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Impacts of Biochar on *Trifolium incarnatum* and *Lolium multiflorum*: Soil Nutrient Retention and Loss in Sandy Loam Amended with Dairy Manure

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Abstract: Biochar has many potential benefits in agroecosystems such as increasing productivity of crops and modifying soil nutrient content. Biochar is sourced from many waste materials which could easily and sustainably remedy current challenges in concentrated agricultural operations that use manure-based fertilizers. However, relatively little is known about its effects on forage species in conjunction with manure or biochar enriched with manure effluent. Our objective was to look at the effect of biochar and dairy effluent soil amendments on a forage legume and a grass. In this study, sandy loam soil was amended with a variety of biochar (BC) in a greenhouse setting. Factors included (1) BC type; (2) BC loading percentage; (3) effluent saturation of BC; and (4) forage inclusion. The study was repeated twice: once with *Trifolium incarnatum* and once with *Lolium multiflorum*. Plant material was assayed for biomass (BM) and C and N content. Soil was assayed for nutrient content and micronutrients. Data were not normally distributed and were consequently analyzed for variance using non-parametric methods in R. Overall, *T. incarnatum* showed a very strong negative ($p \leq 0.05$) impact associated with increasing loading percentages of blend and manure BC on herbage BM, while effluent saturation showed no effect ($p > 0.05$). In contrast, *L. multiflorum* showed a strong ($p \leq 0.05$) positive impact of increasing loading percentages of saturated wood, blend, and manure BC on herbage BM. BC impact on soil nutrients and forage varied greatly depending on type of BC, loading percentage, and forage species included. Results indicated the importance of BC properties and rates, as well as forage species for nutrient tolerances when choosing a BC amendment and loading rate.

Keywords: biochar; *Trifolium incarnatum*; *Lolium multiflorum*; nutrient retention; nutrient loss; sandy loam soil

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1. Introduction

The Natural Resources Conservation Service (NRCS) branch of the United States Department of Agriculture (USDA) uses a matrix called the Conservation Practices Physical Effects (CPPE) to measure how conservation efforts affect natural resources. Within the CPPE, there is a section called "Amendments for Treatment of Agricultural Waste" that describes management practices associated with processing agricultural waste, improving and/or protecting air/water quality, and improving and/or protecting animal health [1]. Amendments for Treatment of Agricultural Waste contains 10 subtopics in which "effects" play out on a single practice scale rather than a system, whereas in natural practice all subtopic areas interplay. Without considering interactions, there is a loss in understanding of how changing single practices affects the entire system.

CPPE practice effects are rated on a scale of 5, Substantial Improvement, to −5, Substantial Worsening [2]. Wastewater degradation rates "excess pathogens and chemicals from manure, bio-solid" effect as a 2, Slight to Moderate Improvement, with the rationale that "amendments can be used to alter the waste stream to remove salts, metals, and some pathogens" [2]. The amendment rating concludes that additional practice reforms need to be made in order to improve the management of agricultural waste. Using cattle manure as a fertilizer is an agricultural practice known across the globe for soils with nutrient deficits, as it is a N-, P-, and K-dense material [3,4]. However, misuse of manure fertilizer can lead to negative environmental effects such as water runoff contamination or groundwater leaching [3].

According to the USDA [5], a dairy cow can produce up to 37 kg wet manure/454 kg animal unit each day. This manure contains approximately 0.03 kg P/day [5]. Extrapolating those numbers, a single dairy cow produces up to 11 kg P/year. Manure waste is either kept in solid form for composting or in a slurry/liquid [6]. Liquid/slurry manure is treated in anaerobic lagoons and later distributed to agricultural fields via liquid manure spreaders or irrigation systems [6]. Continuous liquid manure dispersal results in soil P oversaturation and high runoff probability [7].

Manure application rates target crop nitrogen (N) needs; however, these result in P over-application which creates a build-up of soil P [8]. Excess soil P creates a potential for surface water P runoff and possible leaching [8]. Currently, agronomic soil testing estimates crop response to P, rather than a total measurement [8]. This increases P loss in soils because crops do not always behave in the expected manner; additionally, external factors such as weather and topography can affect P loss [8]. Phosphorus–soil interactions, such as P sorption, have been well documented in laboratory settings [9]. Although laboratory testing is preferred because it controls external factors, to fully understand P behavior in natural systems, in situ experiments must be implemented.

Biochar (BC) has substantial capacity for P adsorption [10–12]. Its P adsorption capacity is correlated with surface area [10]. When comparing biochar of 4.05 $\frac{m^2}{g}$ to 97.20 $\frac{m^2}{g}$ P-sorption increases from 9.46 $\frac{mg}{g}$ to 14.48 $\frac{mg}{g}$ [10]. Adsorption ability is linked to P concentration ([P]), where at high [P] the P-sorption rate slows due to competition for binding sites [10]. The functional groups containing various cations (Ca^{2+}, Mg^{2+}, Al^{3+}, and Fe^{3+}) attached to the main aromatic hydrocarbon structure of BC are pertinent [10–12]. P is adsorbed by BC either through electrostatic attraction or by an anion/cation exchange under various pH, point of zero charge of BC and solution pH [10–12].

BC surface functional groups have a substantial role in the adsorption and desorption rates of P. Common negatively charged functional group binding sites include phenolic, hydroxylic, and multi-dentate carboxylic [13]. Cations in soils such as Ca^{2+}, Mg^{2+}, Al^{3+}, and Fe^{3+} can be taken up by the biochar in an anion/cation exchange and held there as a possible adsorption site for anions [10,13]. Many BC are modified with cations to facilitate P-sorption, especially in soil P remediation [13]. Abiotic factors can influence functional group P binding behavior. Low pH and high temperatures can facilitate release of biochar-adsorbed P species while high pH and low temperatures can inhibit desorption of adsorbed P [13].

BC can have a variety of effects on different kinds of manure-based fertilizers [14]. BC amendments to solid manure have a catalytic effect on composting time and decrease total N_2O emissions by enhancing N_2O-reductase activity of denitrifying bacteria [14]. Experimental amendments of BC to dairy effluent have an approximate 30% recovery of P from lagoons [15].

BC has been tested in the past for its potential in increasing productivity of crops. However, whether BC has a positive effect and what application rate should be used is still in discussion, especially with cool-season forages receiving manure application. Some studies show that composted BC is correlated with an increase in plant growth and soil fertility as the amendment amount increases [16]. Others have reported that although they initially found a compost-BC amendment to be beneficial to plant growth, in a second growth period plants had significant retardation [17]. Furthermore, some studies have indicated that although compost had a positive effect on the biomass of plants, biochar had no effect and may even negatively impact plant growth as amount increases [18]. This contrasts with manure application which tends to increase forage legume [19] and forage grass [20] production as well as N and P content with diminishing positive effects when BC is added, more so for the legume.

There are no published studies looking at *Trifolium incarnatum* response to biochar soil amendment. However, there are studies looking at other *Trifolium* species in which biochar, especially in combination with other amendments, increased aboveground plant biomass [20,21]. In contrast, numerous studies examined the impact of BC amendment on soil and tissue of *Lolium multiflorum.* These observed a decrease in plant tissue yield as well as P content when biochar was applied to soil without the addition of supplemental P [22]. One also observed greater soil NO_3-N retention in the presence of BC [23]. Taken together, this leads us to hypothesize that BC with or without additional organic soil-nutrient amendments affect legumes and grasses differently.

BC may improve agricultural waste management and mitigate negative environmental effects linked to manure fertilization and is therefore a potential improvement to current field fertilization practices. Studying BC within a greenhouse setting allows for control of external factors and to develop baseline data for field trials. Our objectives were to measure changes in soil nutrients with the following soil amendments: with dairy manure effluent, different BC types, a range of loading rates, and two forage species (*T. incarnatum* and *L. multiflorum*) known for their adaptation to low rainfall and cool-season temperatures in sub-tropical regions known to have soils deficient in key nutrients and micronutrients required by annual forages. Improving our understanding of biochar amendments in combination with dairy effluent application and their impacts on plant and soil properties will guide future production decisions concerning use of manure effluent and biochar to enhance nutrient content in annual cool-season pastures.

2. Materials and Methods

2.1. Experimental Design

The study was conducted at the Texas A&M AgriLife Center in Stephenville, TX, USA (32.2454° N, −98.1970° W) utilizing the greenhouses on location over a 120-day period. Each pot was considered an experimental unit and all treatment combinations were replicated four times. This was a four-factorial experiment in which the factors were (1) BC type; (2) BC effluent saturation; (3) BC loading percentage; (4) forage inclusion. The study was carried out simultaneously on *T. incarnatum* and *L. multiflorum*. Cool-season forages were focused because they predominate over row crops in low rainfall regions with mild winters.

2.1.1. Soil Preparation

Soil was collected from the top 20 cm of a Windthorst fine sandy loam [19] in Stephenville, TX, USA homogenized, air-dried under ambient conditions, sifted, and distributed in 3 kg units to 288 1-gallon plastic nursery pots. A sandy loam was selected because it is a common texture in this region

2.1.2. Biochar Preparation

Three types of BC were utilized in this study originating from manure (Ecochar, Evansville, IN, USA), wood (Waste to Energy, Inc., South Slocomb, AL, USA), or a manure/wood blend (50% each). BC was ground using a Thomas Wiley Mill (Swedesboro, NJ, USA) fitted with a 2 mm screen and used as received (S^-) or saturated (S^+) in dairy manure effluent collected from the 2nd Lagoon at Tarleton State University's Southwest Regional Dairy Center that feeds a total mixed ration in a confined animal operation. The saturation process consisted of combining BC and dairy effluent in a 1:1 ratio to create a slurry of S^+ BC. Slurries were homogenized every day for 14 days and allowed to evaporatively dry at ambient temperatures. Once dry, the S^+ BC was sifted to allow for proper incorporation into soils. All BC was incorporated in pots on a dry matter weight percent soil replacement.

2.1.3. Treatments

Each forage species was considered a separate experiment. Each experiment included three factors: biochar source, biochar saturation with manure effluent, and biochar loading percentage. Thirty-eight distinct treatment combinations resulted: (1) Soil (control); (2) Soil + forage (control); (3–5) 2/5/10% manure S^- BC; (6–8) 2/5/10% manure S^- BC + forage; (9–11) 2/5/10% manure S^+ BC; (12–14) 2/5/10% manure S^+ BC + forage; (15–17) 2/5/10% wood S^- BC; (18–20) 2/5/10% wood S^- BC + forage; (21–23) 2/5/10% wood S^+ BC; (24–26) 2/5/10% wood S^+ BC + forage; (27–29) 2/5/10% blend S^- BC; (30–32) 2/5/10% blend S^- BC + forage; (33–35) 2/5/10% blend S^+ BC; and (36–38) 2/5/10% blend S^+ BC + forage.

2.1.4. Seeding and Watering

In treatments receiving forage, 7 seeds of either *T. incarnatum* or *L. multiflorum* were seeded into each pot. Once seedlings were fully established at 2 weeks, they were thinned down to 2 plants/pot. Pots were watered as needed (~5–7 days) to maintain near field capacity and leachate was recycled back into the soil.

2.2. Sampling and Sample Preparation

2.2.1. Soil

A sub-sample of soil representing 0.5% of total pot soil was taken from each experimental unit (pot) using a small soil probe to minimize root loss and account for a complete cross-section of soil. The samples were allowed to air-dry under ambient conditions until weight stabilized, then sifted.

2.2.2. Forage

Plants were sheared at soil level to separate above-ground herbage from roots. Roots were washed with water to remove all remaining soil. All samples were dried in a forced-air oven at 55 °C until weight stabilized. Biomass was recorded immediately after removal from the oven. All samples were ground though a 1171H10 Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) fitted with a 1 mm screen.

2.3. Sample Analysis

2.3.1. Soil

Soil samples were assayed for the determination of permanganate oxidizable carbon using a method adapted by Culman et al. [20]. Soil samples were additionally assayed for micronutrients by Texas A&M AgriLife Extension Service—Soil, Water, and Forage Testing Laboratory using extractants described by Mehlich [21]. Data received from this lab included pH, conductivity, P, K, Ca, Mg, S, Na, Fe, Zn, Mn, and Cu values. Additionally, soil NO_3-N data were provided using a Cd reduction [22,23].

2.3.2. Forage

Plant samples were assayed for C and N content via CN828 elemental analysis by combustion (LECO Corporation, St. Joseph, MI, USA). For herbage and root samples, the percentages given by the assay were multiplied by the total weight of samples to determine total weight of C and N in grams.

2.4. Statistical Analysis

Data were analyzed using R (R Core Team, 2021). Independent variables consisted of BC type, BC saturation, BC loading percentage and forage inclusion. Dependent variables consisted of soil and plant-captured N, P, and C, as well as other nutrient and soil health indicators such as dry weight herbage and root yields.

Data collected were not normally distributed, and no adequate adjustments were found to correct this, so non-parametric data analyses were used. A Kruskal–Wallis test was used to test for significant ($p \leq 0.05$) differences among dependent variables grouped by treatment. In cases where differences were discovered though the Kruskal–Wallis test, a Dunn's post-hoc test was performed to determine specific differences. For crimson clover the Benjamini–Hochberg method was used to decrease the false discovery rate and for Italian ryegrass no method was applied to adjust for *p*-values. Other non-parametric methods such as the Spearman's correlation were used to analyze and identify trends in the data. We considered significance at $p \leq 0.05$ and did not report individual probabilities in text unless they were $p \leq 0.05$ and relevant to the discussion.

3. Results

3.1. Herbage Biomass

3.1.1. *Trifolium incarnatum*

Blend and manure S^+ and S^- BC affected *T. incarnatum* herbage dry weight (H-DW), while wood BC showed no influence. Similarly, blend and manure S^- BC affected root dry weight (R-DW), while wood, blend, and manure S^+ BC produced no differences. Many differences occurred between control and higher BC loading percentages. For instance, control had 100% more H-DW compared to 10% blend S^- BC (Table 1).

Table 1. *p*-values for *Trifolium incarnatum* herbage dry weight vis-á-vis control pots with no biochar.

Comparison	*p*-Value
Control—10% Blend S^- BC *	0.041
Control—10% Blend S^+ BC	0.029
Control—5% Manure S^- BC	0.036
Control—5% Manure S^+ BC	0.043
Control—10% Manure S^- BC	0.034
Control—10% Manure S^+ BC	0.042
2% Blend S^- BC—10% Blend S^- BC	0.037
5% Blend S^+ BC—5% Manure S^+ BC	0.049
2% Manure S^+ BC—5% Manure S^+ BC	0.048
2% Manure S^+ BC—10% Manure S^+ BC	0.047

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

Other control comparisons, where differences were measured in H-DW, included 10% blend S^+ BC, 5/10% manure S^- BC, and 5/10% manure S^+ BC (Table 1). Loading at 10% for blend and manure BC, S^+ and S^-, led to a complete failure of establishment, as did loading at 5% for manure BC (Table 1). Differences were also clear between 2% loading of blend and manure BC, S^+ and S^-, and higher BC loading percentages (Table 2).

Table 2. *Trifolium incarnatum* herbage dry weight medians according to saturation and loading percent.

Loading Percent	Wood BC *		Blend BC		Manure BC	
	S^-	S^+	S^-	S^+	S^-	S^+
				g		
Control	1.455	1.275	0.885	1.655	1.400	0.975
2%	0.640	0.890	2.095	0.865	0.290	0.935
5%	0.235	0.990	0.435	0.330	0.000	0.000
10%	0.370	1.015	0.000	0.000	0.000	0.000

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

As stated before, 10% loading of blend and manure BC, S^+ and S^-, led to a complete failure of establishment. Additionally, loading at 5% manure S^+ BC led to a complete failure of establishment, but there were no differences between 2% manure S^- BC—5% manure S^- BC ($p = 0.44$).

The only difference across BC types for H-DW occurred between 5% blend S^+ BC—5% manure S^+ BC (Table 1). Establishment and growth did occur at 5% blend S^+ BC with a median H-DW of 0.330 g, where 5% manure S^+ BC failed to establish (Table 1). These differences showed a strong indication that manure BC influenced establishment at high loading rates ≥5%.

Control comparisons where differences were measured in R-DW included 10% blend S^- BC, 10% manure S^- BC, and 5% manure S^- BC (Table 3).

Table 3. *p*-values for *Trifolium incarnatum* root dry weights.

Comparison	*p*-Value
Control—10% Blend S^- BC *	0.044
Control—5% Manure S^- BC	0.044
Control—10% Manure S^- BC	0.043
2% Blend S^- BC—10% Blend S^- BC	0.039

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

Similar to H-DW, there was a complete failure for root establishment at 10% manure BC (Table 4). Additionally, there were differences for R-DW at 2% Blend S^- BC—10% Blend S^- BC in which pots with 2% loading had a median R-DW of 1.68 g and establishment failure at 10% loading with a median R-DW of 0.00 g (Tables 2 and 4).

Table 4. *Trifolium incarnatum* root dry weight medians according to saturation and loading percent.

Loading Percent	Wood BC *		Blend BC		Manure BC	
	S^-	S^+	S^-	S^+	S^-	S^+
				g		
Control	1.175	0.785	1.115	0.855	0.855	0.925
2%	0.450	0.990	1.675	0.810	0.125	0.505
5%	0.230	0.940	0.255	0.195	0.000	0.000
10%	0.295	0.465	0.000	0.000	0.000	0.000

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

When reviewing the results for the *T. incarnatum* growth and biomass production in response to BC type, saturation, and loading percent, it becomes clear that manure BC, when applied at high percentages such as 5% and 10%, regardless of saturation, had an inhibiting factor on germination and establishment. This inhibition was also present in the 10% blend BC, where 5% of the blend BC applied was manure BC.

Initially, the final soil data did not give a strong indication of where this failure could have come from in the germination and establishment period. It appears that the *T. incarnatum* may have suffered from excessive zinc in the soil [24]. A study conducted by Marques et al. [24] reported that *T. incarnatum* germinated in sand with water had a 10% inhibition of germination at 3.9 ppm Zn and a 50% inhibition at 5.3 ppm Zn. Additionally, when the sand was treated with a nutrient solution instead of water the 10% inhibition of germination fell to 2.9 ppm Zn and 50% inhibition to 4.8 ppm Zn [24].

Initial soil data for 5% manure S^- BC showed a 173% Zn increase compared to what Marques et al. [24] used when reporting 50% germination inhibition, while 10% manure S^- BC showed an increase of 443% in regard to their zinc levels (Table 5).

Furthermore, when the manure BC was saturated, the zinc concentrations for 5% manure S^+ BC loading increased to 189% and 10% loading an increase of 474% (Table 5). Differing Zn concentrations in the amended soil came from different levels of Zn in the initial manure, blended, and wood BC (Table 6).

Table 5. Initial soil zinc concentrations in ppm for *Trifolium incarnatum*.

BC Type	Loading Percent 0%	2%	5%	10%
			ppm	
Wood S^- BC *	0.2	0.9282	2.0205	3.841
Wood S^+ BC	0.2	1.003	2.2075	4.215
Blend S^- BC	0.2	3.1996	7.699	15.198
Blend S^+ BC	0.2	4.1694	10.1235	20.047
Manure S^- BC	0.2	5.9146	14.4865	28.773
Manure S^+ BC	0.2	6.2436	15.309	30.418

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

Table 6. Initial biochar characterization.

	Wood BC *	Blend BC	Manure BC
		%	
Nitrogen	0.211	0.290	0.738
Phosphorus	0.004	0.631	1.149
Potassium	0.214	1.767	4.392
Calcium	0.216	3.649	6.389
Magnesium	0.035	0.722	2.615
Sodium	0.059	0.326	0.742
Ash	5.83	22.94	40.05
Fixed Carbon	60.70	42.27	23.83
Volatile Matter	27.84	30.21	32.57
		ppm	
Zinc	36.61	150.18	285.93
Iron	775.36	3721.51	7708.70
Copper	12.62	62.29	153.70
Manganese	139.14	330.85	432.47
Sulfur	13.70	3943.97	3167.22
Boron	2.32	6.22	29.74
pH	8.8	9.4	10.2

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

The manure BC possessed a 154.60% higher Zn concentration than the wood BC while the blended BC also had 121.602% higher Zn concentration than the wood BC (Table 6). This indicated great impacts of BC properties on crop productivity which was consistent with what Niruala et al. reported [25].

Excessive phosphorus is another possible contributor but is discussed in Section 3.2 found below. Final H-DW showed a strong negative correlation to increasing soil Zn concentrations regardless of saturation or loading percent, with the exception of wood BC as it had no differences amongst H-DW (Figures 1 and 2).

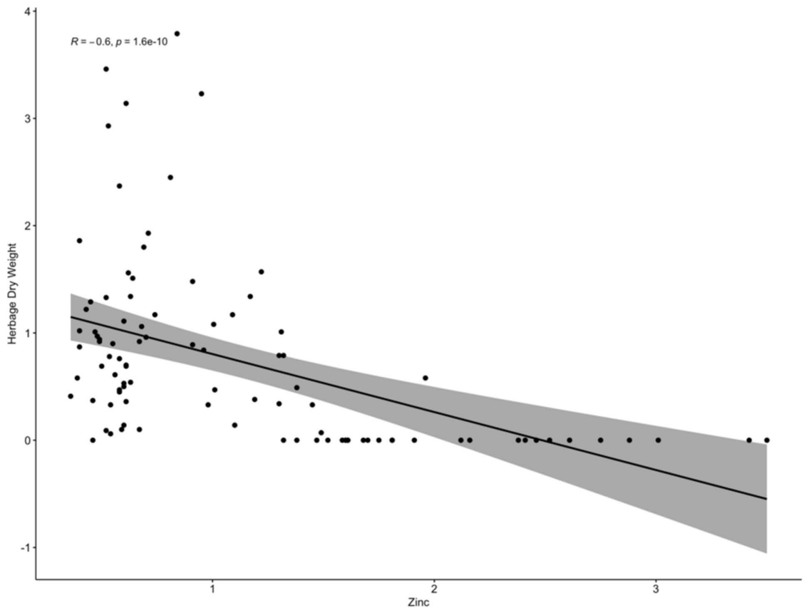

Figure 1. Herbage dry weight (H-DW) (g) × Zn (ppm), Spearman's correlation for *T. incarnatum*.

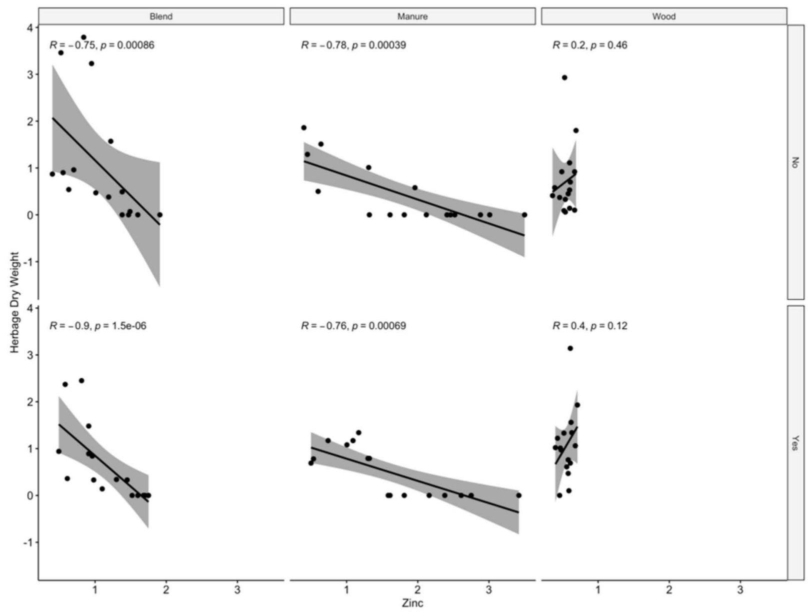

Figure 2. Herbage dry weight (H-DW) (g) × Zn (ppm), Spearman's correlation for *T. incarnatum* by BC type and saturation (yes for saturated).

R-DW, like herbage, had a strong negative correlation to increasing soil zinc concentrations regardless of saturation or loading percent, with the exception of wood BC as it had no differences amongst root weight (Figures 3 and 4).

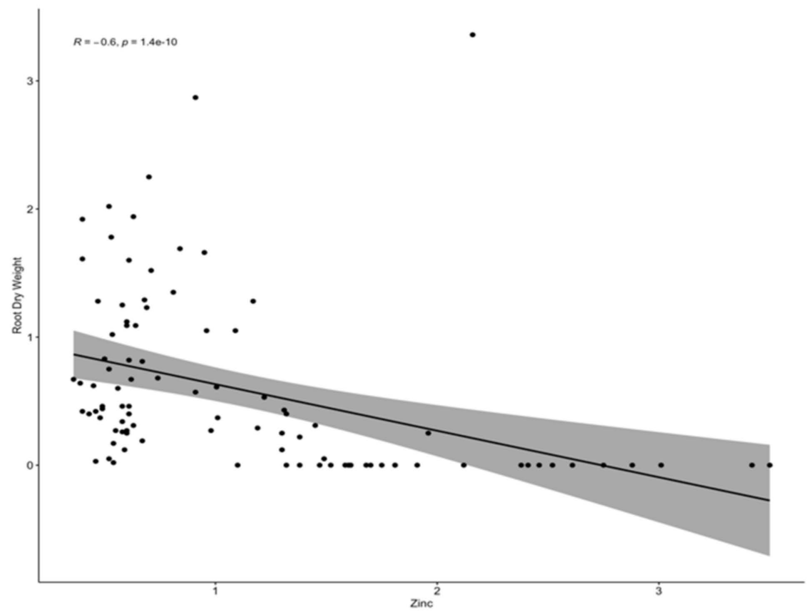

Figure 3. Root dry weight (R-DW) (g) × zinc (ppm), Spearman's correlation for *T. incarnatum*.

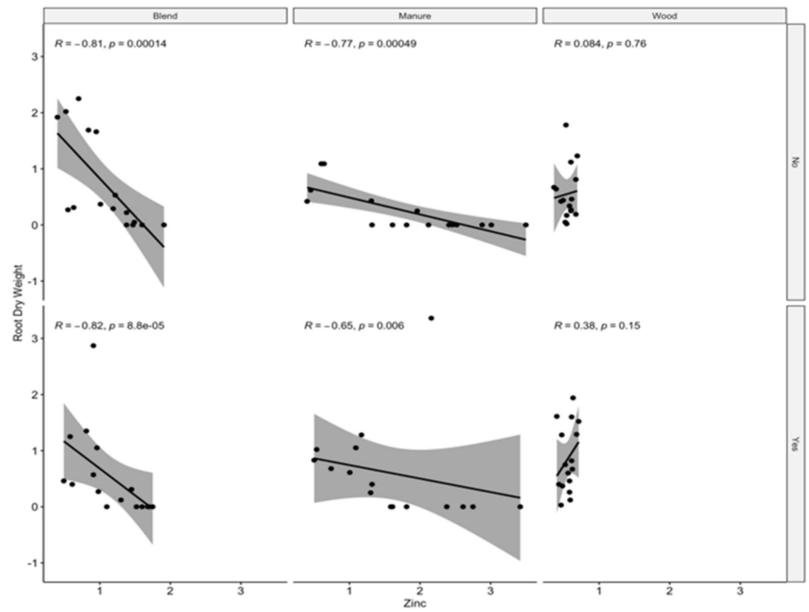

Figure 4. Root dry weight (R-DW) (g) × Zn (ppm), Spearman's correlation for *T. incarnatum* by BC type and saturation (yes for saturated).

Median H-DW and R-DW indicated a well-defined negative impact of loading $\geq$5% manure BC, regardless of saturation. The best performing amendment was 2% blend S^- BC loading with a median H-DW of 2.10 g and a median R-DW of 1.68g (Tables 1 and 2).

3.1.2. *L. multiflorum*

Wood, blend, and manure, S^+ and S^- BC affected *L. multiflorum* H-DW. Many of the differences occurred between control and higher BC loading percentages. Control comparisons where differences were measured included 2% manure S^+ BC, 5% manure S^+ BC, 10% manure S^+ BC, 10% blend S^+ BC, 5% manure S^- BC, and 10% wood S^+ BC (Table 7).

Table 7. Significant *p*-values for *Lolium multiflorum* herbage dry weight.

Comparison	Percent Difference	*p*-Value
Control—10% Wood S^+ BC *	+92.90%	0.035
Control—10% Blend S^+ BC	+82.24%	0.018
Control—2% Manure S^+ BC	+89.40%	0.038
Control—5% Manure S^- BC	+97.30%	0.043
Control—5% Manure S^+ BC	+114.59%	0.016
Control—10% Manure S^+ BC	+139.85%	0.017
5% Wood S^+ BC—10% Wood S^+ BC	+93.85%	0.030
10% Wood S^- BC—10% Wood S^+ BC	+135.06%	0.005
2% Manure S^- BC—2% Wood S^- BC	−122.65%	0.027
5% Manure S^- BC—5% Wood S^- BC	−129.59%	0.009
5% Manure S^+ BC—5% Wood S^+ BC	−91.75%	0.029
10% Manure S^- BC—10% Manure S^+ BC	+134.81%	0.047

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

Additionally, there was a difference between 5% wood S^+ BC—10% wood S^+ BC.

Lolium multiflorum H-DW also responded to differences between saturations of the same BC type and loading percent. Saturation had a similar effect on growth in wood BC sets at high loading percent, increasing median H-DW 422%, as it did on manure BC sets in which saturation increased median H-DW 413% (Table 8).

Table 8. *Lolium multiflorum* herbage dry weight (g) medians according to saturation and loading percent.

Loading Percent	Wood BC *		Blend BC		Manure BC	
	S^-	S^+	S^-	S^+	S^-	S^+
				g		
Control	0.920	0.830	0.755	0.625	0.755	0.600
2%	0.345	1.270	0.900	0.895	1.460	1.570
5%	0.465	0.820	1.145	0.760	2.200	2.205
10%	0.435	2.270	1.065	1.510	0.660	3.385

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

Lastly, differences were observed between BC types of the same saturation and loading percent (Table 8). No comparisons include blend BC in this category as blend BC are 50/50% manure/wood combinations. Overall, loading percent had a strong positive correlation to HDW when associated with all S^+ BC types (Figure 5).

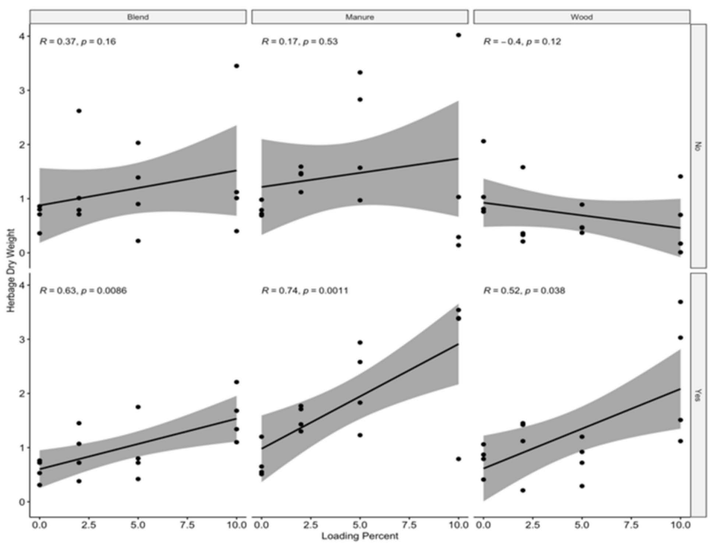

Figure 5. H-DW x loading percent, Spearman's correlation for *L. multiflorum* by BC type and saturation (yes for saturated).

The best performing amendment was 10% manure S^+ BC with a median H-DW of 3.39 g (Table 5). This value, however, was not performatively different than other S^+ BC at 10% loading [*p*-value 0.67 (10% blend S^+ BC) and 0.98 (10% wood S^+ BC)]. This is not unexpected as *L. multiflorum* is well suited to a variety of soils, given proper moisture levels and responds well to high soil nutrient content [26].

The Kruskal–Wallis test for *L. multiflorum* R-DW indicated no difference across the data with a *p*-value of 0.509. Root systems at harvest were robust with shoots growing out of the drainage holes. If pots had been larger or deeper, there may have been a difference among treatments, as the pot size in this trial may have been a limiting factor for root growth.

3.2. Soil Phosphorus

Post-experimental soil + BC + forage bio-available Mehlich III [21] were compared with initial soil + BC bio-available P to determine P loss (uptake by plants) and retention. Data indicated that the most soil-P loss occurred at 10% S^+ BC for all types. The 10% blend S^+ BC loading lost an average of 72% Mehlich III soil-P (*p*-value 0.57), 10% wood S^+ BC loading lost an average of 33% (*p*-value 0.59), and 10% manure S^+ BC lost an average of 64% (*p*-value 0.81). None of these pre-trial to post-trial comparisons were considered significant, but there were differences post-experimental. Soils that retained the most P varied by BC type, but all were S^-. 2% blend S^- BC loading retained an average of 60% P, 2% manure S^- BC loading retained an average of 66% P, and 10% wood S^- BC loading retained an average of 87% P. These comparisons had *p*-values = 0.61, 0.77, and 0.79, respectively; however, they were important for soil-P retention pre- and post-trial.

Initial soil with no amendments was low in P at 12 ppm; consequently, most P retained or lost came from the BC application. The S^+ BC-treated soils contained 4% higher P content than the S^- BC, S^+ manure BC, S^+ blend BC had a 41% increase, and S^+ wood BC had a 280% increase. Increases in P content increase the competition for binding sites. This is one explanation for why the S^+ BC soils lost P at a higher rate [10]. This "falling off" action of P from the BC binding sites may allow for more Mehlich III P to be available for uptake by the forage. However, to support this argument further testing would need to be carried out on the BC to characterize surface area and functional groups through processes such as hydrolysis pyrolysis and/or solid state ^{13}C nuclear magnetic resonance [27].

To verify that the BC was impacting the soil P and not the action of the forage, comparisons were made between post-trial pots treated with forage + BC, no forage + BC, and forage + no BC. No differences were found between forage + BC and no forage + BC for either *T. incarnatum* or *L. multiflorum* (Tables 9 and 10).

Table 9. Impact of biochar and forage application on soil phosphorus for *Trifolium incarnatum*. Green highlighted cells are significant ($p \leq 0.05$) values.

Wood S^- BC *			**Wood S^+ BC**		
Loading	**BC Effect *p*-Value**	**Forage Effect *p*-Value**	**Loading**	**BC Effect *p*-Value**	**Forage Effect *p*-Value**
2%	0.946	0.771	2%	0.960	0.936
5%	0.607	0.869	5%	0.730	0.602
10%	0.974	0.928	10%	0.953	0.635
Blend S^- BC			**Blend S^+ BC**		
Loading	**BC effect *p*-value**	**Forage effect *p*-value**	**Loading**	**BC effect *p*-value**	**Forage effect *p*-value**
2%	0.177	0.897	2%	0.079	0.981
5%	0.048	0.940	5%	0.014	0.990
10%	0.010	0.953	10%	0.003	0.912
Manure S^- BC			**Manure S^+ BC**		
Loading	**BC effect *p*-value**	**Forage effect *p*-value**	**Loading**	**BC effect *p*-value**	**Forage effect *p*-value**
2%	0.0732	0.962	2%	0.127	0.894
5%	0.007	0.984	5%	0.012	0.931
10%	0.002	0.994	10%	0.002	0.994

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated. Green are significant *p*-values.

Table 10. Impact of BC and forage application on phosphorus in soil for *L. multiflorum*. Green highlighted cells are significant values.

Wood S^- BC *			**Wood S^+ BC**		
Loading	**BC Effect *p*-Value**	**Forage Effect *p*-Value**	**Loading**	**BC Effect *p*-Value**	**Forage Effect *p*-Value**
2%	0.925	0.891	2%	0.380	0.652
5%	0.829	0.442	5%	0.832	0.602
10%	0.799	0.748	10%	0.854	0.431
Blend S^- BC			**Blend S^+ BC**		
2%	0.077	0.993	2%	0.052	0.978
5%	0.015	0.943	5%	0.009	0.978
10%	0.003	0.974	10%	0.001	0.9406
Manure S^- BC			**Manure S^+ BC**		
2%	0.027	0.953	2%	0.046	0.953
5%	0.002	0.959	5%	0.002	0.820
10%	0.0004	1.00	10%	0.0005	0.978

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

Differences were found between forage + BC and forage + no BC, indicating that the BC impacted the soil P rather than the forage (Tables 9 and 10). Wood BC, both S^+ and S^-, did not affect soil P for *T. incarnatum* or *L. multiflorum*. *T. incarnatum* had large differences in soil P at 5% and 10% blend, S^+ and S^-, BC compared with control containing no BC which registered at a median of 14 ppm P (Tables 9 and 11).

Table 11. Soil phosphorus ppm median values for pots growing *Trifolium incarnatum*.

Wood S^- BC *		**Wood S^+ BC**	
Loading	**Median**	**Loading**	**Median**
	ppm		ppm
0%	13	0%	16.5
2%	12.5	2%	16
5%	14	5%	15.5
10%	12	10%	17
Blend S^- BC		**Blend S^+ BC**	
	ppm		ppm
0%	14	0%	13.5
2%	73.5	2%	85
5%	138.5	5%	162
10%	219.5	10%	248
Manure S^- BC		**Manure S^+ BC**	
	ppm		ppm
0%	16.5	0%	15.5
2%	161	2%	103.5
5%	323	5%	227.5
10%	637.5	10%	442

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

When compared to control plants, 5% blend S^- BC contained an 890% increase in P, 10% blend S^- BC contained a 1468% increase, 5% blend S^+ BC contained a 1100% increase, and 10% blend S^+ BC contained a 1737% increase (Table 11).

T. incarnatum also had differences for 5% and 10% manure, S^+ and S^-, BC. When manure BC were compared to control an even larger difference in P concentration occurred, 5% manure S^- BC contained a 1858% increase in soil P, 10% manure S^- BC contained a 3764% increase, 5% manure S^+ BC contained a 1368% increase and 10% manure S^+ BC contained a 2752% increase.

Excessive P in soils growing *T. incarnatum* could be another explanation for establishment failure at 5% and 10% manure BC, and 10% blend BC. Excessive Zn and P in soils are expressed similarly through chlorosis in young plants, which can make it difficult to determine the cause of establishment failure [24,28–30]. On the other hand, excessive soil P can cause Zn deficiencies in the plant itself by blocking micronutrient uptake, by quickly converting Zn into plant-unavailable forms [29]. Very strong positive correlations exist within the data collected for P and Zn soil concentrations that would indicate excessive P blocking Zn uptake or Zn transformation (Figures 6 and 7).

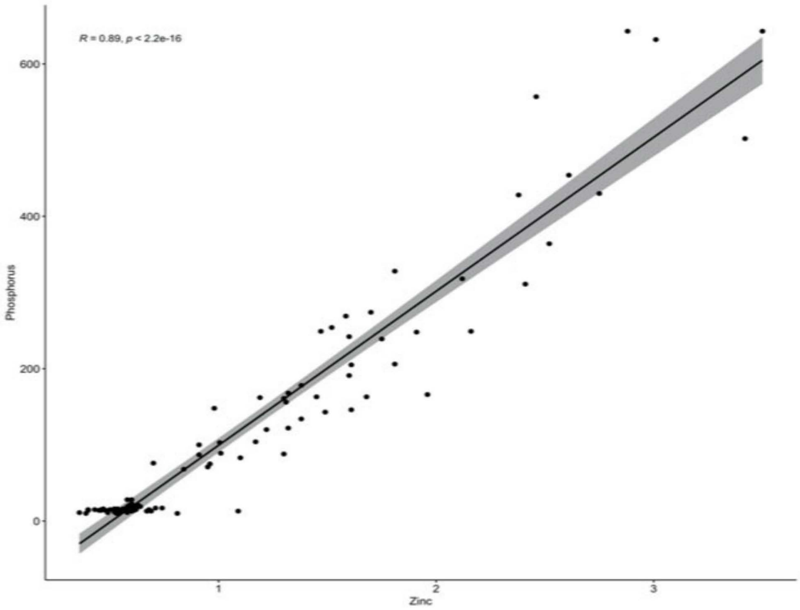

Figure 6. P (ppm) × Zn (ppm), Spearman's correlation for *Trifolium incarnatum* soils.

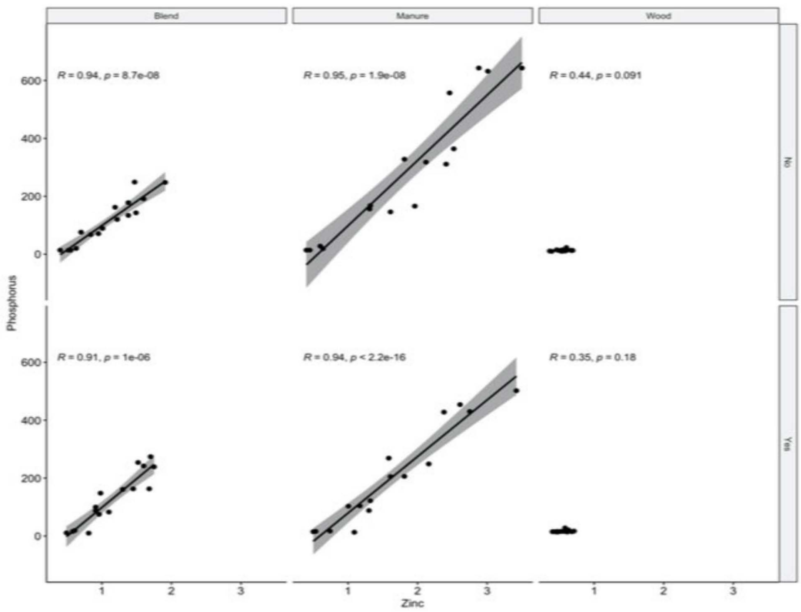

Figure 7. P (ppm) × Zn (ppm), Spearman's correlation for *Trifolium incarnatum* soils by biochar type and saturation (yes is saturated).

To determine which is the primary cause, further nutrient testing on herbage is required. If low levels of zinc were found in lower loading levels of BC, then excessive soil P could be considered a primary cause. Soil containing between 150 and 200 ppm P can have issues supporting plants for 3 to 5 years and any P values above 330 ppm require special treatment [29]. In a study conducted by Pang et al. [31], most perennial legumes exhibited reduced biomass production when P exceeded 192 ppm. In severe cases of excessive P, *T. subterraneum* expressed reduced growth followed by sudden necrosis [28].

Median soil P ppm for 5%, manure, 10% manure and 10% blend BC, all of which had *T. incarnatum* establishment failure, were over the 192-ppm maximum tolerance level proposed by Pang et al. [31]. *Lolium multiflorum* had large differences in soil P at 5% and 10% blend, S^+ and S^-, as well as 2% blend S^+ BC when compared with control containing no BC which registered at a median of 13 ppm P (Tables 10 and 12).

Table 12. Soil phosphorus ppm median values for *Lolium multiflorum*.

Wood S^- BC *		**Wood S^+ BC**	
Loading	**Median**	**Loading**	**Median**
	ppm		ppm
0%	13	0%	15
2%	14	2%	18
5%	11.5	5%	16
10%	14	10%	14.5
Blend S^- BC		**Blend S^+ BC**	
	ppm		ppm
0%	13	0%	13
2%	86	2%	84.5
5%	157	5%	160.5
10%	237.5	10%	252.5
Manure S^- BC		**Manure S^+ BC**	
	ppm		ppm
0%	14	0%	13.5
2%	161	2%	107
5%	313.5	5%	264.5
10%	634	10%	428.5

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

When compared to control 5% blend S^- BC contained a 1108% increase in P, 10% blend S^- BC contained a 1727% increase, 2% blend S^+ BC contained a 550% increase, 5% blend S^+ BC contained a 1135% increase, and 10% blend S^+ BC contained a 1842% increase (Table 12). *Lolium multiflorum* also had differences for 2%, 5%, and 10% manure biochar, saturated and unsaturated (Table 10). When manure BC were compared to control, large differences in soil P resulted in a 1050% increase in P for soils receiving 2% manure S^- BC, a 2139% increase for 5% manure S^- BC, a 4429% increase for 10% manure S^- BC, a 630% increase for 2% manure S^+ BC, a 1859% increase for 5% manure S^+ BC, and a 3074% increase for 10% manure S^+ BC (Tables 10 and 12).

As stated previously, *L. multiflorum* is a tolerant species in regard to unbalanced or excessive soil nutrient content [26]. This resilience to high nutrient content, specifically high soil P content, was quantified by Sharma and Sahi [32] in a study which tested two types of *L. multiflorum* in soils enriched with 0–20,000 ppm P. Herbage biomass increased as P content increased until 20,000 ppm where there was a negative significant impact [32]. In our study, when *L. multiflorum* is compared to *T. incarnatum*, the grass is a better option when working with soils and BC where P-content may already be high as it has a higher tolerance for nutrient load.

3.3. Soil Oxidizable Carbon

There were no differences in soil oxidizable C content among post-trial control and treated receiving manure, blend, and wood BC, saturated or unsaturated, when growing *T. incarnatum* at all loading levels. Additionally, there were no differences in *L. multiflorum* post-trial containing wood and blend BC, saturated or unsaturated, at all load levels. Differences in soil oxidizable C did occur between 5%, saturated and unsaturated, manure BC for *L. multiflorum*. Although, there was only one difference among post-trial for either *T. incarnatum* or *L. multiflorum*, strong positive correlations existed between increasing loading percent of manure BC and soil oxidizable C; this correlation does not exist for blend BC or wood BC (Figure 8).

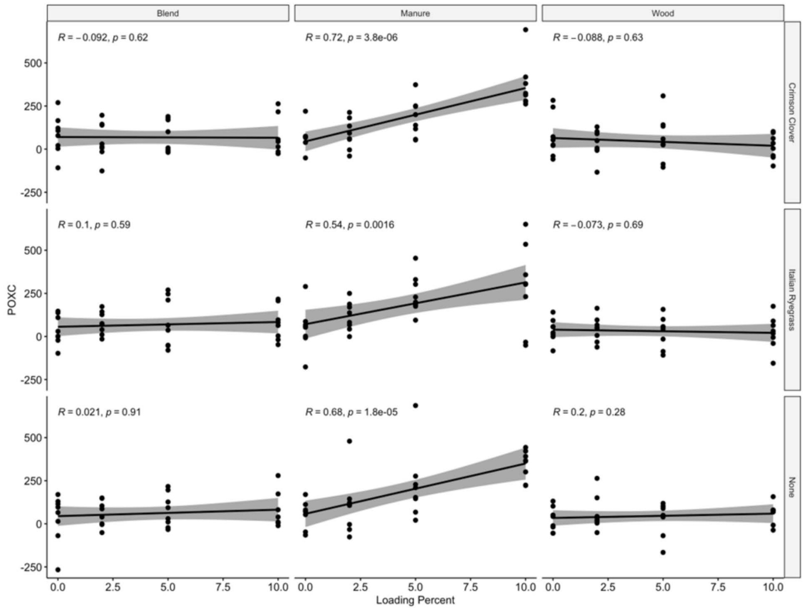

Figure 8. Soil oxidizable C (ppm) × Loading Percent, Spearman's correlation for forage by BC type.

An explanation for the difference in soil oxidizable C of BC types is that BC made from plant material are low in nutrient materials where manure-based BC have a positive effect on soil biochemical properties [33].

3.4. Forage and Soil Nitrogen

Post-experimental soil + BC + forage bio-available soil NO_3-N values were compared with initial soil + BC to determine bio-available N change. Post-experimental soil N content indicated ≥97% N reduction in all BC amendments regardless of saturation or loading percent. Even though BC can help to retain nutrients such as soil N, retention is highly variable depending on the physical and chemical properties such as pH, surface area, and functional groups on the BC surface [34].

Differences in total N content for *T. incarnatum* H-DW occurred between 2% blend S^- BC—2% manure S^- BC for an increase of 471% N content and 5% blend S^+ BC—5% wood S^+ BC for an increase of 515% N content (Table 13).

Table 13. Median total nitrogen content for *Trifolium incarnatum*.

Loading Percent	Wood BC *		Blend BC		Manure BC	
	S^-	S^+	S^-	S^+	S^-	S^+
			mg			
Control	24.17	41.18	22.17	46.91	29.42	23.13
2%	12.26	20.07	32.56	13.19	5.70	17.88
5%	6.89	21.75	8.18	3.54	0.00	0.00
10%	5.72	17.44	0.00	0.00	0.00	0.00

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

There was also a single difference of control and saturated blend BC 5% blend S^+ BC for an increase of 1227% N content (Table 13). Differences in *L. multiflorum* H-DW total N content occurred between 5% manure S^- BC—5% wood S^- BC for an increase of 473% N content and 5% manure S^- BC—5% blend S^- BC for an increase of 1248% N content (Table 14).

Table 14. Median total nitrogen content for *Lolium multiflorum*.

Loading Percent	Wood BC *		Blend BC		Manure BC	
	S^-	S^+	S^-	S^+	S^-	S^+
			mg			
Control	2.37	1.22	4.28	6.56	2.53	3.03
2%	3.62	4.21	0.41	7.47	5.00	1.28
5%	2.12	5.58	0.90	4.45	12.15	6.44
10%	0.25	7.68	−0.41	4.87	4.37	13.15

* BC = biochar; S^+ = effluent saturated; S^- = effluent unsaturated.

Nitrogen content in forage has a direct relation to crude protein as a forage quality indicator because crude protein is equal to 6.25 × N content [35–41]. The greatest N content observed in *T. incarnatum* was with 2% blend S^- BC, while *L. multiflorum* crude protein peaked in plants amended with 10% manure S^+ BC (Tables 13 and 14). Correlation data demonstrate that increasing loading percentage for manure, wood, and blend BC has a significant strong negative impact on *T. incarnatum* forage quality, while increasing loading for *L. multiflorum* correlates a moderate positive impact for manure BC (Figure 9).

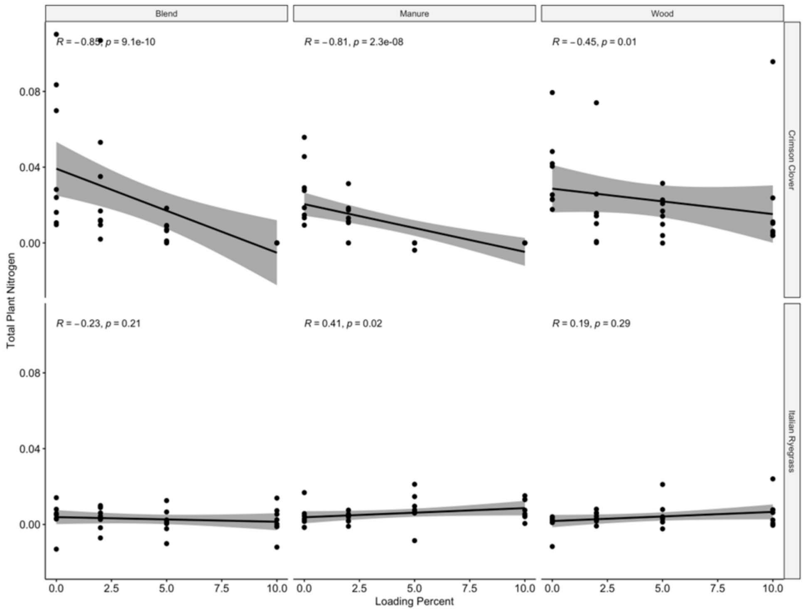

Figure 9. Total Plant N (g) × Loading Percent, Spearman's correlation for *T. incarnatum* and *L. multiflorum* by BC type.

This pattern is similar to the herbage BM data above; not only did increasing BC loading percentage impact BM production negatively but also forage quality, while *L. multiflorum* benefited from an increasing loading percent while also improving forage quality.

4. Discussion

Biochar made from different parent material applied at increasing loading rates and effluent saturation produced differences in forage BM and soil nutrient content in sandy loam soil. *Trifolium incarnatum* BM overall responded negatively to increasing loading percentages of manure, wood, and blend BC, while saturation did not develop a discernable role in forage herbage and root production. By contrast, *L. multiflorum* BM overall responded positively to increasing BC loading percentages of manure, wood, and blend BC. This difference between annual forage grass and legume responses to soil amendment with biochar has been reported elsewhere [20–22]. However, our research was unique in that it was the first to show a negative effect of BC on *T. incarnatum.* It is also unique in that it showed that saturating BC prior to incorporation into soils mitigated the negative effect because saturated BC did not decrease legume herbage or root production. It also minimized NO_3-N removal by BC, which we observed and others have reported [23].

Soil P content increased with increasing loading percentage of blend and manure BC, while wood BC did not increase soil P content. This has been observed with other biochars and cropping systems in which manure was added [22]. Loading levels for $\geq$5% of manure BC, regardless of saturation, became toxic to *T. incarnatum* leading to establishment failure, Zn possibly being a compounding factor. Unlike *T. incarnatum*, *L. multiflorum* responded positively to increased soil P, which can utilize high levels of Zn and P without any inhibition as supported by the literature.

Soil oxidizable C showed no changes due to BC addition but positive correlations existed for increasing soil oxidizable C and increasing loading percentage of manure BC since the manure BC had higher volatile carbon and lower fixed carbon than the wood and blended BCs (Table 6). Soil forage-available N content increased with initial addition of BC; however, $\geq$97% N reduction resulted from all BC amendments, regardless of loading percent or saturation. Soil N was either used by forage for growth or converted to unavailable forms. Total soil N assays would need to be conducted to determine exact nutrient cycling. Total N for *T. incarnatum* BM was negatively impacted by increasing loading percent, regardless of saturation, for manure, blend, and wood BC. Total N for *L. multiflorum* BM was positively impacted by increasing loading percent of manure BC, regardless of saturation. Total N increase was not observed in wood or blend BC.

BC impact on soil nutrient retention/loss and forage varied greatly depending on type of BC, loading percent, and forage species included. When choosing a BC to ensure forage establishment, wood BC performed the best regardless of forage species or loading percentage. However, it was not the best for forage BM or N content. Overall, *T. incarnatum* developed best in soils amended with 2% blend S^- BC, while *L. multiflorum* responded best to 10% manure S^+ BC. This stresses the importance of choosing a BC which suits the forage species. *T. incarnatum* establishment failure at $\geq$5% manure BC loading was possibly caused by excessive soil P and Zn, or interactions of the two nutrients, indicating the importance of plant tolerance and BC properties prior to seeding. Saturation did not affect BM accumulation even though effluent should have acted as a fertilizer, providing N, P, oxidizable C, and other micronutrients to the plants. Further research on other cool-season legumes and grasses is necessary to address this question.

Further research should include BC effects on forage, soil, and nutrient content looking at a greater number of parameters, especially effects on the soil microbiome. This would have been helpful for addressing atmospheric N_2 fixation in *T. incarnatum* together with whether BC promoted microbial colonization. More in-depth testing on BC such as characterization of its surface area, pore size, and attached functional groups would allow better understanding of how nutrients are adsorbed or released, especially for P retention. Herbage and root samples should be assayed for total P content to determine P nutrient cycling and ability of forage species to use excess P, especially in *L. multiflorum* which performed well in high P soils. Lastly, adjustment of BC loading rates should be considered. The loading of 10% BC is not currently a realistic rate when moving into a real-world field application, due to cost as well as negative effects of certain BC types on forage such as *T. incarnatum*.

5. Conclusions

The first notable conclusion was that BC absent saturation with dairy manure effluent nutrients was detrimental to forage growth, especially the legume. Our soil data indicated that unsaturated biochar bound soil nutrients crucial for plant development which, we hypothesize, were then unavailable for plant development. Regardless of what occurred, the fact remains that saturating biochar with dairy manure nutrients prior to soil incorporation may be a feasible means of avoiding the short-term detrimental effect of biochar on forage seedling development.

The fact that there was a difference between grass and root reaction to the presence of unsaturated BC in the soil is our second major conclusion. Why there was a difference between the grass and legume needs further elucidation, especially a close examination of root rhizosphere exudates that may have played a stronger role in one species than in the other. In any case, nutrient saturation of BC prior to use in forage seedling establishment appears to be more important in legumes than in grasses. We hypothesize that this may be due in part to differences in root architecture. The fibrous nature of grass roots may facilitate nutrient uptake vis-à-vis the less fibrous legume root system, a difference that may be particularly important for short-lived annual forages.

We also conclude with an acknowledgement that this study had two limitations when it comes to applying results to field operations. The first is that it was a very short-term trial whereas BC contributions to soil-nutrient characteristics are well known as having a long-term effect. Even though both the forages studied are normally ephemeral annuals, longer-term studies could examine the effect of initial BC application on subsequent years. The second obvious limitation is that this trial took place in a controlled greenhouse environment in potted soil that had been thoroughly homogenized, something that does not happen as extremely in a cultivated and especially in a no-cultivation field situation. Longer-term field trials comparing the same species, BC and effluent applications are therefore merited.

Future research could also include repeating this greenhouse experiment with other annual forage legume and grass species, as well as warm-season forage, and adjusting to lower BC loading rates. Field experiments should follow to assess the effect of BC in situ because this will be important information moving forward to production applications using BC for soil amendments, especially treated with manure effluent to enhance nutrient content.

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