements (Appendix 4).

Table 4.4. Pearson correlation coefficients for select biochar physical and chemical properties (using only original biochar data) and rhizobia survival on biochar using data from day 21 of the *Rhizobium* survival study 1 (Fig. 4.2).

Property	r	P value
pH	-0.3458	0.3620
EC	-0.3775	0.3165
%N	-0.2704	0.1725
%C	0.3453	0.0777
C:N ratio	0.4066*	0.0358
BET surface area	-0.2934	0.4436
Pore width	-0.0048	0.9902
Pore volume	-0.1482	0.7035
Water holding capacity	-0.0774	0.8432
Inherent moisture content	-0.0474	0.9036
Hydrophobicity	-0.0120	0.9524
Available water	0.4335	0.1064
Volatile matter	-0.0703	0.8574
Ash	-0.1721	0.6580

*Correlations significant at the 0.05 level
Biochar elements were examined for correlation with *Rhizobium* survival.
Biochar elemental composition (Appendix I) was examined for correlation with *Rhizobium* survival with no significant relationships (p=0.05) being found.

Table 4.5. Pearson correlation coefficients for select biochar physical and chemical properties (using only original biochar data) and rhizobia survival on biochar using data from day 84 of the *Rhizobium* survival study 1 (Fig. 4.3).

Property	r	P value
pH	-0.0345	0.3620
EC	-0.3775	0.3165
%N	-0.3482	0.0751
%C	0.4482*	0.0190
C:N ratio	0.4553*	0.0170
BET surface area	-0.1633	0.6746
Pore width	0.0685	0.8609
Pore volume	-0.1482	0.7035
Water holding capacity	-0.0773	0.8432
Inherent moisture content	-0.0474	0.9036
Hydrophobicity	-0.0880	0.6624
Available water	0.3460	0.2065
Volatile matter	-0.0702	0.8574
Ash	-0.1720	0.6580

*Correlations significant at the 0.05 level
Biochar elements were examined for correlation with *Rhizobium* survival.
Biochar elemental composition (Appendix I) was examined for correlation with *Rhizobium* survival with no significant relationships (*p*=0.05) being found.

Table 4.6. Linear regression and slope analysis of *Rhizobium* population on biochar (SPF2, FB, FFB2, FFB1, BMB, SPF2) over time using rhizobia population data from *Rhizobium* survival study 1.

Biochar comparison† (a vs. b)	Regression equation of biochar a	Regression equation of biochar b	Z value	P value
SPF2 vs. FB	y=7.822 - 0.0364x	y=7.9736 - 0.0323x	-0.44	0.3264
SPF2 vs. FFB2	y=7.822 - 0.0364x	y=8.6242 - 0.0465x	1.07	0.8531
SPF2 vs. BMB	y=7.822 - 0.0364x	y=7.9244 - 0.0437x	0.59	0.7257
SPF2 vs. SPF1	y=7.822 - 0.0364x	y=8.9424 - 0.0211x	-1.70	0.0446
SPF2 vs. FFB1	y=7.822 - 0.0364x	y=8.3934 - 0.0279x	-0.88	0.1841
FB vs. FFB2	y=7.9736 - 0.0323x	y=8.6242 - 0.0465x	2.52	0.9938
FB vs. BMB	y=7.9736 - 0.0323x	y=7.9244 - 0.0437x	1.18	0.8849
FB vs. SPF1	y=7.9736 - 0.0323x	y=8.9424 - 0.0211x	-2.30	0.0107*
FB vs. FFB1	y=7.9736 - 0.0323x	y=8.3934 - 0.0279x	-0.74	0.2266
FFB2 vs. BMB	y=8.6242 - 0.0465x	y=7.9244 - 0.0437x	-0.29	0.3821
FFB2 vs. SPF1	y=8.6242 - 0.0465x	y=8.9424 - 0.0211x	-4.98	ND‡
FFB2 vs. FFB1	y=8.6242 - 0.0465x	y=8.3934 - 0.0279x	-3.04	0.0014*
BMB vs. SPF1	y=7.9244 - 0.0437x	y=8.9424 - 0.0211x	-2.42	0.0082*
BMB vs. FFB1	y=7.9244 - 0.0437x	y=8.3934 - 0.0279x	-1.59	0.0548
SPF1 vs. FFB1	y=8.9424 - 0.0211x	y=8.3934 - 0.0279x	1.07	0.8531
SPF2 vs. FB	y=7.822 - 0.0364x	y=7.9736 - 0.0323x	-0.44	0.3264

*Correlations significant at the 0.05 level.

† The source for each biochar is listed in Table 3.1.

‡No data for P value corresponding to Z value.

4.5 Biochar phytotoxicity bioassay

FHB caused an increase in germination four to six times that of the water control, varying by extract dilution (Figure 4.4 (A)). The undiluted extract of biochars BMB, FB, FFB1, FFB2, OHB, WB and SPF1 resulted in lower PG than the control. At the 3x dilution, the six of these biochars (excluding BMB) exhibited a PG comparable to that of the control. BMB was the only treatment to negatively impact PG at the 3x dilution. The 10x dilution showed biochars FFB2, WB and SPF2 with a lower PG than that of the control.

At the undiluted rate, several of the biochars decreased PRL relative the control including FB, FFB1, FFB2, WB and SPF1 (Figure 4.4 (B)). Treatments BMB, FB, FFB1, FFB2 and OHB caused noticeable increases in PRL at both the 3x and 10x dilutions with FHB and SPF1 having slight increases relative to the control. FFB1 and FFB2 increased PRL at all three of the treatment dilutions ranging from six to nine times that of the control.

The GI is on overall index for the bioassay as it encompasses both PG and PRL into one measure. FFB1 and FHB both had an increase in GI at all extract dilutions, ranging from six to ten times that of the control (Figure 4.4 (C)). Treatments WB, SPF1 and SPF2 displayed similar results in that both the undiluted and 10x dilution caused decreases in the GI while the 3x dilution caused an increase. Treatments BMB, FB, and OHB displayed various responses in GI compared to the control with the 10x dilution causing GI to increase in all three treatments.

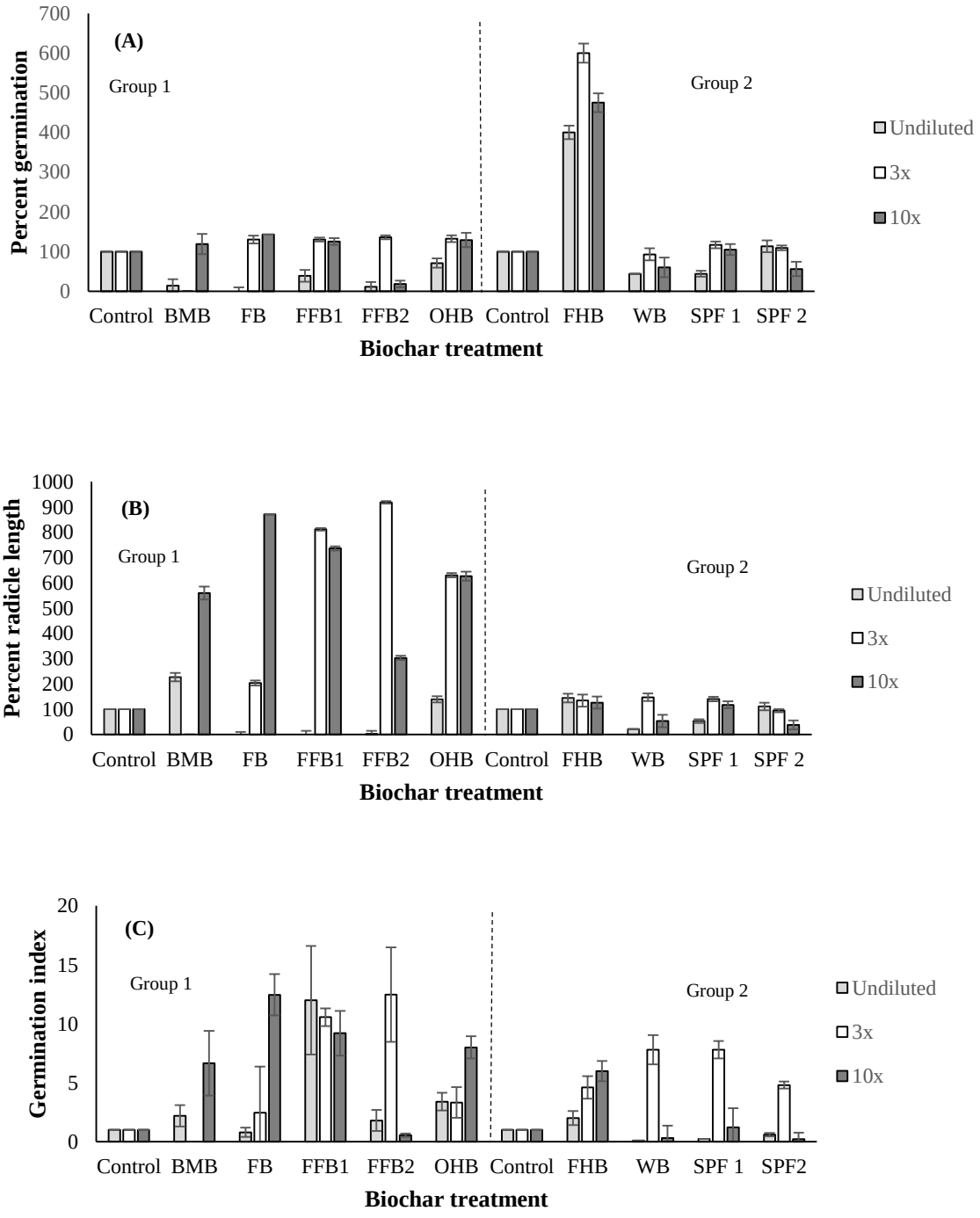

Fig. 4.4. Assays for the effect of water extracts from biochars on seed germination (A), radicle growth (B), and germination index (C) of garden cress seeds. Percent germination (PG) and percent radicle length (PRL) were calculated for each treatment relative to the water control. The germination index was an overall measure for phytotoxicity combining PG and PRL. Biochar treatments of bone meal biochar (BMB), fish biochar (FB), Flin Flon biochar 1 (FFB1), Flin Flon biochar (FFB2), oat hull biochar (OHB), flax hull biochar (FHB), wheat biochar (WB), spruce/pine/fir biochar (SPF1) of Out of Ashes BioEnergy Inc. and spruce/pine/fir biochar (SPF2) of DiaCarbon Energy Inc. Group 1 and Group 2 biochars were analyzed at different times with the calculations being based on the water control at that time. Error bars indicated standard error of the mean (n=5).

4.5 Plant biomass and nodule enumeration

4.5.1 Study 1

The ability of biochars BMB, FB, FFB1, FFB2, SPF1, SPF2 and a commercial inoculant to deliver rhizobia to pea seed in a pot study using field soil was assessed (Table 4.6). Despite screening the soil for native rhizobia, all of the uninoculated controls were nodulated suggesting that the treatments were also affected by native rhizobia populations. The biomass values for the uninoculated pea plants were comparable to the pea inoculated with the commercial peat inoculant and other treatments and therefore, do not indicate any differences in plant growth. The commercial peat inoculant control resulted in the lowest number of nodules although average shoot, root and seed weight were comparable to the biochar and additional control treatments.

4.5.2 Study 2

The ability of biochars FB, SPF1 and a commercial inoculant to deliver rhizobia to pea seed in potting mix was assessed (Table 4.7; Table 4.8). Pea in the uninoculated control and sterile biochar treatments (FB and SPF1) were nodulated suggesting *Rhizobium* presence in the potting mix or *Rhizobium* contamination via another source. The uninoculated pea control did statistically differ from all other treatments as it had the lowest seed weight per pot and N content per dried biomass although it was comparable to other pea treatments in nodule weight, root biomass weight and shoot biomass. There were no observable trends in statistical differences in pea biomass among the inoculated biochar, sterile biochar, and commercial peat inoculant treatments. FB and the commercial peat inoculant treatments caused a significantly higher number of nodules in pea when compared to SPF1 treatments although a similar trend was not observed in nodule weights for the respective treatments. Although not statistically different, pea treated with FB had a higher average seed weight, %Ndfa and average N content derive from the atmosphere per pot when compared to pea treated with SPF1. Due to an error during the study, the ^{15}N-urea (10 atom %) was not applied to the uninoculated control pea treatment resulting in a lack of the appropriate data necessary to calculate % Ndfa for that treatment.

The ANOVA results indicate a significant difference between the treatments applied to pea in the average N content of biomass derived from the atmosphere per pot although the Tukey

HSD test indicated no differences between any of the treatments (Table 4.8). This can be attributed to the ANOVA test being more sensitive to differences which can lead to the test suggesting there is a significant difference between treatments when there is not. Alternatively, the Tukey HSD is a more conservative test therefore, it did not indicate differences among treatments.

Table 4.7. Ability of biochars inoculated with *Rhizobium leguminosarum* bv. v*iciae* to promote nodulation of field pea (*Pisum sativum*) in field soil.

	Treatment†	Average number of nodules per treatment pot	Average nodule dry weight per treatment pot (g)	Average Shoot biomass per treatment pot (g)	Average root biomass per treatment pot (g)	Average seed weight per treatment pot (g)
Inoculated	Bone Meal Biochar; **BMB**	170bc	0.51bc	6.98a	1.12a	3.13b
	Fish Biochar; **FB**	192abc	0.68ab	7.05abc	1.29a	3.59ab
	Unknown Flin Flon 1; **FFB1**	238abc	0.56bc	6.74b	0.94a	3.47ab
	Unknown Flin Flon 2; **FFB2**	326ab	0.46c	6.35bc	0.97a	3.66ab
	Spruce/Pine/Fir 1; **SPF1**	362a	0.60abc	6.65b	1.44a	3.45ab
	Spruce/Pine/Fir 2; **SPF2**	231abc	0.68abc	7.16ab	1.19a	4.01ab
Sterile‡	Spruce/Pine/Fir 1; **SPF1**	298ab	0.67abc	6.51bc	1.16a	4.17ab
	Unknown Flin Flon 2; **FFB2**	331a	0.50bc	5.66c	0.85a	3.50b
Controls	Uninoculated Control§	216abc	0.78a	6.38bc	1.40a	3.57ab
	Nodulator XL ®¶	81c	0.06d	7.71a	0.99a	4.62a
P value		<0.0001	<0.0001	<0.0001	0.0438	0.0070

Means with the same letter within a column were not significantly different at P<0.05 (Tukey's HSD).

† The source for each biochar is listed in Table 3.1.

‡ Biochar was sterilized and was not inoculated with *Rhizobium leguminosarum* bv. *viciae.*

§ No inoculant or biochar added.

¶ Sourced from Becker Underwood, Saskatoon, SK.

Table 4.8. Ability of biochars inoculated with *Rhizobium leguminosarum* bv. v*iciae* to promote nodulation of field pea (*Pisum sativum*) in potting mix.

	Treatment†	Average number of nodules per treatment pot	Average nodule dry weight per treatment pot (g)	Average Shoot biomass per treatment pot (g)	Average root biomass per treatment pot (g)	Average seed weight per treatment pot (g)	Average N content per dried biomass (mg g^{-1})
Inoculated	Fish Biochar; **FB**	1135ab	0.27a	16.62ab	1.81a	9.30a	150ab
	Spruce/Pine/Fir 1; **SPF1**	523c	0.13b	17.79a	1.83a	8.44ab	130b
Sterile‡	Fish Biochar; **FB**	1008abc	0.27a	16.81ab	1.57a	8.83a	150ab
	Spruce/Pine/Fir 1; **SPF1**	533bc	0.22ab	15.12bc	1.63a	5.66bc	150ab
Controls	Uninoculated Control§	481c	0.18ab	12.98c	1.45a	3.32c	75c
	TagTeam ®¶	1401a	0.23ab	17.73ab	1.60a	8.65a	160a
P value		0.0001	0.0103	<0.0001	0.1694	<0.0001	<0.0001

Means with the same letter within a column were not significantly different at P<0.05 (Tukey's HSD).
† The source for each biochar is listed in Table 3.1.
‡ Biochar was sterilized and was not inoculated with *Rhizobium leguminosarum* bv. *viciae*.
§ No inoculant or biochar added.
¶ Sourced from Novozymes, Saskatoon, SK.

Table 4.9. Ability of biochars inoculated with *Rhizobium leguminosarum* bv. v*iciae* to promote nodulation of field pea (*Pisum sativum*) in potting mix: Nitrogen results.

	Treatment†	Average N content per dried biomass (mg g^{-1})	Nitrogen derived from atmosphere (%)	Average N content derived from atmosphere per pot (mg pot^{-1})
Inoculated	Fish Biochar; **FB**	150ab	77.37a	11.67a
	Spruce/Pine/Fir 1; **SPF1**	130b	71.67ab	9.48a
Sterile‡	Fish Biochar; **FB**	150ab	72.47ab	10.74a
	Spruce/Pine/Fir 1; **SPF1**	150ab	66.50b	9.61a
Controls	Uninoculated Control§	75c	ND	ND
	TagTeam ®¶	160a	76.22ab	12.57a
P value		<0.0001	0.0372	0.0425

Means with the same letter within a column were not significantly different at P<0.05 (Tukey's HSD).
† The source for each biochar is listed in Table 3.1.
‡ Biochar was sterilized and was not inoculated with *Rhizobium leguminosarum* bv. *viciae.*
§ No inoculant or biochar added.
¶ Sourced from Novozymes, Saskatoon, SK.

5.0 DISCUSSION

5.1 Biochar physical and chemical characteristics relating to *Rhizobium* survival

The overall variability in physical and chemical characteristics was supported by previous findings (Amonette and Joseph, 2009; Downie et al., 2009; Lehmann et al., 2011; Enders et al., 2012; Mukome et al., 2013; Hale et al., 2015). Although production data was not available for the biochars characterized in this study, it is well documented that biochar properties are linked to the feedstock source, and the temperature and duration at which biochar is manufactured (Amonette and Joseph, 2009; Downie et al., 2009; Mukome et al., 2013). It is important to note that biochar produced in the same batch can exhibit high variability within a tested parameter and thus, testing multiple replicates from a batch is suggested (Amonette and Joseph, 2009; Downie et al., 2009).

Temperature is known to be a precursor to biochar physical properties including surface area, pore volume and pore diameter (Day et al., 2005; Downie et al., 2009). Surface area, pore diameter and porosity did not correlate with *Rhizobium* survival although these properties play a role in providing habitat for microorganisms. The porosity of biochars can be complex to study due to the heterogeneous nature of pore sizes and structure. Scanning electron micrographs of the original and manipulated biochars captured the diversity of biochar surfaces (Appendix 2). The surface morphology of biochars is linked to surface area. Higher surface area biochars typically contain slit-shaped pores with vesicles, and lower surface area biochars result in more plate-like particles with slit-shaped pores (Mukome et al., 2013). The vesicles form via volatile matter being released from the biochar pores (Mukome et al., 2013). This pattern was not observed in the biochar SEMs obtained for this study probably due to the biochars being ground and sieved to 200 mesh (75 µm) prior to analysis. The grinding of the samples would have altered each biochars particle structure and the sieving would have limited particle size; although, varying particle sizes and shapes were observed within each biochar sample. The average pore width of the original biochars ranges from 9.58 to 95.32 nm which would have limited rhizobia bacteria, typically 500 to 3000 nm in size (Somasegaran and Hoben, 1994), from entering pore space. Subsequently, the rhizobia may have attached to the external surfaces of the biochar leaving them more vulnerable to drying out, temperature changes and external stresses.

There was no correlation between WHC, inherent moisture content, A_W, or the hydrophobicity index and *Rhizobium* survival.

The chemical properties of biochar are influenced by both feedstock and production conditions (Amonette and Joseph, 2009). The EC of the biochars did not correlate with *Rhizobium* survival. Salinity effects on *Rhizobium,* not only in the inoculant carrier but in the soil it is applied to, are important to understand when producing and applying inoculant. One study found that when a culture solution was raised from 1.2 mS cm^{-1} to 6.7 mS cm^{-1} or to 13.1 mS cm^{-1} the growth (mean doubling time) of all *Rhizobium* strains and species being tested decreased, with several strains failing to grow at the latter value (Singleton et al., 1982). Although not typical, certain strains of *Rhizobium* species have been found to survive in environments up to 43.0 mS cm^{-1} (Singleton et al., 1982). This suggests that many strains of *Rhizobium* can survive in salt concentrations which are adverse to most agricultural legumes (Singleton et al., 1982). The EC ranges of the biochars used in this study did not surpass the upper EC values suggested by Singleteon et al. (1982) to cause adverse effects to *Rhizobium*. While EC was not a key property relating to *Rhizobium* survival in this study, it is evident that it can have adverse effects on rhizobia at higher levels. The pH, volatile matter and ash content of the biochars also did not correlate with *Rhizobium* survival. The volatile matter content ranged from 15% to 55% and the ash content ranged from 6% to 64%, both of which are in range with values from previous literature (Lehmann et al., 2011; Enders et al., 2012). Consistent with other biochar properties, there was a large range of elemental compositions between the biochars (Appendix 1).

Biochar C:N ratio was positively correlated to *Rhizobium* survival using both day 21 (r= 0.4066) and day 84 (r=0.4553) of *Rhizobium* survival data while %C showing a positive correlation (r= 0.4482) with only day 84. In a similar study examining a plant growth promoting rhizobacteria (PGPR), the C:N ratio was found to negatively correlate with the initial inoculum density, and positively correlate with inoculum density following 4 weeks of incubation in soil (Hale et al., 2015). The C:N ratio is known to largely influence the composition of the soil microbial community as it determines the N supply available to microbes (Hogberg et al., 2006). Generally, a ratio of 20:1 is the critical limit above which immobilization of N occurs and N is unavailable for plant uptake (Chan and Xu, 2009). Based on this, typically C:N ratios higher than 20:1 create competition among microorganisms for the available N. It is important to note

that the chemical stability of a large fraction of a particular biochar effects how much of C and N are readily available for microorganisms to use as an energy source (Lehmann et al., 2011). Additionally, the C in biochars is often biologically very recalcitrant organic C therefore, it is expected that N immobilization is negligible or transient despite high C:N ratios (Chan and Xu, 2009). Biochar application to soil has shown to increase soil microbial biomass (SMB) carbon and SMB C:N ratio with application resulting in reduced temporal variability in the soil environment subsequently reducing temporal fluctuations in C and N dynamics (Zhang et al., 2014). Conversely, biochar additions to soil have been found to significantly decrease soil microbial community activity through decreased soil organic matter decomposition and N mineralisation (Dempster et al., 2012). Similarly, the addition of biochar to composting systems resulted in a decrease in microbial biomass (Jindo et al., 2011).

A correlation with biochar %C and *Rhizobium* survival occurred when using day 84 data. It is possible that at this time the *Rhizobium* became reliant on the biochar for C as an energy source. *Rhizobium* were applied to the biochar in a nutrient solution that contained C and, after being stored for 84 days, the rhizobia may have exhausted this C as an energy source resulting in the biochar becoming the primary C source. Carbon has been shown to both increase and decrease microbial biomass depending on the existing C availability, the magnitude of C change, and the microorganism group being examined (Lehmann et al., 2011).

There was no significant correlation between biochar %N and *Rhizobium* survival. This differs from Hale et al. (2015) findings, as they observed a positive correlation with %N and initial PGPR cell densities. The author's suggested that inoculant preparation plays a role as the inoculum in their study was prepared in a non-buffered, sterile saline solution with no N supplement (Hale et al., 2015). For *Rhizobium* survival studies 1 and 2 the inoculum was prepared in a Yeast Mannitol broth where yeast acts as an N source. This would have decreased the dependence on biochar to supply N to the *Rhizobium* upon their application. These results indicate that the carbon and nitrogen dynamics, particularly the C:N ratio, of biochar in various biological environments play an important role in microbial community composition and activity.

Biochars OHB, FHB and WB failed to support *Rhizobium* after 21 days in survival study 1. Furthermore, WB failed to support *Rhizobium* after seven days survival study 2. These biochars exhibited the lowest BET surface areas at 0.11, 2.99 and 2.92 $m^2 g^{-1}$ of biochar,

respectively, and the lowest pore volumes at 0.003, 0.004 and 0.005 cm^3 g^{-1} of biochar, respectively. Although it should be noted that SPF1, the highest performing biochar in survival study 1, had a relatively low pore volume of 0.005 cm^3 g^{-1} of biochar. Biochars OHB and FHB were the only two original biochars to have MED index of 6. Both OHB and FHB failed to support *Rhizobium* after 21 days suggesting that, qualitatively, the extreme hydrophobicity of the biochars may have negatively impacted the bacteria's survival. However, SPF1 had the strongest performance in *Rhizobium* survival study 1 but had the same MED index of 3 as OHB, which did not support *Rhizobium*. Therefore, this suggests that hydrophobicity is not a key property in *Rhizobium* survival but extreme hydrophobicity may impact microorganism survival. Hale et al. (2015) had similar results proposing that hydrophobicity does not appear to be a crucial property when examining biochar as an inoculant carrier although it is important to ensure sufficient time for a liquid inoculum to infiltrate biochar material.

A pivotal challenge in formulating new inoculant carriers is recognizing the live nature of the active ingredient and formulating carriers that can reach the high standards for efficacy and long storage lives (Xavier et al., 2004). Commercial peat inoculant producers provide a guaranteed minimum number of rhizobia cells per gram of their product as long as storage and application procedures are followed properly with a typical shelf life lasting 9-12 months. When using their peat-based inoculants, BASF© (2015) provides a guaranteed minimum of 1.0×10^9 rhizobia per gram for Nodulator© XL while Novozymes© (2015) provides a guaranteed minimum of 7.4×10^8 *Rhizobium leguminosarum* viable cells per gram for TagTeam©. This minimum guaranteed number provides distributors and producers with confidence that the product they are applying will be effective. Following day 84 of *Rhizobium* survival study 1, the enumeration of rhizobia on the six biochars ranged from 6.83×10^4 to 1.77×10^7. This range highlights the varying abilities that biochars have in supporting rhizobia bacteria and, at present, the difficulty in predicting *Rhizobium* survival on biochars.

The characterization of these biochars highlighted the variability in physical and chemical properties. When producing an inoculant carrier consistency is an absolute requirement in a carrier substrate (Stephens and Rask, 2000). There have been several studies examining production conditions and feedstock as they relate to the resulting biochars properties (Sharma et al., 2004; Azargohar and Dalai, 2008; Ozcimen and Ersoy-Mericboyu, 2009; Spokas

et al., 2011; Mukome et al., 2013). If these relationships can be established and understood then the ability to predict biochars properties could be utilized to create a more uniform product. This would be beneficial in furthering its production as an inoculant carrier.

5.2 Biochar manipulation: Property changes and *Rhizobium* survival

The pH of biochar is an important factor because the bacteria live in close spatial proximity to the biochar surface (Lehmann et al., 2011). Following biochar manipulation, the pH of biochars FB, SPF1 and WB increased by 15%, 41% and 26%, respectively. The inability of the manipulated biochars FBM, SPFM1 and WBM to support *Rhizobium* was probably largely influenced by this increase in alkalinity. The optimal pH conditions for rhizobia growth are between 6.0 – 7.0 (Somasegaran and Hoben, 1994) with some species being observed to grow at pH 4.5 and pH 11 (Singh et al., 2015). Maintaining the pH of a carrier at the optimum range for the inoculant organism not only supports the target organism but helps to discourage contaminant growth in the product (Paau et al., 1998). In order to adapt to a pH change, bacteria must first sense this. In alkaline conditions, rhizobia implement certain strategies to maintain intracellular pH including decreased membrane permeability, internal buffering, amelioration of external pH, proton extrusion/uptake, and prevention of metal ion toxicity (Dilworth and Glenn, 1999). Following this, species dependent genes are triggered to facilitate cell function adaptations in order for the bacteria to survive and grow (Hirsch, 2010). Few genes in *Rhizobium* spp. have been found that are induced under alkaline conditions partly due to few studies examining alkaline tolerance (Hirsch, 2010). Under both acidic and alkaline conditions, enzyme activity is inhibited and most metabolic processes are impaired (Thies and Rillig, 2009). Biochars FBM, SPFM1 and WBM had pH's of 11.14, 12.30 and 11.17 resulting in the *Rhizobium leguminosarum* bv. *viciae* species being applied to extremely alkaline conditions. On inoculation day, the biochars were sampled with all three of the manipulated biochars having no *Rhizobium* colonies being observed. Even though the pH was not significantly correlated to *Rhizobium* survival there may be a threshold pH above which rhizobia will not grow or survive. The results from the *Rhizobium* survival study indicate this upper threshold to be at a pH greater than 10 as SPF2 was able to support *Rhizobium* at a pH of 10.01 while none of the manipulated biochars were able to support *Rhizobium* at pH's greater than 11. It is probable that the alkaline conditions of the manipulated biochar's caused adverse effects to the bacteria resulting in their lack of survival.

The relationship between surface area and pyrolysis temperature has been fairly well studied with an overall consensus that increased temperatures result in increased surface areas (Day et al., 2005; Azargohar and Dalai, 2008). Once the biochars are activated they, by definition, become activated carbon (Anderson et al., 2013) which is characterized by extensive surface area, a high degree of surface reactivity and favorable pore size distribution (Alaya et al., 2000). This was observed when biochars FB, SPF1 and WB were manipulated via steam activation with each biochar's surface area increasing 4454%, 13544% and 20294%, respectively. Collectively, this increase in surface area coincided with an increase in pore volume ($cm^3\ g^{-1}$) and a decrease in average pore width (nm). This parallels previous findings where increased surface area resulted in increased pore volume particularly that of micro-pores which by nature have smaller pore width (Alaya et al., 2000; Downie et al., 2009). Following biochar activation, biochars can develop complex internal surface area which is largely dependent on the activation conditions used to manipulate the biochars (Azargohar and Dalai, 2008). Biochars made at high temperatures have been found to lack structure due to the melting of cell structure and by plastic transformations (Downie et al., 2009) which would have particularly affected the biochars post-manipulation. Surface area, porosity and pore size all relate to bacterial adhesion to the biochar. Bacteria attach to the biochar surface via several mechanisms including hydrophobic attraction or electrostatic forces (Lehmann et al., 2011). Adhesion may also be dependent on pore size and shape as the organism needs to successfully fit and attach to the pore space (Lehmann et al., 2011). The average pore width of the manipulated biochars ranges from 4.91 to 7.89 nm which would have limited rhizobia bacteria, typically 500 to 3000 nm in size (Somasegaran and Hoben, 1994), from entering pore spaces leaving the bacteria unprotected.

The biochars pre-and post-manipulation were analyzed for functional group composition with changes observed following manipulation particularly in the range 600 to 1000 cm^{-1}. As previously stated, steam manipulation can increase surface area by removing constituents in the effluent gas (Azargohar and Dalai, 2008) which would subsequently change the functional group composition of each biochar. In addition, steam activation can promote the release of volatile matter via partial de-volatilization and enhance crystalline C formation (Alaya et al., 2000). The shift in the baseline from 600 to 1000 cm^{-1} following manipulation was probably due to an increased carbonised component (Sharma et al., 2003). Following manipulation, all three of the

biochars lost peaks associated with lignin probably due to the de-volatilization of lignin during pyrolysis (Sharma et al., 2003). Lignin materials previously processed with an acid-wash, removing inorganic components such as sodium and potassium, had higher rates of lignin de-volatilization and subsequent decrease in char yield (Sharma et al., 2003). As this was the second processing for the biochars, the first pyrolysation could have acted as a pre-process by removing inorganic components and allowing for the lignin to be further degraded.

5.3 Biochar phytotoxicity bioassay

The phytotoxicity of the biochars on garden cress seed varied by biochar and dilution rate. No chemical analyses were done on the biochar extracts but mobile compounds in the extract could result in positive or negative effects on microorganisms (Buss and Masek, 2014). Bastos et al. (2014) found the water extract from soil amended with biochar contained cadmium, cobalt, chromium, copper, manganese, zinc, nickel, lead, arsenic and mercury metals as well as 16 priority PAHs listed by the U.S. Environmental Protection Agency. Potential toxic elements and compounds are a function of biochar feedstock and processing conditions thus, great variability can exist between biochars. Additionally, PAHs are rather immobile in biochars making them unlikely to be the cause of toxicity in germination tests (Buss and Masek, 2014).

The undiluted extract was generally the only extract dilution to cause adverse effects on the garden cress seed. This could be due to the presence of certain nutrients and/or concentration of the nutrient within the biochar extract. Similarly, Buss and Masek (2014) found that water extracts at the highest concentration had very strong negative effects on germination and found that seeds in direct contact with biochar exhibited greater toxicity than seeds only in contact with leachate. The 3x and 10x dilutions typically caused stimulation in percent germination and radicle length although this varied by treatment. Biochar has previously shown positive effects on plant growth particularly root growth (Lehmann and Joseph, 2009; Buss and Masek, 2014). Biochar can effect plant root growth as a direct nutrient source and/or through impacts on nutrient availability (Prendergast-Miller et al., 2013).

5.4 Evaluating the ability of biochar to deliver nodulating *Rhizobium* to pea crop

There was no significant difference in pea growth between the inoculated and uninoculated treatments but there were observable differences in pea growth between the two biochar treatments. When applied to pea, both the inoculated and sterile FB treatments caused

higher results in average seed weight, %Ndfa and average %Ndfa per pot when compared to SPF1 treatments. Biochar can affect crop biomass and yield through direct and indirect nutrient effects. Biochar can directly supply nutrients to a crop, although it is more common for plants to benefit from indirect nutrient responses such as storing nutrients from fertilizers or improving fertilizer-use efficiency (Chan and Xu, 2009). The elemental composition of the biochars revealed that FB had an N content 18 times that of SPF1. Although it should be noted that the total elemental concentration of many nutrients may not reflect the actual availability of these nutrients to plants especially those organically bound (Chan and Xu, 2009). FB and SPF1 had C:N ratios of 12:1 and 345:1, respectively. Generally, a ratio of 20:1 is the critical limit above which immobilization by N occurs and N is unavailable for plant uptake (Chan and Xu, 2009). It is possible that the higher, more available N in FB enhanced pea growth when compared to SPF1. Additionally, the uninoculated control caused significantly lower average N content per dried pea biomass when compared to that of the commercial peat inoculant and the biochar treatments. The biochar treatments may have directly enhanced pea N content by providing nutrients for growth or by enhancing the rhizobia's ability to fix nitrogen through increased nutrient availability.

The *Rhizobium* survival studies indicated that each biochar had a different capacity to support the bacteria. To account for this, it was decided to apply *Rhizobium* at a rate of 1.0 x 10^6 per pea seed when inoculating the biochar. At this rate, varying amounts of biochar had to be applied based on each biochars capacity to support *Rhizobium* populations (Appendix 4). The amount of inoculated biochar applied ranged from 0.005 to 3.4 g per seed. This is another example of the variability that is encountered when using various biochars as inoculant carriers. From a production perspective, it would advantageous to produce biochars with predictable properties that have predictable relationships with rhizobia survival and growth.

6.0 Conclusion and Future Research Direction

6.1 Conclusion

The nine biochars examined in this study displayed a wide range of values corresponding to physical and chemical properties. This range can be attributed to the use of different feedstocks and varying production conditions. Six (BMB, FB, FFB1, FFB2, SPF1 and SPF2) of the nine biochars inoculated with *Rhizobium* were able to support the bacteria over an 84 day period. The C:N ratio and %C of each biochar was found to correlate with *Rhizobium* survival which agrees with previous studies examining biochar as an inoculant carrier (Hales et al., 2015). These findings suggest that biochar does have the ability to effectively support rhizobia with biochar properties largely affecting their ability to do so.

Following this initial survival study, three biochars were chosen to be manipulated via steam activation. The manipulated biochars displayed unanimous increases in surface area, pore volume and pH while pore diameter decreased. The pre- and post-manipulated biochars were used to evaluate the ability of each biochar to support *Rhizobium* over a period of 28 days. All of the biochars were enumerated on inoculation day. The manipulated biochars were immediately unable to support *Rhizobium*. The increase in alkalinity of the manipulated biochars was probably toxic to the *Rhizobium* resulting in the bacteria being unable to survive upon application. The two original biochars that support *Rhizobium* over the 28 day period were subsequently used in a pot study examining each biochars ability to deliver *Rhizobium* to pea seed in pot study using soilless potting mix. The uninoculated control was successfully nodulated in this study indicating the potting mix carried *Rhizobium* or *Rhizobium* contamination via another source. Each respective biochar had a sterile treatment as well as an inoculated treatment in this study. Although there were no significant differences between the inoculated biochar and sterile biochar treatments there were observable differences between the biochars. Biochar FB, in both inoculated and sterile treatment, were comparatively higher in in average seed weight, %Ndfa and average N content derived from the atmosphere per pot. This suggests the biochar itself had an impact on pea plant growth.

Each biochar's potential phytotoxicity was examined using a garden cress phytotoxicity bioassay. Biochar's effect on garden cress varied by biochar and dilution rate. Typically, decreases in PG, PRL and GI were greatest in the undiluted extracts. There was observed

stimulation in PG and PRL at the diluted extracts of each biochar although this varied by treatment and dilution rate. It is hypothesized that increased nutrient availability caused the stimulation in germination and radicle growth. Similarly, excess nutrients leading to toxicity and/or the presence of toxic compounds caused decreases in the PG, PRL and GI.

The results from these studies indicate that biochar has the potential to be an inoculant carrier for *Rhizobium spp.* but further research is needed to identify key biochar properties relating to rhizobia survival. Additionally, it would be necessary to produce biochars with desirable properties which can be challenging when considering the variability not only among feedstocks but within a specific feedstock sample. Production conditions also vary between sources, not only in temperature and pyrolysis duration, but in production equipment as well. This relationship between biochar feedstock and production conditions, and the subsequent biochar properties needs to be thoroughly understood for biochar to move forward as a commercial inoculant.

6.2 Future research

Current inoculant carriers, including peat and clay, are slowly renewable resources that are subject to availability and have associated environmental impacts with acquiring them. Biochar has a suite of physical and chemical properties commonly characterized and it would be advantageous to understand how each of the properties affect not only *Rhizobium* but the target crop and the soil environment it is being applied to. If the relationship between feedstock and production conditions, and the resulting biochar properties is more intricately understood then a more predictable and uniform biochar can be produced. It would be prudent moving forward to set-up studies that aim to bridge the gap in research examining biochar as an inoculant carrier.

Biochars effects on microbial community, microbial function, root function, enzyme interaction, and research examining biochar as an inoculant have been identified as priority research areas relating to biochar (Lehmann et al., 2011). Biochar, in various forms, has previously been studied as an inoculant carrier (Beck, 1991; Ogawa and Okimori, 2010; Hale et al., 2015) but the studies lack biochar diversity or comprehensive knowledge on biochar properties and production conditions; which is indicative of biochar research in general (Lehmann et al., 2011). It would benefit future research to adhere to standard analysis procedures so research results can better be collectively compared. The IBI (2013) has defined

basic utility properties and toxicant reporting guidelines that could be useful in setting up a standard framework for analyses.

Additionally, it would be wise to consider formulating biochar into a more user friendly form. Previous literature has suggested that the main issues in commercializing new microbial inoculants is formulating a viable, cost-effective, and user-friendly final product (Xavier et al., 2004). Powdered biochar is not easily handled and can be quite messy. An adhesive that works well with the char material would have to be examined. An alternative would be creating biochar pellets ultimately acting as a granular inoculant. This would ease application and make the product more user friendly.

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APPENDICES

APPENDIX I

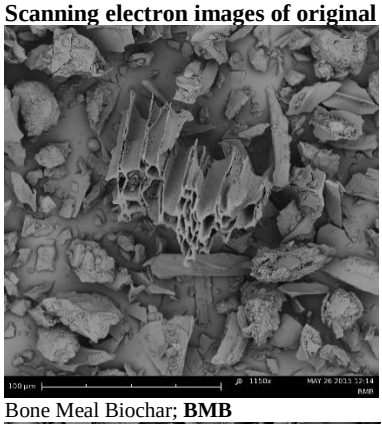
Table A. Elemental composition of biochars (mg kg^{-1}) (n=1)[†].

Element	Biochar											
	BMB	**FB**	**FFB1**	**FFB2**	**OHB**	**FHB**	**WB**	**SPF1**	**SPF 2**	**FBM**	**WBM**	**SPFM1**
Li	10.5	3.1	9.9	6.1	1.7	2.7	0.8	0.7	5.1	6.0	1.6	2.7
Na	6184	9140	6315	7622	612	1098	211	1148	1784	18938	480	3201
Mg	9826	6227	9200	6981	4421	6069	1839	1630	1433	9122	3447	2437
Al	21013	4396	18489	11714	263	1499	803	2428	2728	8949	1503	4081
P	24101	32823	34733	40791	5830	2387	1276	489	558	55636	2393	714
K	14723	9488	15032	26629	27525	13727	30006	4263	2666	14327	55791	5555
Ca	69823	55363	86616	81609	5118	16195	3414	9225	5225	93634	6575	15149
Sc	3.4	0.8	3.2	2.5	ud	0.3	ud	0.5	0.7	2.5	0.4	1.1
Ti	1253	229	1202	790	19	87	56	136	169	406	105	273
V	36.1	7.3	31.0	17.3	1.6	3.7	2.0	4.1	5.3	11.3	3.2	7.9
Cr	188.1	79.2	115.3	61.4	108.4	38.4	35.6	34.5	58.8	83.5	78.7	32.3
Mn	440	203	426	271	255	152	87	417	372	307	162	573
Fe	28987	8043	28076	11794	2897	1989	1757	2322	4364	11997	3456	3365
Co	6.0	1.6	5.2	2.5	0.9	0.9	0.5	0.8	1.1	3.6	2.4	2.6
Ni	49.9	25.7	36.9	28.4	47.9	15.9	14.0	13.8	21.4	55.1	57.0	49.4
Cu	204	30	131	19	64	11	11	16	216	52	132	16
Zn	178	194	162	118	78	32	15	63	193	102	40	5
Ga	3.8	0.9	3.9	1.9	ud	0.3	ud	0.5	0.6	1.9	0.3	0.9
Ge	0.6	ud	0.6	ud	ud	ud	ud	ud	ud	ud	ud	ud
As	7.6	ud	6.7	3.3	ud	ud	ud	ud	ud	1.8	ud	ud
Se	ud	ud	ud	ud	ud	ud	ud	ud	ud	ud	ud	ud
Rb	27	10	25	21	20	6	10	4	3	15	19	6
Sr	188	179	167	99	22	84	22	65	35	309	41	109
Y	6.7	1.3	6.3	4.5	0.1	0.5	0.3	0.5	0.7	2.1	0.5	1.0
Zr	41.5	8.8	44.3	30.4	3.3	4.4	2.0	2.9	6.3	11.5	6.7	12.2
Nb	5.1	0.9	5.2	2.9	0.2	0.4	0.4	0.2	0.8	1.7	0.6	0.5
Mo	7.9	4.8	5.8	3.4	9.9	1.4	1.6	2.4	3.5	76.8	115.7	93.1
Ag	ud	ud	ud	ud	ud	ud	ud	ud	ud	0.2	0.1	0.1
Cd	0.1	0.4	0.1	0.2	ud	1.2	0.2	ud	0.4	ud	ud	0.1
Sn	34.3	2.0	26.3	2.3	6.0	0.6	0.4	1.1	28.4	7.1	4.0	1.6
Sb	1.4	0.3	1.2	0.6	0.1	0.1	ud	0.1	9.7	0.9	0.1	0.2
Cs	1.2	0.5	1.0	0.6	0.1	0.2	0.2	0.2	0.3	0.4	0.4	0.4
Ba	322	73	316	193	34	66	134	64	52	134	253	104
La	11.1	2.6	11.6	7.0	0.1	1.1	0.6	0.5	0.6	3.6	0.9	3.1
Ce	23.5	5.1	23.8	14.9	0.2	2.3	1.3	0.9	1.2	7.2	1.7	1.3
Pr	2.8	0.6	2.9	1.8	0.0	0.3	0.1	0.1	0.2	0.9	0.2	0.2
Nd	10.6	2.1	10.6	6.8	0.1	1.1	0.6	0.6	0.7	3.2	0.8	0.7
Sm	2.1	0.3	1.9	1.3	ud	0.1	0.1	0.1	0.1	0.6	0.1	0.1
Eu	0.4	0.1	0.3	0.2	ud	ud	ud	0.0	ud	0.1	ud	ud
Gd	1.7	0.3	1.7	1.2	ud	0.2	0.0	0.1	0.1	0.4	0.1	0.2
Tb	0.2	0.0	0.2	0.2	ud	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Dy	1.1	0.2	1.0	0.8	0.0	0.1	0.0	0.1	0.1	0.3	0.1	0.2
Ho	0.2	0.0	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Er	0.6	0.1	0.5	0.4	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.1
Tm	0.1	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Yb	0.6	0.1	0.5	0.4	0.0	0.0	0.0	0.0	0.1	0.2	0.1	0.1
Lu	0.1	ud	0.1	0.0	ud	ud	ud	ud	ud	0.0	ud	ud
Hf	0.2	0.1	0.3	0.1	ud	ud	ud	ud	ud	0.1	0.1	0.0
Ta	0.2	0.0	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
W	3.2	1.4	3.2	0.8	0.4	ud	ud	ud	0.3	19.0	25.6	18.4
Tl	0.1	ud	0.1	0.0	ud	ud	ud	ud	ud	ud	ud	ud
Pb	13.4	2.2	7.8	4.0	1.5	0.4	0.4	1.5	17.8	0.8	0.3	0.6
Th	1.8	0.4	2.8	1.4	ud	0.3	0.1	0.1	0.1	0.9	0.2	0.2
U	0.8	0.2	0.8	0.4	0.0	0.2	0.1	0.1	0.1	0.3	0.1	0.1

† All measurements were made on ICP-MS

APPENDIX 2

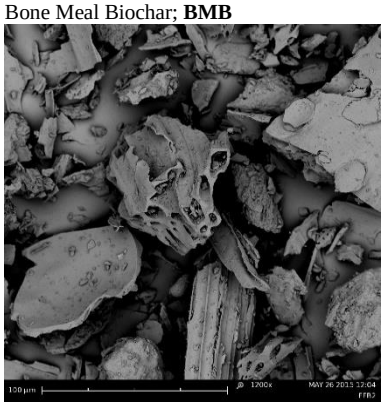
Scanning electron images of original and manipulated biochars at an approx. 100 µm scale.



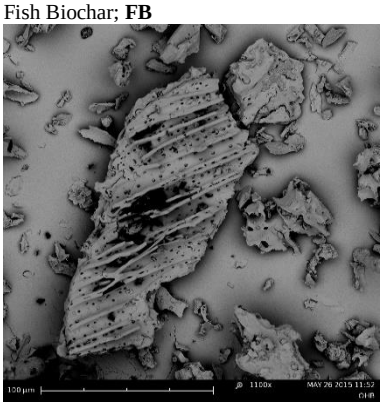
Bone Meal Biochar; **BMB**

Fish Biochar; **FB**

Flin Flon biochar 1; **FFB1**



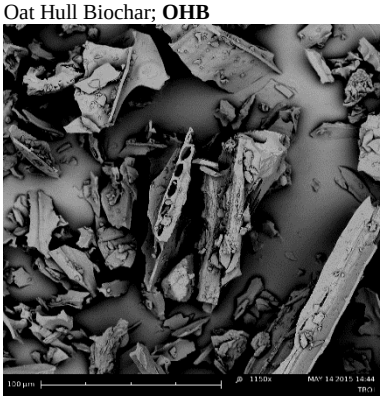
Flin Flon biochar 2; **FFB2**



Oat Hull Biochar; **OHB**

Flax Biochar; **FHB**

Wheat Biochar; **WB**



Spruce/Pine/Fir 1; **SPF1**

Spruce/Pine/Fir 2; **SPF2**

Fish Biochar Manipulated; **FBM**

Wheat Biochar Manipulated; **WBM**

Spruce/Pine/Fir 1 Manipulated; **SPFM1**

APPENDIX 3

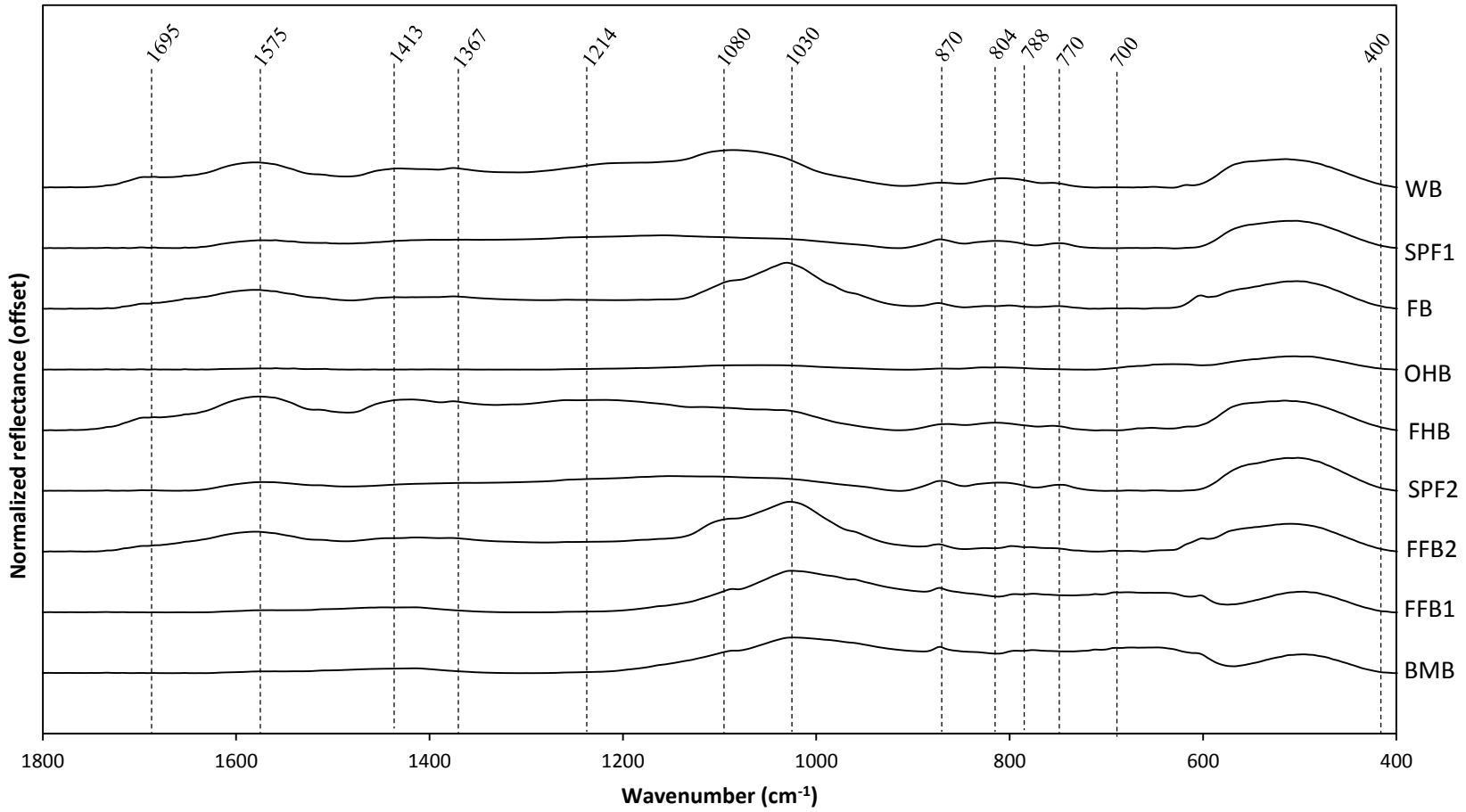


Fig. A. FTIR-ATR spectra for biochar treatments of bone meal biochar (BMB), fish biochar (FB), Flin Flon biochar 1 (FFB1), Flin Flon biochar (FFB2), oat hull biochar (OHB), flax hull biochar (FHB), wheat biochar (WB), spruce/pine/fir biochar (SPF1) of Out of Ashes BioEnergy Inc. and spruce/pine/fir biochar (SPF2) of DiaCarbon Energy Inc. Refer to Table 4.1.3 for functional groups corresponding to the identified wavenumbers.

APPENDIX 4

Table B. Survival study 1: Survival of *R. Leguminosarum* bv. *viciae* in biochar over an 84 day period.

	Log_{10} CFU per gram of biochar after day:												
Biochar†	0	7	14	21	28	35	42	49	56	63	70	77	84
SPF2	8.36(7.81)‡	8.17(7.62)	6.79(6.71)	7.01(6.62)	7.31(7.55)	4.52(4.76)	7.01(6.17)	5.37(5.24)	6.05(6.17)	5.65(5.58)	4.80(4.74)	5.51(5.47)	5.22(5.35)
FB	8.31(8.01)	8.24(7.60)	7.22(7.17)	7.32(6,83)	6.87(6.44)	6.25(6.19)	7.00(6.94)	5.97(5.49)	6.23(6.31)	5.80(5.49)	5.46(4.96)	5.91(5.62)	5.45(5.08)
FFB2	8.56(8.20)	8.28(8.01)	8.30(8.25)	7.72(7.62)	6.99(7.11)	6.16(5.89)	7.33(6.88)	6.75(5.99)	5.79(5.73)	5.87(5.63)	5.18(5.16)	4.74(5.65)	5.00(4.76)
BMB	8.09(7.97)	8.13(7.88)	6.17(6.11)	6.35(6.21)	6.42(6.31)	7.59(7.06)	7.16(5.46)	5.75(5.75)	5.04(5.08)	5.29(5.43)	4.20(4.15)	4.69(7.34)	4.83(4.83)
SPF1	8.31(8.12)	8.95(8.40)	8.47(8.32)	8.56(8.53)	8.59(8.30)	7.78(7.55)	8.44(7.12)	7.54(7.10)	7.81(7.15)	7.67(7.79))	7.33(7.30)	7.49(7.32)	7.25(6.68)
FFB1	8.14(7.91)	8.46(8.19)	7.73(7.62)	7.97(7.80)	8.10(7.86)	7.45(7.55)	7.92(6.33)	6.43(6.58)	6.33(5.89)	6.28(6.23)	6.35(6.14)	6.48(6.28)	6.39(6.35)
FHB	8.14(7.79)	7.32(7.28)	0.00(0.00)	0.00(0.00)	0.00(0.00)	ND$^{\delta}$	ND	ND	ND	ND	ND	ND	ND
WB	8.53(7.84)	6.54(6.75)	0.00(0.00)	0.00(0.00)	0.00(0.00)	ND	ND	ND	ND	ND	ND	ND	ND
OHB	7.35(6.95)	0.00(0.00)	5.92(6.16)	0.00(0.00)	0.00(0.00)	ND	ND	ND	ND	ND	ND	ND	ND

† Biochar sources can be found in Table 3.1.
‡ Number in brackets is the standard deviation of the mean of 3 replicates.
δ ND indicates no data as sampling was terminated for the treatment.

Table C. Survival study 2: Survival of *R. Leguminosarum* biovar *viciae* in biochar over a 28 day period.

	Log_{10} CFU per gram of biochar after day:			
Biochar†	0	7	14	21
SPFM1	ND$^{\delta}$	ND	ND	ND
FBM	ND	ND	ND	ND
WBM	ND	ND	ND	ND
SPF1	7.50(6.99)‡	5.90(6.04)	6.96(7.16)	5.56(5.64)
FB	6.78(6.37)	7.17(7.04)	6.74(6.52)	5.87(5.00)
WB	7.18(6.67)	ND	ND	ND

† Biochar sources can be found in Table 3.1.
‡ Number in brackets is the standard deviation of the mean of 3 replicates.
δ ND indicates no data as sampling was terminated for the treatment.