EARLY PRODUCTION OF SWITCHGRASS (*PANICUM VIRGATUM* L.) AND WILLOW (*SALIX* SPP.) INDICATES CARBON ACCUMULATION POTENTIAL IN APPALACHIAN RECLAIMED MINE AND AGRICULTURAL SOIL

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Abstract

The production of bioproduct feedstocks such as switchgrass (*Panicum virgatum* L.) and willow (*Salix* spp.) on marginal lands provides an attractive option to grow dedicated bioenergy crops with carbon capture and storage capabilities to help achieve climate change mitigation goals while reducing competition with land for food production. However, how the production of these crops alters plant-soil-microbe interactions that govern stable soil C accumulation in highly degraded soil is underexplored. The objectives of this study were to examine select biological and chemical properties related to stable SOM production from the growth of switchgrass and willow on marginal soil over two growing seasons and whether biochar amendment can positively affect these parameters. To address our objectives, paired former surface mined lands and non-mine impacted marginal agriculture sites were selected across West Virginia, USA and biochar and unamended control treatments were imposed. Through the first two growing seasons, microbial activity and demand for C increased and was accompanied by a shift in extracellular enzyme investment from the decomposition of labile to more recalcitrant C sources. There was a destabilization of mineral-associated organic matter in the mine sites, indicative of high N demand. Biochar amendments did not impact microbial activity but did increase the C:N of SOM. Overall, our results suggest that the early growth of switchgrass and willow can result in C accumulation in highly degraded lands, while it may take several more years to see these benefits in less degraded soil.

1. Introduction

Rising issues related to climate change mitigation and the global energy supply have led to increased interest in the use of bioproduct crops (i.e., plant-based feedstock) for energy production and carbon capture (U.S. Department of Energy, 2011). However, diverting farmland dedicated to the supply of food for human consumption towards producing feedstock for bioproducts is potentially of ethical concern (Barnard, 1983; Stoy et al., 2018; Hasegawa et al., 2018). Marginal lands, that is, land with soils that have physical, chemical, and biological properties that make them unsuitable for conventional agricultural production, provide an attractive option for bioproduct crop production as they do not compete with land used for food production and may serve to maintain or improve soil health conditions (Blanco-Canqui, 2016; Li et al., 2022; Mehmood et al., 2017). The Appalachian region of the U.S.A. is poised to take advantage of this need, as there are over sixty million hectares of marginal land (Lemus & Lal, 2005; Emery et al., 2017). Examples of the agronomically challenging soil characteristics of reclaimed lands include the presence of rock fragments (can be ~50% of mineral soil), compaction, lack of topsoil, low microbial activity, and the inability to store plant available water and essential nutrients (Johnson and Skousen, 1995; Zipper et al., 2011; Haering et al., 2004). Though, highly disturbed soils can sequester carbon (C) at higher rates compared to less disturbed soils (Blanco-Canqui, 2016). For example, bioproduct systems in the Mid-Atlantic region can increase soil organic matter (SOM) by 20-125% after ten years whereas systems in Europe in arable soil increased SOM by only 0.2-0.5% after six to eight years (McLaughlin & Kszos, 2005; Beuch et al., 2000). Thus, marginal lands are promising targets for not only bioproduct crop production, but also for soil carbon capture and storage (Lal, 2004).

The formation of SOM through plant-soil-microbe interactions is an important process for soil multifunctionality such as nutrient and water storage, erosion reduction, and aggregate stability (Ferrarini et al., 2021; Sahu et al., 2017; Oldfield et al., 2015; Overstreet & DeJong-Huges, 2009). Plants provide labile SOM to the soil through components of litter, roots, and root exudates which contain compounds with essential nutrients such as C, N, P, and S. These organic compounds are mineralized by soil microbes which release nutrients into the soil where they may be assimilated into microbial and plant biomass (Sahu et al., 2017). For example, microbes produce the β-Glucosidase (BG) enzyme to degrade cellulose, a component of plant cell walls, which is a relatively labile C source and often decomposed in the early stages of plant decomposition (Xia & Wander, 2021; Bell et al., 2019; Stege et al., 2010; Lv et al., 2023). Peroxidase (PER) enzymes depolymerize lignin, which is highly chemically recalcitrant and gives plants their rigid structure (Sinsabaugh, 2010; Lebo et al., 2002; Burns et al., 2013; Sinsabaugh and Shah, 2011). While these enzymes are typically associated with C acquisition, lignocellulose decomposition may also be favored in N-limited environments, as soil microbes could plausibly depolymerize lignin to acquire N rich lignin-bound proteins (Rinkes et al.; Craine et al., 2007; Moorhead and Sinsabaugh, 2006; Dyckmans et al., 2002). The combination of decomposed plant material and microbial residues plays a vital role in the formation of stable SOM (Cotrufo et al., 2012; Kästner & Miltner, 2018; Bradford et al., 2013). Microbial respiration and permanganate oxidizable C (POXC) are commonly used to assess SOM formation as they measure labile C which is closely associated to nutrient cycling and C accrual and are sensitive to land management changes (Culman et al., 2012; Hurisso et al., 2016).

Recent frameworks conceptualize SOM as two functionally distinct fractions: particulate organic matter (POM) and mineral-associated organic matter (MAOM). POM is defined by undecomposed plant-derived fragments > 53 µm, no general protection mechanism, a C:N of 10-40, and a cycling time of years to decades. MAOM is defined by highly processed, low molecular weight compounds < 53 µm, protection by sorption to mineral surfaces, a C:N of 8-13, and a cycling time of decades to centuries (Lavallee et al., 2020). POM material is degraded and assimilated into microbial biomass, resulting in microbial turnover, and the resulting decomposition products, in addition to root exudates <53 µm, sorb with minerals in the soil, producing MAOM (Sokol et al., 2019; Cotrufo et al., 2013).

The bioproduct crops switchgrass (*Panicum virgatum* L.) and willow (*Salix* spp.) are particularly promising for production on marginal lands due to their high water and nutrient use efficiencies, robust root systems, and ability to produce viable yields in marginal soil (Scalgline-Mellor et al., 2018; Marra et al., 2013; Kuzovkina and Volk, 2009). Switchgrass production has resulted in accumulation of soil C, maintenance of soil N, and increased microbial diversity, biomass, and activities (Liebig et al., 2005; Dirks & Jackson, 2020; Jesus et al., 2016; Li et al., 2022). Willow production boasts some of the same benefits and has generally been used to control soil erosion and N leaching (Volk et al., 2006; Zumpf et al., 2021; Ferrarini et al., 2017).

One underexplored management practice that has potential to maximize the fertility and C sequestration capacity of soil is the application of biochar (Fike et al., 2017; Adegbidi et al., 2003). Biochar is a pyrolyzed organic material with unique features like high C content, cation exchange capacity, large surface area, and the promotion of the stabilization of plant C inputs and microbial products (Lehmann & Jospeh, 2015; Wang & Wang, 2019; Joseph et al., 2021). Biochar can increase microbial activity while decreasing the mineralization of native SOM by providing a source of readily available C (Zhang et la., 2018; Wang et al., 2016; Whitman et al., 2014; Pokharel et al., 2020). While biochar may be a promising soil amendment to maintain or even enhance soil C, its promise in enhancing soil biological parameters related to soil chemical and biological properties in bioproduct cropping systems remains underexplored.

There is a gap in understanding related to the responses of soil microbial and chemical processes that govern SOM formation to the production of switchgrass and willow in the marginal lands of the Appalachian region and if biochar application helps enhance these processes. To address this, experimental plots of switchgrass and willow were established on reclaimed mined land as well as non-mine impacted marginal land in West Virginia, U.S.A and biochar was applied to a sub-set of plots at rates common to production systems in the region (Hass et al., 2012). Across the first two growing seasons, we assessed biological and chemical parameters that are associated with SOM formation, namely, permanganate oxidizable carbon (POXC), microbial respiration, PER and BG enzyme activities, and POM and MAOM (Amat et al., 2020; Culman et al., 2012). We hypothesize that switchgrass and willow production over two growing seasons on marginal lands will support C accumulation through 1) increased microbial respiration and POXC 2) greater microbial investment in extracellular enzymes associated with C decomposition, and 3) increased POM with no change in MAOM. Further, we hypothesized that 4) biochar amendments would increase microbial activity, 5) there would not be significant changes to measured soil properties compared to previous land use, and 6) the parameters measured would be associated with MAOM weight.

2. Materials and Methods

2.1 Site Description and Experimental Design

Experimental plots were established in the spring of 2021 at six sites, three former mine sites and three marginal agriculture sites, across West Virginia, U.S.A. The reclaimed mine sites were named Allstar Mine 1 (39°03’46” N 80°17’52” W), Allstar Mine 2 (39°03’17” N 80°18’08” W), and Goshen Road (39°31’50” N 79°58’45” W). All the former mine sites were reclaimed in a similar fashion and seeded with tall fescue (*Lolium arundinaceum* (Schreb.) S.J. Darbyshire), orchardgrass (*Dactylis glomerata* L.), birdsfoot trefoil (*Lotus corniculatus* L.), and clovers (*Trifolium* spp.; Scagline-Mellor et al., 2018). The non-mine impacted marginal agriculture sites were at Jackson’s Mill (39°05’31” N 80°28’30” W), Reedsville Farm (39°30’18” N 79°48’31” W), and Agronomy Farm (39°39’30” N 79°54’12” W). The Reedsville and Agronomy farms are part of the West Virginia University Farms System and Jackson’s Mill is owned by West Virginia University Extension. More details about site locations, previous land use, and soil characteristics can be found in Table 1.

Table 1 Summary of Site Location and Characteristics

Site	Location	Previous Land Use	Texture	pH	Nutrient Limitation
Mine Sites					
AllStar 1	Jane Lew	Pasture	Silt loam	5.3	Low P
AllStar 2	Jane Lew	Pasture	Silt loam	6.6	Low P
Goshen Road	Morgantown	Fallow field	Silt loam	5.5	Low P
Agriculture Sites					
Agronomy Farm	Morgantown	Hayfield	Silt loam	6.2	Low P
Reedsville Farm	Reedsville	Hayfield	Silt loam	6.0	Low K
Jackson's Mill	Weston	Pasture	Silt loam	No data	No data

Land Use Definitions
Pasture: Land covered with grass and other low laying plants suitable for grazing animals.
Hayfield: Land covered with taller grasses and plants that are grown for harvesting purposes.
Fallow field: Arable land not under crop rotation.

At all sites, existing vegetation was killed by the application of glyphosate at 4.68 L ha^{-1} in the early spring 2020. P and K deficiencies were reconciled through fertilizer amendments according soil test results from the West Virginia University Soil Test Lab. At each site, 24 plots (18 for Allstar Mine 2 site due to space limitations) were created, upon which crops of two bioproduct feedstocks (that is, hybrid willow [*Panicum virgatum* L.] and switchgrass [*Salix* spp.]) were established. Willow and switchgrass planting occurred in late May and early June 2021. Prior to planting, the soil was prepared by chisel plowing to a depth of 25.4-30.5 cm followed by disking. Hybrid willow clones (Preble cultivar; 25.4 cm cuttings) were planted in 16 x 15.8 m plots at 61 x 76 cm spacing using double row plantings with 1.5 m between the double rows. To manage the growth of undesired plant species, two pre-emergent herbicides (Oxyfluorfen and pendimethalin) were applied over the cuttings at a rate of 0.44 L ha^{-1}. Additional weed management included the use of Assure II plus surfactant at 0.73 L ha^{-1} for grass competition and Transline at 0.85 L ha^{-1} for broadleaf competition. Persistent and tolerant weeds were also controlled by rototilling and mowing during the first growing season. Switchgrass (BoMaster cultivar) was planted using a no-till seed drill at a rate of 5.6 kg of seed ha^{-1} in 12.1 x 15.8 m plots. Triclopyr was applied for broadleaf weed management. Persistent and tolerant weeds were controlled by mowing during the first growing season. Both willow and switchgrass establishment practices were similar to those used in production systems in the region.

The experimental design consisted of the aforementioned plots arranged in a randomized split plot design with crop type (willow or switchgrass) and soil amendment (i.e., biochar and an unamended control) as treatments. Plots at each site were split evenly for the crop treatment and contained four replicates for the biochar treatment and eight unamended replicates as a control (Allstar Mine 2 contained six replicates for the control treatment and three replicates for the biochar treatment). The biochar amendment was produced from softwood residues and supplied by Pacific Biochar (see Table 2 for biochar characteristics). Plots with the biochar amendment received an application of 11209 kg ha^{-1} which was incorporated via disking prior to planting.

Table 2 Proximate Analysis of Biochar

Parameter	As Received	Dy Weight Basis
Moisture, % [A]	75.1	0.0
Bulk Density, g/cc [A]	0.47	0.12
Carbon, % [B]	22.2	89.2
Hydrogen, % [B]	0.4	1.6
Nitrogen, % [B]	0.2	0.9
Oxygen, % [Calc.]	0.6	2.3
Ash, % [A]	1.5	6.0
Volatile Matter, % [A]	5.8	23.2
Butane Activity, g/100 g [C]	2.7	10.7
Surface Area Correlation, m^2/g [E]	118.2	474.1
Organic Carbon, % [Calc.]	22.2	88.8
Hydrogen/Organic Carbon [Calc.]	0.22	0.22
Carbonates (as $CaCO_3$), % [D]	0.81	3.25

Test Method
A: ASTM D 1762-84; B: Dry Combustion; C: ASTM D 5742-95; D: ASTM 4373; E: Butane Activity Surface Area Correlation Based on McLaughling et al., (2012); Calc.: Calculated using the dry weight results of other parameters.

2.2 Soil Sampling

To determine the soil chemical and biological consequences of initial bioproduct crop establishment on previously mined and marginal agriculture land in Appalachia, soil samples were collected in June 2021 after site preparation but before crop establishment and again during May (i.e., early growing season) and August (i.e., late growing season) 2022. For establishment year sampling, soil samples were obtained by digging with a trowel to 10 cm depth at five random locations per plot. For the year 1 samples, soil samples were similarly collected within 10 cm of a growing willow or switchgrass. Off-plot samples were also collected during the May 2022 sampling period for comparison to the previous land use. After sampling, the soil was homogenized, and a subsample placed in a Ziploc bag and stored on ice for transportation to the laboratory. Upon return to the laboratory, aliquots of the homogenized sample were stored at i) 4 °C for assessments of microbial reparation, ii) -80 °C for enzyme activity, and iii) airdried for chemical analyses.

2.3 Permanganate-Oxidizable Carbon (POXC)

To determine the effect of crop growth and biochar amendment on putatively bioavailable carbon, permanganate-oxidizable carbon (POXC) was estimated following Culman et al. (2012) and Weil et al. (2003). Briefly, air dried soil was ground to a fine powder using a Retsch MM 400 ball mill (Haan, Germany; 30 frequencies s^{-1} for 1 minute). Two and a half grams of the pulverized soil was aliquoted into three separate 50 ml polypropylene screw-top centrifuge tubes per sample. Eighteen milliliters of deionized water and 2 ml of 0.2 *M* $KMnO_4$ stock solution were added to each tube. The samples were then shaken on an oscillating shaker table (Eppendorf New Brunswick Scientific Excella E10; Hamburg, Germany) for 2 minutes at 240 oscillations $minute^{-1}$ at which point the tubes were left to settle in a dark cabinet for 10 minutes. After settling, 0.5 ml of the supernatant was transferred into another 50 ml centrifuge tube containing 49.5 ml of deionized water and mixed. An aliquot (300 µl) of each tube was transferred into a clear 96-well plate containing three deionized water blanks, standard stock solutions (0.005, 0.01, 0.015 and 0.02 *M* $KMnO_4$), as well as soil-free control samples.

The plates were read using a microplate reader (BMG Labtech FLUOstar Omega; Berlin, Germany) by measuring absorbance at 550 nm. The raw sample readings were corrected using the blanks and a standard curve was constructed to obtain a regression line for calculating POXC values which were expressed in units of mg POXC kg^{-1} soil.

2.4 Microbial Respiration

To determine how microbial activity responded to the growth of bioproduct crops and biochar application, microbial respiration was measured based on the methods from Franzluebbers et al. (1996) and Grandy et al. (2013). Twenty grams of air-dried soil were placed in a 0.473 L mason jar and re-wetted to 50% water holding capacity. Deionized water was added in a circular motion to minimize the breakdown of aggregate structure and to prevent splashing (Grandy & Robertson 2007). After the samples were re-wetted, the jars were sealed with lids containing a rubber septum. The samples were pre-incubated in a dark cabinet at 20 °C for 7 days. After pre-incubation, each jar was uncapped under a fume hood for 5-10 minutes to allow the headspace in the jar to equilibrate with the ambient air. Additional deionized water was added to keep moisture levels equal after initial re-wetting (Grandy et al., 2013). The jars were sealed and stored again, and headspace gas was sampled after 24, 48, and 72 hours. To sample CO_2 in the jars, a 10 ml sample of headspace was injected into an EGM-5 infrared gas analyzer (IRGA; PP Systems; MA, U.S.A; Grandy & Robertson 2007; Wade et al., 2018). Between each measuring period, the jars were uncapped underneath a fume hood to allow the headspace in the jar to equilibrate with the ambient air. CO_2 respired was expressed as mg CO_2-C kg soil^{-1} h^{-1}.

2.5 Extracellular Enzyme Activity

To determine the effects of switchgrass and willow production and biochar application on the activity of extracellular enzymes associated with C acquisition, β-Glucosidase (BG; EC 3.2.1.21) and Peroxidase (PER; EC 1.11.1.7) activities were measured using methods from Saiya-Cork et al. (2002). Two grams of soil for BG or 1 g for PER was combined with 125 ml of 50 *mM* sodium acetate buffer (pH 5) and homogenized for 1 min using a biohomogenizer (BioSpec Products; OK, U.S.A). The substrate used for the BG assay was 4-Methylumbelliferyl β-D-glucopyranoside and for the PER assay was L-dihydroxyphenylalanine (DOPA). The PER plates received an additional 10 µl 0.3% H_2O_2 in each well. The microplates were incubated in a dark cabinet at 20 °C for 5 and 18 hours for BG and PER assays, respectively (DeForest et al., 2004).

The plates were measured using a microplate reader (BMG Labtech FLUOstar Omega). The BG sample reactions were terminated by adding 10 µl of 1 *M* NaOH to each well prior to measuring in the plate reader. BG was measured using fluorescence with 355 nm excitation and 460 nm emission filters. After correcting for negative controls and quenching, activities were expressed in units of nmol h^{-1} g^{-1}. PER activity were quantified by measuring absorbance at 460 nm and expressed in units of nmol h^{-1} g^{-1} after correcting for negative controls (Saiya-Cork et al., 2002).

2.6 SOM Fractionation

To determine the effects of bioproduct crops and biochar application on functionally distinct soil organic matter (SOM) pools, SOM was fractionated to mineral-associated organic matter (MAOM; slower turnover) and particulate organic matter (POM; faster turnover) following Bradford et al. (2008). Prior to SOM fractionation, soil samples were air dried and passed through a 2 mm sieve. Ten grams of sieved soil was then added to an Erlenmeyer flask with 30 ml of sodium hexametaphosphate (NaHMP; 5%) and shaken on an oscillating shaker table (Eppendorf New Brunswick Scientific Excella E10) for 18 hours at 130 oscillations minute^{-1} (Eclesia et al., 2012). The dispersed soil was then passed through a 53 µm sieve using a Fritsch Analysette 3 (Idar-Oberstein, Germany), which sprayed approximately 400 ml of deionized water and shook at 0.8 oscillations s^{-1}. The material that passed through the sieve was operationally defined as MAOM and the material that was retained was operationally defined as POM (Paul et al., 2001). The samples were then dried to a constant weight at 55 °C and ball-milled to a fine powder using a Retsch MM 400 ball mill (30 frequencies s^{-1} for 1 minute) for combustion analysis to measure total C and N of each fraction on a Thermo Fisher Scientific FlashEA 1112 Flash Combustion Analyzer (MA, U.S.A).

2.7 Statistical Analysis

To test our hypotheses, multi-factor ANOVA tests were used with land type (mine or agriculture), date (June 2021, May 2022, and August 2022), treatment (biochar or control), and crop (switchgrass or willow) as independent variables to test the variance of POXC, microbial respiration, microbial enzyme activity, and SOM fraction mass and C:N (R Core Team, 2022). To compare the soil chemical and biological properties of soil under switchgrass and willow production after a year of growth compared to previous land use, multi-factor ANOVA tests were used with crop (switchgrass, willow, and pasture) as the independent variable to test the variance of measured soil chemical and biological properties within mine and agriculture sites. Normal distribution was tested using the Shapiro-Wilk test (Kassambara, 2023). Data that did not fit a normal distribution was power transformed. Homogeneity of variance was tested using Levene’s Test (Kassambara, 2023). Means were compared using Tukey’s Honest Significant Difference (HSD) with a statistical significance accepted at $\alpha < 0.05$ and marginal significance was considered at $0.05 < \alpha < 0.10$ (de Mendiburu, 2021). The Pearson coefficient of correlation was calculated using data from the experiment. Linear regression analysis was used to quantify the explanatory variables of measured data on MAOM weight (R Core Team, 2022).

3. Results

Table 3 p-Values of ANOVA Analysis per Parameter

Effect	POXC	Microbial Respiration	BG	PER	POM Mass	MAOM Mass	POM C:N	MAOM C:N
Land Type	**0.02**	**<0.01**	**0.03**	**0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
Date	**0.01**	**<0.01**	**<0.01**	0.05^	0.71	0.42	0.42	**<0.01**
Treatment	0.82	0.08^	0.22	0.37	0.91	0.65	**<0.01**	**<0.01**
Crop	0.65	**<0.01**	**<0.01**	0.74	0.07^	0.17	0.97	0.13
Land Type x Date	**<0.01**	<0.01	**<0.01**	0.20	**0.04**	**<0.01**	0.06^	0.84
Land Type x Treatment	0.315	0.89	0.68	0.51	0.38	0.21	**0.03**	0.16
Date x Treatment	0.71	0.51	**0.04**	0.56	0.56	0.70	0.29	0.85
Land Type x Crop	0.64	0.90	**0.02**	0.61	0.17	0.72	0.94	0.55
Date x Crop	0.96	0.43	0.38	0.89	0.24	0.98	0.14	0.73
Treatment x Crop	0.39	0.64	0.56	0.77	0.99	0.65	0.88	0.83
Land Type x Date x Treatment	0.48	0.78	0.60	0.11	0.94	0.89	0.79	0.66
Land Type x Date x Crop	0.06^	0.50	**0.01**	0.64	0.68	0.75	0.22	0.92
Land Type x Treatment x Crop	0.46	0.27	0.24	0.34	0.28	0.28	0.11	0.25
Date x Treatment x Crop	0.15	0.88	0.63	0.24	0.43	0.51	0.15	0.51
Land Type x Date x Treatment x Crop	0.46	0.80	0.41	0.41	0.64	0.75	0.18	0.97

Bolded items are statistically significant ($\alpha < 0.05$)
^ Marginally significant ($0.05 > \alpha < 0.10$)

3.1 Permanganate Oxidizable C and Microbial C Mineralization

Permanganate Oxidizable C (POXC) decreased across the two growing seasons in the agriculture sites, with a 16% decrease from the first to third sampling date, whereas there was a marginal increase (14%; $p = 0.06$) during the second growing season in the mine sites (date x land type; $p < 0.05$; Figure 1a). POXC concentrations at each site were similar until the third sampling point where the mine sites exhibited a 20% higher POXC concentration than the agriculture sites (date x land type; $p < 0.05$; Figure 1a). Neither biochar application nor crop type altered POXC concentrations across both marginal agriculture and mine sites (Supplemental Figure S1).

Microbial respiration increased across two growing seasons by 422% and 251% in the mine and agriculture sites, respectively (date x land type; $p < 0.01$; Figure 1b). In comparing respiration trajectories of both land types, microbial respiration was similar in both land types for the first sampling period, whereas mine sites exhibited ~28% higher respiration than the agriculture sites in both the second and third sampling period (date x land type; $p < 0.01$; Figure 1b). The biochar amendment supported marginally greater microbial respiration, with a 5% increase compared to the control treatment (1.25 ± 0.07 vs. 1.16 ± 0.05, respectively; treatment main effect; $p = 0.08$). Across crop types, switchgrass production led to 13% greater microbial respiration as compared to willow across the two growing seasons (crop main effect; $p < 0.01$; Supplemental Figure S1).

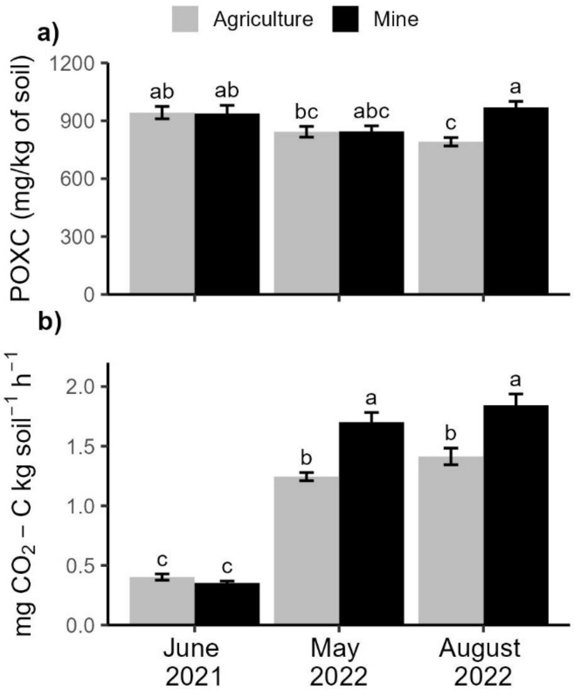


Figure 1. The effect of time and land type on (a) permanganate oxidizable carbon (POXC) and (b) microbial respiration. Means and standard error are presented (N=4) and different letters indicate significant difference ($\alpha < 0.05$; n = 406 for POXC and n =380 for microbial respiration).

3.2 Extracellular Enzyme Activity

The activity potential of two microbial enzymes associated with SOM decay exhibited opposite trends with time. In the agriculture sites, BG activity decreased by ~30% for both switchgrass and willow across the two growing seasons (land type x crop x date; $p < 0.01$; Figure 2a). In the mine sites, switchgrass production led to a 19% decrease in BG activity (crop main effect; $p < 0.05$) whereas BG activity was unchanged throughout the two growing seasons by willow production. Both treatments decreased throughout two growing seasons, with a 31% and 19% decrease in BG activity from the first to third sampling period for biochar and control plots, respectively ($p < 0.01$; Supplemental Figure S3). Peroxidase (PER) activity exhibited a 28% increase across the two growing seasons (date main effect; p

< 0.05; Figure 2b). The mine sites supported 23% greater PER activity compared to the agriculture sites (land type main effect; $p < 0.05$; Figure 2c). Neither the biochar treatment nor crop type affected PER activity.

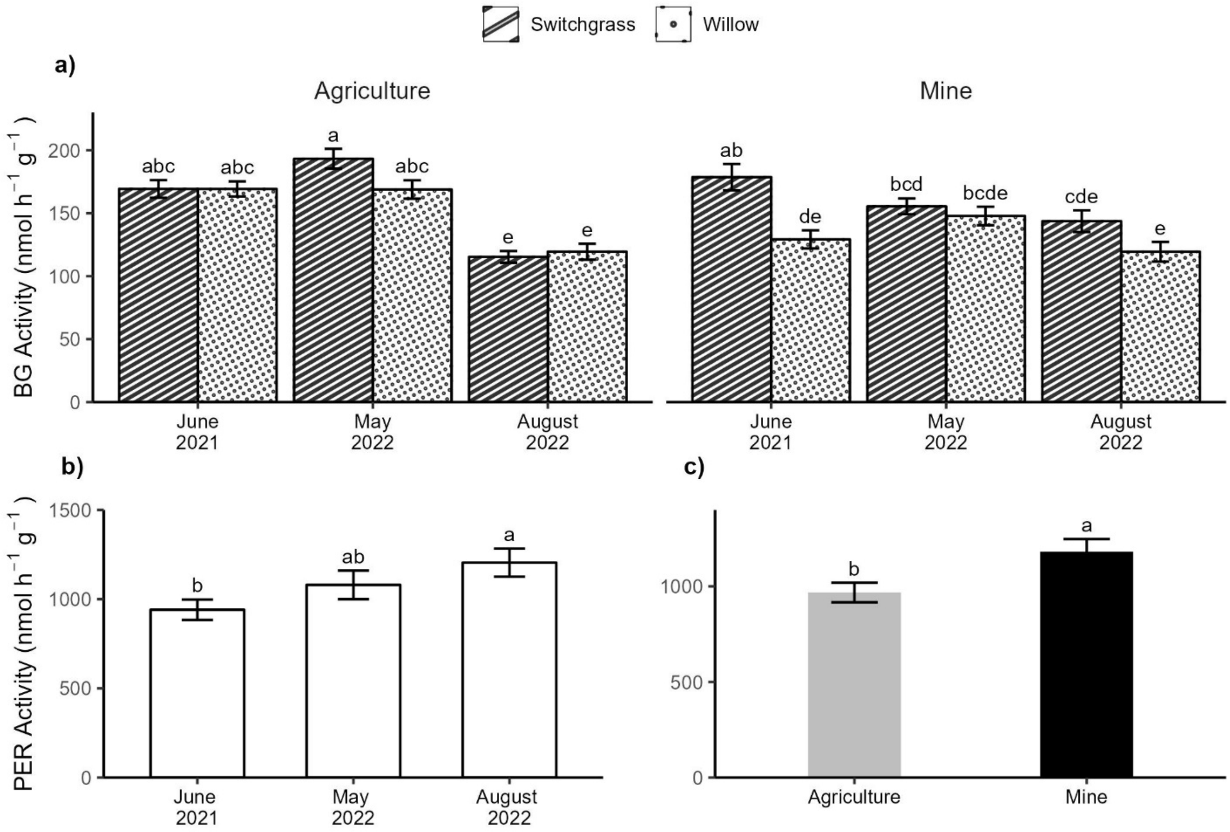


Figure 2 The effect of time, crop type and lane use on soil microbial enzymes associated with the decay of organic matter, including β-Glucosidase (BG; panel a) and peroxidase (PER; panels b-c). Means and standard error are presented (n = 395 for BG and n = 325 for PER) with different letters indicating a significant difference ($\alpha < 0.05$).

3.3 POM and MAOM

There were no significant changes over time for the POM weights. POM in the agriculture sites weighed 42% and 52% less per unit mass soil than the mine sites in the first and second growing seasons, respectively (land type x date; $p < 0.05$; Figure 3a). There were no significant differences in POM weights between the biochar amended samples and control. Switchgrass production resulted in a marginally significant 4% increase in POM weights per unit mass soil as compared to willow samples (crop main effect; $p = 0.07$; data not shown).

MAOM weight per unit mass soil decreased by 4% between the first growing season and the second growing season for the mine sites, with no significant change for the agriculture sites (land type x date; $p < 0.01$; Figure 3b). MAOM in the agriculture sites weighed 15% and 24% more per unit mass soil than the mine sites in the first and second growing seasons, respectively (land type x date; $p < 0.05$; Figure 3b). There were no significant differences between the biochar amended samples and control and the switchgrass and willow samples.

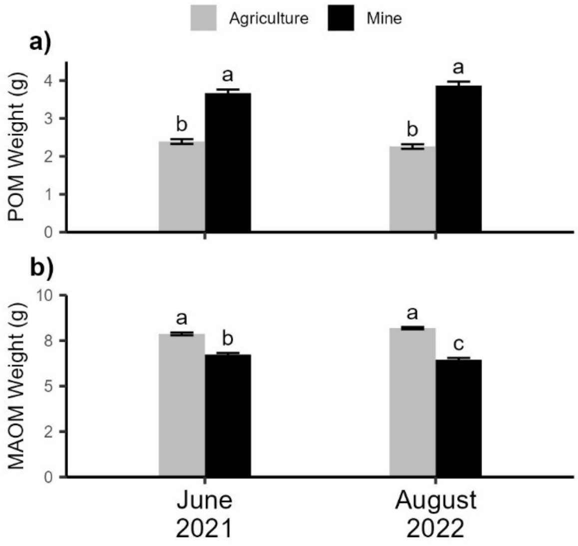


Figure 3 Weights of functionally distinct soil organic matter fractions including (a) particulate organic matter (POM) and (b) mineral associated organic matter (MAOM) differed by land use type and were generally consistent after one year of crop production. Means and standard error (n = 253 for POM and n = 264 for MAOM) are presented and different letters indicate a significant difference ($\alpha < 0.05$).

There were no significant changes over time for the POM C:N. POM C:N in the mine sites was 14% higher than the agriculture sites in the first growing season but was more similar in the second growing season (land type x date; $p = 0.06$; Table 4). POM C:N was 35% and 24% higher in the biochar treatment compared to the control in the agriculture and mine sites, respectively (land type x treatment; $p < 0.05$; Table 4). Both switchgrass and willow production led to similar POM C:N.

MAOM C:N decreased by 6% from the first to the second growing season (date main effect; $p < 0.01$; Table 4). MAOM C:N was also 7% higher in the mine sites than in the agriculture sites (land type main effect; $p < 0.01$; Table 4). MAOM C:N was 37% higher in the biochar samples compared to the control samples (treatment main effect; $p < 0.01$; Table 4). Both switchgrass and willow production led to similar MAOM C:N.

Table 4 ANOVA Summary of POM and MAOM C:N

	POM C:N		MAOM C:N	
Effect	Significance	Mean values	Significance	Mean values
Land type	<0.01		<0.01	
Agriculture		17.21^{a} (0.37)		9.76^{a} ǂ (0.11)
Mine		19.12^{b} (0.41)		10.43^{b} (0.08)
Treatment	<0.01		<0.01	
Biochar		21.86^{a} (0.48)		10.84^{a} (0.13)
Control		16.22^{b} (0.24)		9.71^{b} (0.07)
Date	NS§ (0.42)		<0.01	
June 2021		18.32 (0.39)		10.40^{a} (0.10)
August 2022		17.92 (0.41)		9.78^{b} (0.09)
Land type x treatment	0.03		NS	
Mine				
Biochar		22.30^{a} (0.74)		11.37 (0.20)
Control		17.50^{b} (0.39)		9.94 (0.10)
Agriculture				
Biochar		21.47^{a} (0.64)		10.31 (0.14)
Control		15.06^{c} (0.23)		9.50 (0.08)
Land type x date	0.06		NS	
Mine				
June 2021		19.60^{a} (0.52)		10.76 (0.15)
August 2022		18.62^{ab} (0.64)		10.11 (0.16)
Agriculture				
June 2021		17.32^{bc} (0.51)		10.06 (0.12)
August 2022		17.11^{c} (0.54)		9.47 (0.09)

± Mean contrasts were based on box-cox transformed data.

ǂ Values are means with SE in parentheses. Different letters for average values for effects are significant.

§ Not significant.

Table 5 Summary of Parameter Means per Crop at Each Land Type

Parameter	Mine			Agriculture		
	Switchgrass	Willow	Pasture	Switchgrass	Willow	Pasture
POXC (mg/kg of soil)	815^a (42)	873^a (37)	843^a (57)	871^a (43)	813^a (33)	867^a (96)
Microbial Respiration (mg CO_2-C kg soil^{-1} h^{-1})	1.69^a (0.08)	1.71^a (0.14)	1.86^a (0.16)	1.34^a (0.05)	1.15^b (0.04)	1.52^a (0.11)
BG (nmol h^{-1} g^{-1})	155^a (6)	148^a (7)	200^b (19)	193^a (8)	169^b (7)	170^{ab} (12)
PER (nmol h^{-1} g^{-1})	1224^a (135)	1398^a (229)	No Data	838^a (109)	869^a (126)	1118^a (855)
POM Weight (g)	3.81^a (0.12)	3.80^a (0.10)	4.20^a (0.31)	2.43^a (0.07)	2.27^a (0.06)	2.47^a (0.15)
MAOM Weight (g)	6.50^a (0.12)	6.54^a (0.09)	6.27^a (0.24)	7.93^a (0.08)	8.01^a (0.08)	8.12^a (0.05)
MAOM C:N	10.41^a (0.16)	11.92^a (1.37)	9.14^b (0.14)	9.66^a (0.11)	9.81^a (0.13)	8.61^b (0.05)
POM C:N	19.67^a (0.62)	19.96^a (0.76)	14.58^b (0.43)	17.49^a (0.72)	17.74^a (0.66)	12.93^b (1.16)

± Mean contrasts were based on transformed data if data was not evenly distributed and are separated by land type.

ǂ Values are means with SE in parentheses. Different letters for average values for effects are significant.

3.4 Comparison to Prior Land Use

In comparing the previously established pasture to newly established switchgrass and willow systems after two growing seasons, in the mine sites, there was relatively reduced BG activity (-25% and -30%, in switchgrass and willow, respectively) and a greater C:N of both POM (+30% and +31%) and MAOM (+13% and +26%). In the agriculture sites, there was increased POM C:N (+30% and 31%, in switchgrass and willow, respectively) and MAOM C:N (+12% and 13%) compared to the nearby pasture (Table 5).

3.5 Correlation and Regression Analysis

To determine the associations between the soil chemical and biological properties observed in this study, correlation analyses were performed. Robust and significant correlations of $r > 0.7$ were observed between MAOM C:N and POM C:N ($r = 0.75$) as well as MAOM weight and POM weight ($r = 0.94$). POXC exhibited significant but lower strength correlations with microbial respiration ($r = 0.34$), BG activity ($r = 0.43$), MAOM weight ($r = 0.45$), and POM weight ($r = 0.44$). Microbial respiration was associated with BG activity ($r = 0.39$), MAOM weight ($r = 0.49$), MAOM C:N ($r = 0.23$), and POM weight ($r = 0.53$). A summary of the results is found in Supplemental Figure S4.

As MAOM is a primary indicator of stable soil C, a linear regression model with MAOM weight as the dependent variable and POXC, BG, PER, MAOM C:N, and POM C:N as independent predictor variables was produced and resulted in an adjusted r^2 value of 0.40 with POXC, microbial respiration, and MAOM C:N as the strongest predictors. A goodness-of-fit test on the predicted vs. observed values from the linear model resulted in an r^2 of 0.44 and RMSE of 0.12. A summary of the regression statistics is in Supplemental Table S2.

4. Discussion

To better understand the short-term soil chemical and biological responses to switchgrass and willow crop production on marginal agriculture and reclaimed mine land, we assessed soil properties related to soil C cycling over two growing seasons. Permanganate oxidizable C (POXC) varied over the two growing seasons in the mine sites and decreased in the agriculture sites while microbial respiration increased over two growing seasons across both land types, partially supporting Hypothesis 1. There was an overall decrease in β-Glucosidase (BG) and increase in peroxidase (PER) enzyme activities over two growing seasons, partially supporting Hypothesis 2. Hypothesis 3 was not supported, as there were no detectable changes in POM and MAOM weights in the agriculture sites and a decrease in MAOM weight in the mine sites. The biochar treatment did not lead to an observable difference in microbial activity, though biochar application was associated with an increased C:N in both POM and MAOM fractions, partially supporting Hypothesis 4. Similarly, Hypothesis 5 was partially supported as switchgrass and willow production produced equal to or slightly less desirable soil chemical and biological properties than the adjacent pasture. Lastly, Hypothesis 6 was partially supported as microbial respiration, POXC, and MAOM C:N were strongly correlated with MAOM weight.

4.1 Functionally Distinct SOM Fractions are Variable Across Time, Sites, and Treatments

The production of switchgrass and willow across two growing seasons decreased the weight of the MAOM fraction in the mine sites, which is potentially indicative of the utilization of MAOM-bound N to satisfy the N demand of these sites. While MAOM is generally considered to be a temporally stable SOM fraction, recent evidence indicates that it may also be more dynamic than previously thought and may represent an important source of organic N in soil (Gentsch et al., 2015; Torn et al., 2013; Jilling et a., 2021). The mechanisms that drive MAOM destabilization are likely a result of interactions with organic acids in root exudates as well as with microbial enzymes (Li et al., 2021; Jilling et al., 2021). MAOM may store a majority of SOM-N (80% in grasslands) and generally exhibits low C:N of ~9:1, as we see here (Jilling et al., 2020; Giannetta et al., 2018; Denef et a., 2013). While switchgrass and willow can produce high yields in N deficient systems, N is often the most limiting nutrient for these crops during the establishment phase, especially on marginal lands (Liu et al., 2022; Berlin et al., 2014). Under low N conditions, enzymes produced by mineral-associated microbes within proximity to N substrates can be effective at accessing it (Jilling et al., 2021). Furthermore, switchgrass root exudate chemistry is strongly dependent on N availability, with a greater abundance of organic acids (which can directly destabilize MAOM) under low N conditions (Smercina et al., 2020). Taken together, it is likely that due to plant and microbial efforts to alleviate N limitation, MAOM was destabilized to release MAOM-bound N. In the agriculture sites, while there was no significant difference in the MAOM weight over time, there was a trend towards increased MAOM, putative of stable C accumulation in the future.

MAOM C:N decreased over the two growing seasons in both the mine and agriculture sites, which is potentially indicative of high microbial turnover. MAOM can support relatively high rates of microbial enzyme activity, growth, and respiration as compared to POM (Kandeler et al., 1999; Kandeler et al., 2019). Increased microbial activity and the resulting production of dead microbial biomass (i.e., necromass) is a potential explanation for the observed decrease in C:N since bacterial C:N are typically 5-7 which is substantially lower than the initial MAOM C:N in these sites of ~11 (Madigan & Martinko, 2006).

Biochar amendments led to increased C:N in both the MAOM and POM fractions, likely a result of the high C:N of biochar (>100; Dong et al., 2016; Plaza et al., 2016; El-Naggar et al., 2018). Biochar application can result in a 4-fold increase in soil dissolved organic carbon (DOC; Jones et al., 2011; Mukherjee & Zimmerman, 2013), which can interact with minerals thus has the potential to increase MAOM C:N (Kaiser & Kalbitz, 2012; Malik & Gleixner, 2013; Zhuiykov 2018). Biochar application can also increase N limitation in systems, likely contributing to the high C:N ratio in POM fractions as POM-N may be preferentially mineralized under these conditions (Chen et al., 2022; Gao et al., 2019; Luce et al., 2016). POM C:N in the mine soil was higher compared to the agriculture soil, likely due to greater C in the mine soils from leftover coal from previous mining activities and potential N limitation in the system (Ganjegunte et al., 2009; Rumpel & Kögel-Knabner, 2002).

Mine sites exhibited relatively lower MAOM and higher POM weights than the agriculture sites. This is likely a result of the time since reclamation (<50 years) as microbial communities inhabiting these soils have had less time to depolymerize complex compounds in POM and turnover themselves, a process which ultimately produces MAOM (Midwood et al., 2021). MAOM weight and soil age are highly associated and the mean age of C in MAOM is 100-500+ years (Su et al., 2022; Pierson et al., 2021). There is evidence that low N systems support relatively more POM than MAOM possibly due to lower microbial nutrient use efficiency under low nutrient conditions that would normally support POM decomposition towards MAOM in more nutrient rich systems (Cotrufo & Lavallee, 2022; Manzoni et al., 2012). This is supported by the higher POM C:N in the mine sites, which would likely reduce its substrate use efficiency (Cotrufo, 2012). Additionally, microbial carbon use efficiency is reduced as the degree of soil degradation increases (Kane et al., *In Review*), further supporting the observations of higher POM and potential N limitation in the mine systems.

4.2 Microbial Respiration and POXC are Variable Across Time, Sites, and Crops

POXC and microbial respiration are both indicators of putatively microbially-accessible C (Culman et al., 2012; Hurisso et al., 2016; Burke et al., 2019), and may help illuminate the early plant-soil-microbial C dynamics in these systems. Here, microbial respiration increased throughout two growing seasons at both land types, suggestive of increased microbial activity and demand for C. POXC decreased throughout the two growing seasons in the agriculture sites and marginally increased in the mine sites during the second growing season, potentially indicating variability of the balance between C inputs and microbial C demand between the two systems (Xia & Wander, 2021; Culman et al., 2012; O'Neill et al., 2020). However, there is conflicting evidence regarding the nature of POXC which renders the consideration of the relationship between POXC and microbial respiration a challenge. For example, some observations suggest that POXC represents a more stabilized C pool of smaller but relatively heavier C fractions that are more physically protected from microbial decomposition (O'Neill et al., 2020; Culman et al., 2012; Morrow et al., 2016), whereas other evidence suggests that POXC is indicative of an active carbon pool that is readily available for microbial decomposition (Weil et a., 2003; Thoumazeau et al., 2020; Liptzin et al., 2022; Morgan et al., 2020). In a meta-analysis, Hurriso et al. (2016) determined that POXC may be more representative of SOM building while microbial respiration represents the mineralization of organic matter (i.e. a loss of organic matter as CO_2; Liptzin et al., 2022). Therefore, in the agriculture sites it is plausible that the decrease in POXC along with the increase in respiration across two growing seasons indicate that the microbial C decomposition has surpassed the rate of crop net primary production (NPP) and labile C inputs to the soil (Frene et al., 2020; Crowell et al., 2022). In the mine site, the decrease in the first growing season and then increase in the second season of POXC with increased respiration could potentially be indicative of variability in the relationship between plant NPP and microbial C demand. Although, both POXC and respiration can exhibit high intra-lab and intra-year variability, which may also partially explain these results (Wade et al., 2020; Pérez-Guzmán et al., 2021; Maltais-Landry et al., 2021; Diederich et al., 2019). Microorganisms in switchgrass plots respired more CO_2 as compared to in willow plots, which is likely a result of greater root biomass of early-establishment switchgrass crops as compared to that of willow and pastures, which likely contribute easily accessible carbohydrates that contribute to enhanced microbial respiration (Lemus & Lal, 2005; Cunnif et al., 2015; Pugliese et al., 2019; Bhattacharyya et al., 2013). In the long term, it is expected that continued plant growth will support increased POXC and microbial respiration in both the mine and agriculture sites, ultimately resulting in C accumulation (Kane et al., In Review; McLaughlin & Kszos, 2005; Bazrgar et al., 2020).

While we did not measure microbial biomass in this effort, it is often associated with microbial respiration, so the increased respiration over time in this study is likely reflective of an increase in microbial biomass (Franzluebbers et al., 2000; Hurisso et al., 2016; Wade et al., 2018). The resulting turnover of biomass into necromass is a major source of SOM formation (Cotrufo et al., 2012; Miltner et al., 2012; Sokol et al., 2022).

4.3 Shifts in Resource Allocation Through Extracellular Enzyme Activities

The shift in microbial investment from BG to PER across two growing seasons may indicate a shift from a chemically labile to a recalcitrant C economy (Lv et al., 2023; Burns et al., 2013; Berg, 2000; Sinsabaugh and Shah, 2011). Alternatively, the shift in enzyme investment may be due to increased N limitation as BG often decreases with N limitation, while PER activities increase, as we observe here (Sinsabaugh et al., 2002; Burns et al., 2013; Sinsabaugh et al., 2005). Since lignin degradation is energetically expensive and often results in little C and energy yield (Couteaux et al. 1995), the decomposition of lignin could be a strategy to "mine" N when it is deficient in systems (Rinkes et al., 2016; Craine et al., 2007; Moorhead and Sinsabaugh 2006). The greater PER activity in the mine sites as compared to the agriculture sites supports that these systems have a greater degree of N deficiency.

4.4 Towards Maximizing Ecosystem Services of Bioproduct Agroecosystem

In comparing select soil chemical and biological properties after two growing seasons of switchgrass and willow production to that of the previously established pasture, the bioproduct crop system supports similar to perhaps slightly less desirable soil characteristics than the nearby pasture. The most striking difference is the greater POM and MAOM C:N in the switchgrass and willow plots as compared to in the pasture. This is likely attributed to the greater N demand of the bioproduct crops than the pastures to support their relatively greater biomass production (Lemus & Lal, 2005). Further work should determine the N contributions of N-fixing microbial communities, which are active in switchgrass systems (Smercina et al., 2019; Roley et al., 2019) and have been shown to contribute more than 16% of the N demand in other bioproduct crops (Keymer & Kent, 2014).

The initial establishment years of switchgrass and willow systems may not yet support greater C storage than is supported by native succession vegetation (Ma et al., 2000; Tariq et al., 2018; Juyal et al., 2021). However, our research provides evidence that net soil C sequestration may be possible in the early establishment years on especially degraded land such as mine soil. Looking towards stand maturation, which often occurs after 3-4 growing seasons (Skousen et al., 2014; Kopp et al., 2001), our regression results suggest that the stable C pool in the soil (MAOM) will increase if certain soil chemical and biological properties measured here (for example, POXC, microbial respiration, and MAOM C:N) continue to increase as demonstrated in previous research (Ferrarini et al., 2021; Fu et al., 2022; Kantola et al., 2017). It is likely that longer-term bioproduct crop production can result in C sequestration (Blanco-Canqui, 2016; Ma et al., 2000; Rytter et al., 2015) with higher potential in mine soils.

Future work should increase the length of the experiment duration as this study spanned the first two growing seasons of early establishment switchgrass and willow production systems and there are conflicting data about whether early establishment soil changes are predictive of the longer-term trajectory (Ma et al., 2000; McLaughlin & Kszos, 2005; Kahle et al., 2007; Kantola et al., 2017). In addition, to further uncover the plant-soil-microbial dynamics of these systems, continued monitoring through stand maturity and beyond is needed. To better understand soil C dynamics, samples should be collected at depths deeper than 10 cm as >50% of soil carbon may be stored at depths deeper than 20 cm (Harrison et al., 2011; Shahzad et al., 2019). This will be important in the agriculture sites; however, future work should also examine how shallow soil, such as those in the mine sites, interact with soil C processes. Further work would benefit from estimations of other potentially predictive parameters of future accumulation of long-lived soil C like microbial necromass production and carbon use efficiency (Kane et al., 2022). Lastly, the focus of this effort was on the soil C consequences of initial switchgrass and willow production, though future efforts to incorporate plant traits like yield, root biomass, and tissue C:N will enhance a holistic understanding of the effect of switchgrass and willow production on reclaimed mined and marginal agriculture soil in Appalachia.

Conclusions

After two growing seasons, the production of switchgrass and willow on marginal agriculture and reclaimed mine land in Appalachia stimulated greater C demand and potential C accumulation in the mine sites, as well as showed signs of C sequestration in the future in both the mine and marginal agriculture sites. There are indicators of N deficiency in these systems, which will require reconciliation to maximize the ecosystem services of bioproduct crop production. Our results support the idea that heavily degraded lands are poised to benefit from the growth of bioproduct crops during early establishment, while it may take several years to see benefits in less degraded soil.

Appendix
Supplemental Material

Supplemental Table 1 Correlation Coefficients

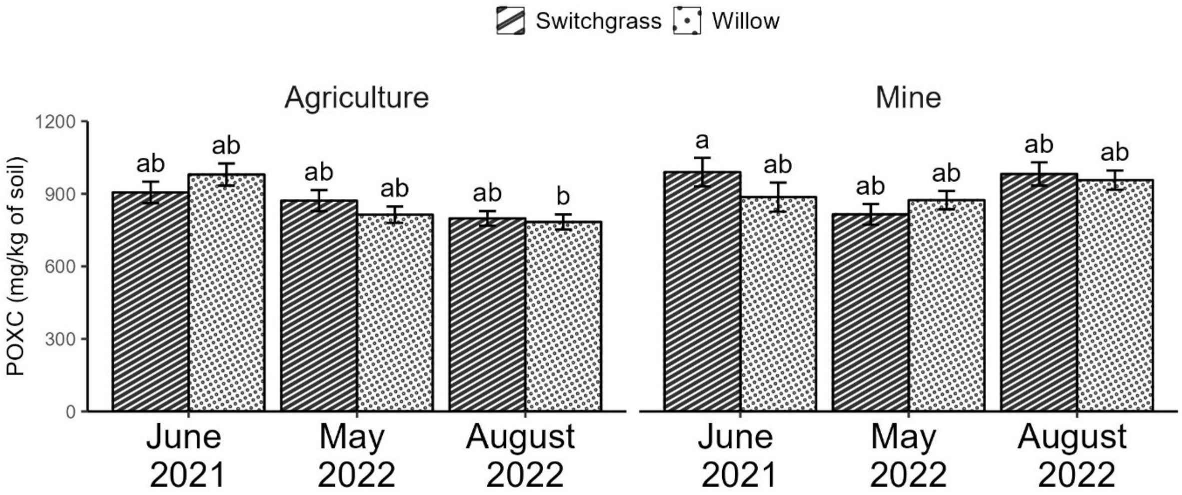
	POXC	Microbial Respiration	BG	PER	POM Weight	MAOM Weight	POM C:N	MAOM C:N
POXC	1.0	0.34	0.43	< -0.01	0.44	0.45	-0.04	0.05
Microbial Respiration	0.34	1.0	0.39	0.03	0.53	0.49	0.02	0.23
BG	0.43	0.39	1.0	0.11	0.17	0.15	0.11	0.02
PER	< -0.01	0.03	0.11	1.0	-0.11	-0.11	0.04	-0.13
POM Weight	0.44	0.53	0.17	-0.11	1.0	0.94	0.19	0.43
MAOM Weight	0.45	0.49	0.15	-0.11	0.94	1.0	0.16	0.39
POM C:N	-0.04	0.02	0.11	0.04	0.19	0.16	1.0	0.75
MAOM C:N	0.05	0.23	0.02	-0.13	0.43	0.39	0.75	1.0

Note: Parameters are power transformed

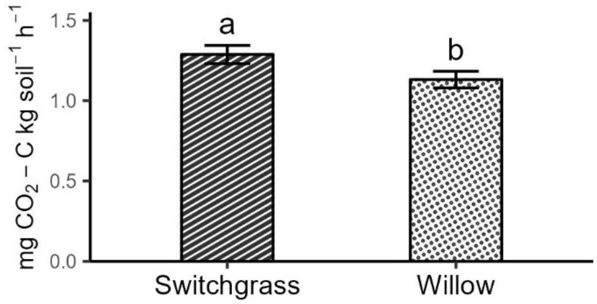
Supplemental Table 2 Summary Statistics of Regression Analysis

	Estimate	Standard Error	t value	p value
Intercept	-2.75×10^{1}	1.23×10^{1}	-2.25	0.03
POXC	2.70×10^{-4}	6.98×10^{-5}	3.87	<0.01
Respiration	1.49×10^{-1}	4.56×10^{-2}	3.27	<0.01
BG	-6.12×10^{-4}	4.55×10^{-4}	-1.35	0.18
PER	-7.04×10^{-4}	1.16×10^{-3}	-0.61	0.55
POM C:N	-5.59×10^{-2}	1.01×10^{-1}	-0.55	0.58
MAOM C:N	5.85×10^{1}	2.52×10^{1}	2.32	0.02

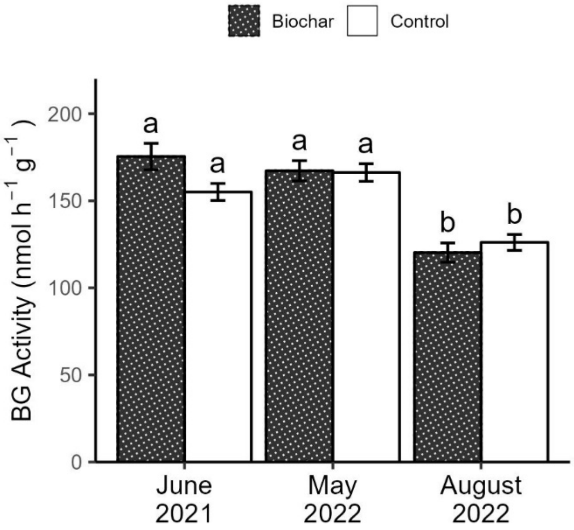
Note: Parameters are power transformed



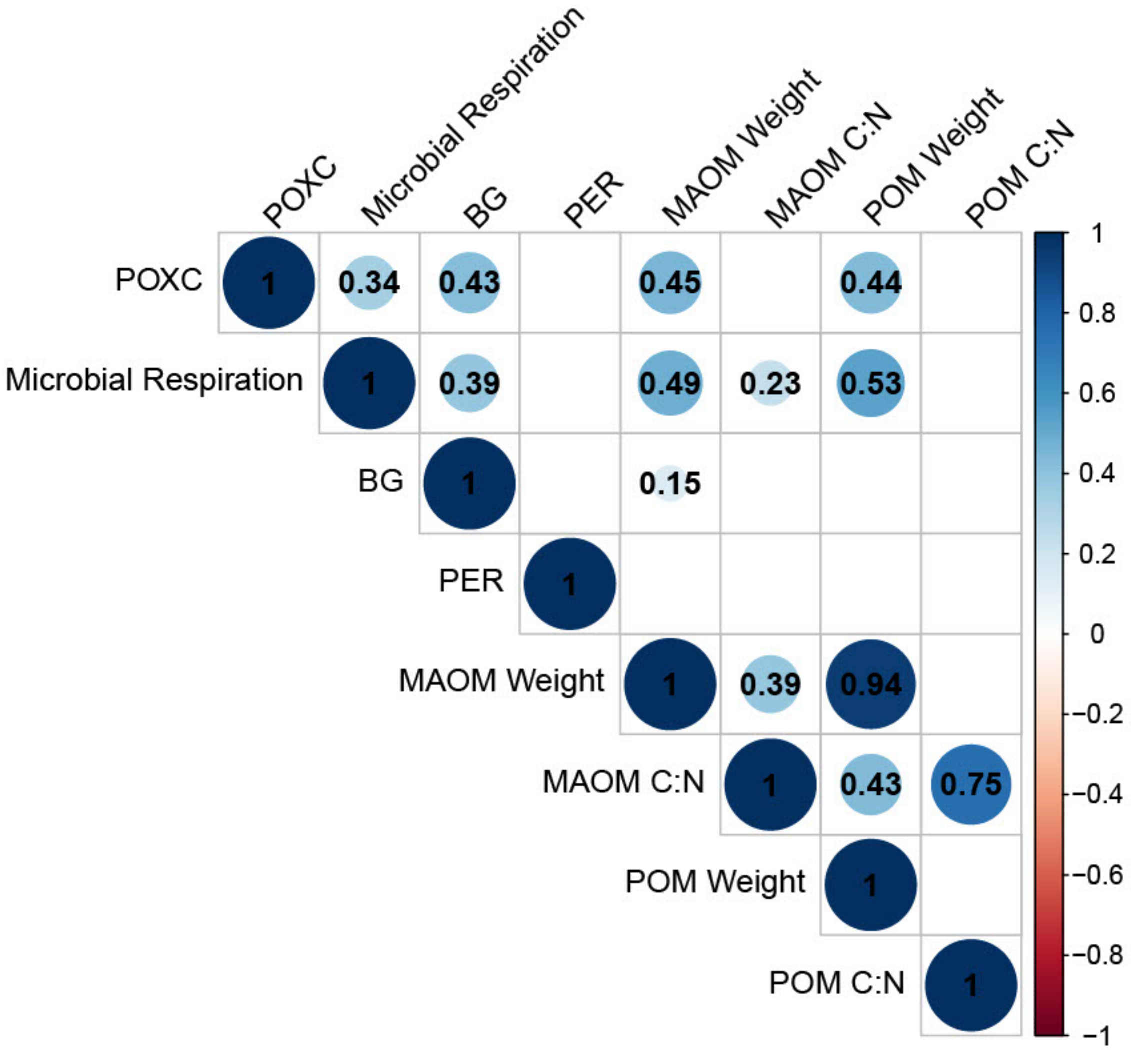
Supplemental Figure S1 Land type, crop, and date interactions on permanganate oxidizable carbon (POXC). Different letters indicate a significant difference ($\alpha < 0.05$). Error bars indicate standard error of the means ($n = 406$)



Supplemental Figure S2 Microbial respiration means of switchgrass and willow. Different letters indicate a significant difference ($\alpha < 0.05$). Error bars indicate standard error of the means ($n = 380$)



Supplemental Figure S3 Treatment and date interaction for β -Glucosidase mean values. Different letters indicate a significant difference ($\alpha < 0.05$). Error bars indicate standard error of the means ($n = 325$).



Supplemental Figure S4. Correlation matrix of the soil chemical and biological properties data collected. Crossed out boxes indicate that correlations were not statistically significant (α < 0.05). Size and color of the circle indicates strength of correlation, in addition to the labeled correlation values.

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