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# Control strategies for the invasive plant kudzu (*Pueraria montana*) only minimally impacts soil activity, chemistry, and bacterial and fungal communities

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ABSTRACT

Invasive plant species pose serious threats to biodiversity and stability of native ecosystems. Kudzu (*Pueraria montana* var. *lobata*) is an abundant and highly aggressive invasive plant in the Southeast United States. Herbicides, bioherbicides, and cultural practices are integral parts of integrated management of kudzu, yet few studies have evaluated the impact of kudzu management strategies on soils and their biological and chemical properties. To examine whether kudzu management options impact edaphic chemistry and/or soil microbial communities, we implemented a randomized complete block design (RCBD) with kudzu control treatments, which included synthetic, biological, and combined herbicide applications as well as mowing. Changes in edaphic chemistry, soil activity, and in bacterial and fungal communities were then measured across a single growing season. Treatments included the herbicides glyphosate and aminopyralid, the fungal bioherbicide *Albifimbria verrucaria*, mowing, as well as the combined treatments of aminopyralid and *A. verrucaria*, glyphosate and mowing, and two controls (untreated control and the surfactant used as a carrier for aminopyralid and *A. verrucaria* spores). Soils were collected at multiple points across the growing season between May and September. Soil enzymatic activity and edaphic chemistry were generally stable across treatments and time. Further, our community analyses indicates that the interaction between treatments and time structures fungal and bacterial soil communities, but only weakly. This study suggests that soil microbial communities are generally stable in response to different management strategies and had no discernable adverse non-target effects. We conclude that land managers likely can use any control strategies that are best suited for their circumstances without undue concern about how kudzu control strategies might impact soils.

## 1. Introduction

Invasive plants pose serious threats to landscapes and native ecosystems and they impact more than 40 million hectares of land in the United States (National Invasive Species Council, 2001). Annual costs to manage invasive plants can exceed 120 billion dollars in the United States alone (Harron et al., 2020). Mechanical, cultural, biological, and chemical methods are common management strategies for invasive plants, which might include prescribed fire, herbicides, mowing, and/or disc harrowing, but these often have limited efficacy (Simmons et al., 2007; Shelton, 2012; Twidwell et al., 2012).

Kudzu (*Pueraria montana* var. *lobata*) is a semi-woody, trailing or climbing, perennial leguminous vine that can grow up to 20–30 m over a single growing season (Sasek and Strain, 1988). Kudzu was actively plated as a forage crop and soil stabilizer during the early to mid-20th century (Sturkie and Grimes, 1939; Forseth and Innis, 2004) and between 1920 and 1950, over 1,000,000 ha of kudzu was planted (Miller and Miller, 2005). Kudzu was removed from the list of recommended cover plants in 1953 and shortly thereafter listed as noxious weed by the US Department of Agriculture (Miller and Edwards, 1983; Blaustein, 2001). Kudzu's economic impact is enormous; it can cause annual loses between $100 to $500 million of productive land in the US (Forseth and Innis, 2004). Kudzu can also adversely impact the abundance or diversity of species that are important habitats for native wildlife and may

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alter community dynamics (Westbrooks, 1998; Heckel, 2004). Kudzu increases local nitrogen mineralization and nitrification rates and contributes to ozone pollution by doubling soil emissions of nitric oxide (NO) (Hickman et al., 2010). Kudzu can also endanger native plant communities, for example, Relict Trillium (*Trillium reliquum*) has been listed as endangered in kudzu infested ecosystems in some parts of the Southeast US (Heckel, 2004). Currently, kudzu is distributed within the US from New York to Florida and westward to Oklahoma and Texas, with recent occurrences in Washington and Oregon, however, the heaviest infestations can still be found in the southern states of Alabama, Georgia, and Mississippi (Miller, 1996; Harrington et al., 2003).

Kudzu management strategies are varied and have variable efficacies and may include livestock grazing, prescribed burning, disk harrowing, and/or herbicide applications (Harrington et al., 2003); however, eradication from herbicides can take 10 years or more of repetitive and intensive herbicide applications (Boyette et al., 2002), which may be prohibitively costly or time inefficient for some land managers. However, bio-herbicidal control of kudzu has shown promise as part of an integrated control approach in conjunction with chemical herbicides (Weaver et al., 2009). The generalist fungal foliar pathogen *Albifimbria verrucaria* (Ascomycota, Sordariomycetes, Hypocreales, Stachybotryaceae; formally *Myrothecium verrucaria* (Lombard et al., 2016)) has been demonstrated to be a potentially effective bioherbicide for the control of kudzu (Boyette et al., 2001, 2002) and under the right conditions, can infect leaves and stems in kudzu with as high as 95–100% infection rates within 14 days of inoculation. *A. verrucaria*, in conjunction with herbicides, might more effectively control kudzu (Weaver et al., 2016) than either alone. Since most biocontrol agents and herbicides only reduce the crown of kudzu, whilst minimally impacting the root system, kudzu often rapidly recovers as the deep tap root has ample energy storage for regrowth, even the most aggressive management strategies can fail to reliably elicit kudzu mortality (Weaver et al., 2016).

It is well documented that soil microbial communities can be altered following the introduction of invasive species (Sielaff et al., 2018; Mamet et al., 2019; Torres et al., 2021), which can lead to higher nitrification rates, and alter competitive outcomes in favor of invasive species and against native plants (Hawkes et al., 2005). Kudzu impacts on soil communities have been demonstrated using PLFAs (Wu et al., 2018), but this has not been queried using metabarcoding to our knowledge. Kudzu does alter vegetation (Profetto and Howard, 2021), arbuscular mycorrhizal fungi density (Greipsson and DiTommaso, 2006), and soil nitrogen levels (Hickman and Lerdau, 2013). Invasive plant-mediated shifts in soil properties can further exacerbate microbial community alterations, which can further favor invasive plant establishment (Reinhart et al., 2003; Reinhart and Callaway, 2006). Soil bacteria and fungi play important roles in degrading and transforming allelochemicals during biological invasions (Lankau, 2010; Achatz and Rillig, 2014). Identifying how soil microbial communities and soil function are impacted by management strategies is important for finding more sustainable management approaches of controlling invasive plant growth while maintaining soil biodiversity and minimally impacting soil health over time (Shahrtash and Brown, 2021; James et al., 2022).

In addition, soil health and fertility are greatly influenced by soil biodiversity (Frąc et al., 2018). Any changes in this biodiversity could consequently alter biogeochemical cycling of soil nutrients, and ultimately soil fertility (Neemisha et al., 2020). Although the fate of chemicals in soil depends on multiple physical and chemical edaphic properties (Yadav et al., 2017; Hussain et al., 2018), several studies have suggested that soil microbial biodiversity can be adversely impacted by some agrochemical inputs following the transformation, transport, and decomposition of herbicidal residues (Chowdhury et al., 2008; Biswas et al., 2018). For example, herbicides may interfere with some biological nitrogen fixation and nutrient cycling processes (Druille et al., 2013; Rose et al., 2016). Increased agrochemical inputs in soil can often lead to community selection for microbial strains that can biotransform or otherwise utilize these compounds (Katsoula et al., 2020), or can facilitate increased phage loads that leads to viral mediated microbial metabolism or degradation of these compounds (Zheng et al., 2022). Therefore, maintaining an active and diverse soil biota is important for buffering the potential negative effects of agrochemical use (Altieri, 1999; Degens et al., 2001; Mazzola, 2004; Pugnaire et al., 2019).

To investigate the impacts of different management strategies for the control of kudzu on soil microbial communities, soil chemistry, soil activity, and other edaphic properties, we used a randomized complete block design in a field with long-established kudzu growth and implemented mechanical, chemical and biocontrol management strategies in different combinations. We queried broad soil enzymatic activity, soil community composition, and soil edaphic properties at multiple timepoints across a single growing season in the Southeastern United States.

We speculated that herbicidal and mechanical control of kudzu will impact soil community composition and enzymatic activity, likely via accumulation and degradation of the dead plant tissue within treated plots which would lead to increased carbon and nutrient pools in soils for enzymes to act upon. We further hypothesized that broad soil chemical pools and attributes would likely be unchanged over the course of a single growing season as the timeframe implemented here may be too limited to elicit measurable shifts in these nutrient pools.

## 2. Materials and Methods

### 2.1. Experimental Design and Sampling

This study took place at the Edward J. Meeman Biological Station (University of Memphis; Millington, TN, USA). Using a randomized complete block design, three blocks of 17 m x 17 m were established in the Meeman kudzu fields (35.356569, −90.005284, near Kudzu Pond, a local landmark). Soils at this site are classified as Memphis series silt loam (NRCS, USDA). These kudzu fields have been colonized by kudzu for at least 30 years (Kennedy, *personal communication*). Within each block, eight 3 m x 3 m (9 $m^2$) plots with 2 m wide mowed buffer strips were established in the winter prior to treatment implementation (Fig. S1) and treatments were assigned randomly to plots. Treatments consisted of glyphosate (Roundup PowerMAX® Monsanto St. Louis, MO), aminopyralid (Milestone®, Corteva, MO, USA, mixed with Induce® surfactant, Helena-Agri Enterprises. Collierville, TN, USA), *Albifimbria verrucaria* (AV) strain CABI-IMI 368023 (Weaver et al., 2021, 2022) with the surfactant Induce as a carrier, mowed (44 in. Rough-Cut Mower/Trail cutter, Swisher, Warrensburg, MO, USA; towed by a Utility Task Vehicle (UTV) set at lowest mowing height of 7.5 cm), mowed + glyphosate, Milestone + AV, and two controls; surfactant only control (Induce®; used a carrier for Milestone and AV) and unsprayed plots (Table 1). Roundup PowerMax and Milestone were applied at 3.03 L / plot (equivalent to 34.29 gr/plot active ingredient glyphosate and 1.58 mL/ plot active ingredient aminopyralid, each is 50% maximum label rate) and two controls, one sprayed with the control surfactant was applied at the rate of 23.51 mL/ha. We applied four liters of spore suspension ($1 \times 10^8$ spores/mL) for a total of $4 \times 10^{11}$ spores/plot. The fungi AV was grown on potato dextrose agar at 28 °C for 5 days and spores were collected as described in Weaver et al. (2016). All liquid applied treatments (glyphosate, Milestone, surfactant, spores) were applied via spray using new 1.5-gallon pump sprayers (GroundWork; Tractor Supply Company, Brentwood, TN, USA) and manually sprayed evenly to saturate the plots. Glyphosate and aminopyralid were applied twice at experimental day (D) – D0, and D16, each at 50% label application rate because split application has been demonstrated to maximize effectiveness on kudzu (Weaver et al., 2016). The bioherbicide *A. verrucaria* was applied thrice (D0, D12, D32), Milestone + *A. verrucaria* application was split such that at D0 we applied Milestone at 100% loading rate, and AV conidia were applied on D16 and D32), for glyphosate + mow, glyphosate was applied at 50% loading rate on D0 and D16 after mowing, and mow treatments (mow and glyphosate + mow) was conducted at D0 and every seven days throughout the

**Table 1**
Treatment implementation and sample collection dates from the kudzu removal experiment at the Meeman Biological Station conducted in 2019. Treatment applications were conducted using split design based on best practices as described. ll herbicidal treatments were applied to the seasonal label rate and did not exceed product maxima. Soils were collected each sampling date for metabarcoding (fungi and bacteria) and soil moisture. Soil samples for chemical analysis are indicated by the symbol †, and samples for the Fluorescein Diacetate Hydrolysis (FD) measures are indicated by the symbol ‡. Mowed treatments were mowed weekly. AV = *Albifimbria verrucaria* (bioherbicide).

| Dates of Treatment Application and Samples Collected | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatments | May 31 (D0) | June 2 (D2) | June 4 (D4) | June 8 (D8) | June 16 (D16) | July 2 (D32) | July 23 (D53) | August 13 (D74) | September 2 (D94) | September 24 (D116) |
| Roundup® | 50% Label Rate (Roundup) †‡ | | | | 50% Label Rate (Roundup) ‡ | †‡ | ‡ | ‡ | ‡ | †‡ |
| Milestone® | 50% Label Rate (Milestone) †‡ | | | | 50% Label Rate (Milestone) ‡ | †‡ | ‡ | ‡ | ‡ | †‡ |
| AV | 1st Application (AV) †‡ | | | | 2nd Application (AV) ‡ | 3rd Application (AV) †‡ | ‡ | ‡ | ‡ | †‡ |
| Milestone® + AV | 100% Label Rate (Milestone) †‡ | | | | 1st Application (AV) ‡ | 2nd Application (AV) †‡ | ‡ | ‡ | ‡ | †‡ |
| Mow | †‡ | | | | ‡ | †‡ | ‡ | ‡ | ‡ | †‡ |
| Roundup® + Mow | 50% Label Rate (Roundup) †‡ | | | | 50% Label Rate (Roundup) ‡ | †‡ | ‡ | ‡ | ‡ | †‡ |
| Surfactant Control | 1st Application †‡ | | | | 2nd Application ‡ | †‡ | ‡ | ‡ | ‡ | †‡ |
| Untreated Control | †‡ | | | | ‡ | †‡ | ‡ | ‡ | ‡ | †‡ |

growing season. Treatment applications were conducted to best mimic likely land manager usages and to follow previously demonstrated efficacy (Weaver et al., 2016) so we implemented treatments at uneven intervals and did not include all possible treatment combinations (see Table 1 for dates of applications).

## 2.2. Data collection

### 2.2.1. Sampling timeframe

Samples were collected on a partial $\log_2$ sampling scheme (Table 1) whereby samples were collected at Experimental Days D0 (beginning May 31, 2019), D2, D4, D8, D16, and D32, followed by additional sampling every three weeks until the end of the growing season on D53, D74, D95, and D116 (terminal sampling on September 24, 2019). In this way, we aimed to capture responses in the short-term and across the growing season.

### 2.2.2. Soil Sampling

For each sampling timepoint (n = 10), we collected three soil cores from haphazard locations within each plot (avoiding edge of the plot) using sliding hammer core sampler (AMS Inc., American Falls, ID, USA). Cores were gathered in the top 15 cm of soil and consisted of ~500 $cm^3$ of soils per plot, per sampling event. Soils from each plot were combined and placed into new 1-gallon zip-top plastic bags and placed on ice into a chest cooler in the field and transferred to the lab. On the same day of collection, soils were sieved (Brass #10 sieves, 2 mm mesh) to remove root and/or rock material, homogenized, and subsampled (0.25 g for DNA extraction). Additionally, two 50 mL sterile conical tubes were filled with soil for chemical, soil moisture, and activity measures. All soils were placed at − 20 °C until they were processed for further analyses.

### 2.2.3. Soil Chemical Analysis

Soil samples were collected to quantify soil chemical attributes for three time points (D0 – initial soil conditions, D32, and D116 – terminal sampling date). Soils were tested at the Soil Testing Lab (Kansas State University, Manhattan, KS, USA) following drying (60° C overnight), grinding, and passing through a 2 mm sieve. For each of these samples, we measured soil pH (1:1 soil to deionized water), total nitrogen (TN) and total carbon (TC) as percentages (LECO TruSpec CN Combustion Analyzer), organic nitrogen ($NH^{4+}$ and $NO^{3-}$), exchangeable cations ($K^{+}$, $Mg^{+2}$, $Na^{+}$, $Ca^{+2}$; Flame Atomic Absorption), and phosphorus (Melich III) were quantified in ppm. Further, we calculated C:N using obtained total carbon and nitrogen values.

### 2.2.4. Soil Moisture

Total soil moisture was calculated for all soil samples (n = 240) (*following* Borowik and Wyszkowska, 2016). For this, 5 g of soil was placed into pre-weighed coin envelopes and samples were oven dried at 80° C for 48 h and dry weight of the expressed per unit mass [(Wet Mass-Dry Mass) X 100)/ Dry Mass].

### 2.2.5. Soil Microbial Enzymatic Activity

Broad soil enzymatic activity was measured using a fluorescein diacetate activity (FDA) assay to assess generalized enzymatic capacity (Schnürer and Rosswall, 1982; Adam and Duncan, 2001). This assay measures the amount of fluorescein generated via FDA hydrolysis by microorganisms and is a measure of generalized soil activity potential. FDA (3′, 6′-diacetyl-fluorescein) is hydrolyzed by several enzymes, including proteases, lipases, and esterases (Green et al., 2006). Fluorescein products can be measured calorimetrically at the wavelength 475–510 nm using a spectrophotometer (Tayler and May, 2000; Sánchez-Monedero et al., 2008). Soils (4.0 g) were placed into a 50 mL centrifuge tube and 15 mL of buffer (6.9 g $NaH_2PO^{-4}$ and 41.8 mL of 1 M NaOH to a final volume of 1 L) was added to all samples. Then, 250 μL of FDA stock (2 mg Flourescein Diacetate per mL in acetone) was added. 15 mL of acetone was then added and samples were agitated for five minutes then centrifuged at 5000 x g for 10 min, followed by addition of FDA Buffer/acetone (1:1). Quantification of enzyme activity was performed using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, US).

### 2.2.6. Kudzu Cover

In addition, we measured the effect of control treatments on kudzu coverage for each sample. Kudzu coverage in a plot was assessed by estimating the percent of a kudzu vegetation coverage within a 1 $m^2$ quadrat placed randomly into a plot and the amount of vegetation that was kudzu (percent) was estimated. We did this three time per plot per sampling date and the average values were used in downstream analyses.

### 2.2.7. DNA extraction and sequence generation

Soil samples (250 mg) were ground (Fisherbrand™ Bead Mill 24 Homogenizer; Thermo Fisher Scientific, Waltham, MA, USA) for 1 min at

max speed (6 m/s). After grinding, total soil genomic DNA (gDNA) was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN, Germantown, MD, USA), following standard protocols, and DNA was quantified using a NanoPhotometer N60 (Implen, Munich, Germany). DNAs were normalized to a concentration of 50 ng/μL for each sample. PCRs were conducted using a two-step amplification procedure (Brown et al., 2018) whereby primary PCR was conducted using the primers nexF-N4-fITS7 and nexR-N4-ITS4 that target the ITS2 region of the rRNA operon of fungi (White et al., 1990; Ihrmark et al., 2012) or the primers nexF-N4–515 f and nexR-N4–806r that target the V4 region of the 16S rRNA gene of bacteria (Caporaso et al., 2011); nexF and nexR are the Nextera forward and reverse sequencing primers and N4 are 4 ambiguous base pairs that improve annealing efficiency. Primary PCRs were conducted in 25 μL reactions with the following concentrations: 2 μL DNA template (100 ng), 12.5 μL 2X Phusion Green High-Fidelity PCR Master Mix (Thermofisher Scientific, Waltham, MA, USA), 2.5 μL (10 μM) of each forward and reverse primer, and 5.5 μL molecular grade $H_2O$. PCR parameters were 98 °C for 30 s, 25 cycles of 98 °C for 20 s, 51 °C annealing temperature (fungi) and 52.5 °C annealing temperature (bacteria) for 30 s, and 72 °C for 40 s, with a final extension at 72 °C for 10 min; all ramp rates were 1 °C /s (SimpliAmp Thermal Cycler, Applied Biosystems, Foster City, CA, USA). Gel electrophoresis was used for the verification of amplification. Secondary PCR reactions (20 μL) consisted of the forward primer (P5-i5-overlap) and the reverse primer (P7-i7-overlap), where P5 and P7 are Illumina Adaptor sequences, i5 and i7 are 8 bp unique Molecular Identifiers (MIDs), and overlap is the partial nexF or nexR, which is the annealing site for secondary PCRs. The forward- and reverse-barcoded 2° primers were mixed to generate unique dual-barcoded primers of 10 μM (5 μM for each primer). See Table S1 for primer and MID sequences. The 2° PCR reactions consisted of 2 μL of 1° PCR product, 10 μL 2X Phusion Master Mix, 2.5 μL of mixed primers, and 6.5 μL molecular grade $H_2O$. PCR parameters were 98 °C for 30 s, 8 cycles of 98 °C for 20 s, 50 °C for 30 s and 72 °C for 40 s, followed by a final extension at 72 °C for 10 min. Final library constructs consisted of P5-i5-nexF-N4-primer-{ITS2 or V4}-primer-N4-nexR-i7-P7 with 32 total cycles. Secondary PCR products were cleaned and normalized using Just-a-Plate™ 96 PCR Purification and Normalization kit (CharmBiotech, Cape Girardeau, MO, USA) and samples were pooled into a single tube for the bacterial and fungal library. Negative controls (molecular grade water) were included throughout and were free of observable amplification and appreciable sequences. After pooling, libraries were cleaned once more (DNA Clean and Concentrator™-5 (Zymo Research, Irvine, CA, USA). The pooled amplicons were sequenced using one reaction each for fungi and bacteria using Illumina MiSeq (300PE; at the Integrated Genomics Facility, Manhattan, KS, USA).

### 2.3. Bioinformatics

Sequences were processed using the program mothur (v.1.41.1; Schloss et al., 2009) generally following (Kozich et al., 2013) with modifications. The forward and reverse sequences were contiged, and where a consensus base has a Q-value less than 25, the base was removed. Contigs were screened to remove sequences with ambiguous base pairs and/or sequences with greater than 12 homopolymers. Contigs were merged into a single fasta file, and sequences were pre-clustered to minimize sequencing induced errors and to reduce computational expense for downstream analyses (Huse et al., 2010). Sequences were classified into taxonomic lineages using a Naïve Bayesian classifier (Wang et al., 2007) against the RDP training set for bacteria (v10) and the UNITE non-redundant species hypothesis data set for fungi (v.6; Kõljalg et al., 2013). Non-fungal and non-bacterial sequences were culled. Sequences were demarcated into OTUs using the abundance based VSEARCH (Rognes et al., 2016), at a 3% dissimilarity threshold. OTUs with 10 or fewer sequences were considered potentially spurious and discarded (Brown et al., 2015; Oliver et al., 2015). To estimate the relative OTU richness ($S_{obs}$), diversity (complement of Simpson's diversity index; 1-D), and evenness (Simpson's evenness, $E_D$) of samples we used an iterative subsampling approach (1000 iterations at a subsampling depth of 2500 sequences per sample), and the mean values were used for all analyses. We used this repeated rarefying approach to normalize our data matrices as it allowed maximum sample retention (as few samples had fewer total sequences than anticipated) whilst reflecting the probabilistic nature of amplicon sequence generation and minimizes inclusion of skewed diversity estimates based on a single subsampling event (Cameron et al., 2021). We verified that this subsampling depth was appropriate by examining rarefaction curves (Fig. S2) where we see that at this subsampling depth, these rarefaction curves are well beyond the rarefaction inflection points. Further, we estimated the percentage of presumed total OTUs observed at this subsampling depth by calculating Good's coverage and Boneh estimates estimating the numbers of additional OTUs that might be observed if subsampling depth is doubled (1000 iterations). We see that coverage estimates indicate we have captured the large majority of all potential OTUs (Fungi – 92.6%; Bacteria – 78.7%) at a subsampling depth of 2500 sequences per sample. Further, doubling the subsampling depth would only increase the number of observed OTU by 7.6% for fungi and 9.9% for bacteria. Together, we are confident that this subsampling depth is suitable for all conducted analyses.

### 2.4. Statistical analysis

All statistics were conducted using a combination of JMP Pro v15 (SAS Institute, Cary, NC, USA), R (v.3.3.3) and mothur (v.1.41.1).

To test if kudzu control treatments, time, or their interactions impact kudzu coverage, soil moisture, and soil chemical measures, we used a repeated measure ANOVA framework with plot nested within blocks and treated as a random effect. OTU alpha diversity values were Box–Cox transformed to meet ANOVA assumptions of normality prior to analyses (Bacteria: $S_{obs}$ $\lambda = 1.64$, $E_D$ $\lambda = 1.131$, 1-D $\lambda = 2$ and Fungi: $S_{obs}$ $\lambda = 2$, $E_D$ $\lambda = 0.167$, 1-D $\lambda = 1.978$). Using a repeated measures ANOVA framework, we tested if diversity estimates (Fungi and Bacteria), individual soil property measurements, soil enzyme activity, and kudzu coverage differed with control treatments, time, and their interactions with Kenward-Roger first order approximations with Kacker-Harville corrections (this type of interaction allows for partial degrees of freedom). Visualization included LOESS local regression to generate smoothed lines to see how richness and diversity change over time across treatments. *Post hoc* tests were then conducted where significant to assess treatment effects (Dunnett's Tests using the initial timepoint [T0] and the unsprayed control plots as controls for this test).

To test if control treatments, time, or their interactions alter soil microbial communities (bacteria and fungi analyzed separately), we used a permutational multivariate analysis of variance (PerMANOVA; (Anderson, 2001)) approach using R with the package VEGAN (function *adonis* with 999 iterations, strata=Block to facilitate repeated measures) (Oksanen et al., 2017) on Bray-Curtis dissimilarity values (subsampled to 2500 sequences per sample as above using 1000 iterations). Where significant, post hoc tests were conducted using the package *RVAIDeMemoire* (function *pairwise.perm.manova* with FDR corrections, 999 iterations) (Hervé, 2021). To visualize communities, NonMetric Multidimensional Scaling (NMDS) was conducted using average Bray-Curtis dissimilarity values used above, as implemented in mothur. In all, 1000 iterations were done to find the optimal solutions and both bacterial and fungal communities optimally resolved across 5 dimensions (5-D stress of 0.188 and 0.183 for bacteria and fungi, respectively). Additionally, to explore if soil physiochemical properties and enzyme activity helps drive microbial communities, we correlated these values agains NMDS axes loading scores using Kendall Tau correlations. Further, to examine individual OTU responses to treatments, we used the Linear Discriminant Analysis Effect Size (LEfSe; Segata et al., 2011), as implemented in mothur, and identified biomarker taxa

that are overrepresented in treatments (class=treatments) whilst accounting for the variability associated with time (subclass=time). For fungal biomarkers, where taxonomic resolution allowed, functional roles were determined using the traits database FungalTraits (Põlme et al., 2020).

## 3. Results

After quality control, denoising, and chimera removal, 10,863 bacterial and 6255 fungal OTUs were retained. Fungal OTUs were dominated by members best identified within the phyla Ascomycota (67.1%), Basidiomycota (11.8%) and Mortierellomycota (9.3%), and within the classes Sordariomycetes (31.3%), Dothideomycetes (21.2%), Mortierellomycetes (9.2%).

Bacterial OTUs were dominated by members best identified within the phyla Proteobacteria (28.1%), Acidobacteria (17.5%), Actinobacteria (9.2%) and classes Alphaproteobacteria (18.8%).

Actinobacteria (9.2%), Planctomycetacia (9.2%) (see Table S2 for taxonomic summaries). All sequences have been deposited in the Sequence Read Archive (SRA) at NCBI under the BioProject accession PRJNA860727. Full OTU x Sample matrices along with representative sequence information and taxonomic identities are provided in the supplemental (Table S3).

### 3.1. Treatment Responses

Kudzu removal treatments differentially impacted the kudzu coverage (Treatment: $F_{7,16}$ =55.954, $P < 0.001$, Time: $F_{7,112}$ =30.497, $P < 0.001$, interactions: $F_{49,112}$ =17.604, $P < 0.001$) with all non-control treatments reducing above ground kudzu apart from the bioherbicide AV (Fig. 1; Table S4). Edaphic properties had varied responses to experimental treatments and time (Table S5). pH, nitrate, potassium, and calcium responded to treatments, while total carbon, total nitrogen, and nitrate, ammonium, calcium, and C:N responded to time.

Microbial diversity was seen to have varied responses to treatments, time, or their interactions in our repeated measures ANOVAs (Table 2). Fungal and bacterial richness and diversity were stable across treatments, yet they changed with time, and fungal diversity was responsive to the interaction of treatment and time (Fig. 2). Fungal and bacterial evenness was unchanged with any model components. PerMANOVA tests revealed that shifts occurred in community structure, but several factors only had low effect sizes as indicated by $R^2$ values. Communities shifted with treatment (Bacteria: $F_{7,153}$ 1.329, $P = 0.001$, $R^2$ =0.038; Fungi: $F_{7,149}$ 1.467, $P = 0.001$, $R^2$ =0.039) and time (Bacteria: $F_{9,153}$ 2.047, $P = 0.001$, $R^2$ = 0.076; Fungi: $F_{9,149}$ 4.287, $P = 0.001$, $R^2$ =0.1487), but not with the interaction of treatment and time (Table 3). This small effect size can be visualized (Fig. 3) where it is demonstrated that there is much overlap in ordination space between treatments. This also demonstrated that soil properties are not as influential in driving bacterial communities as with Fungi (Fig. 3). For Bacteria, only FDA fluorescence was significantly associated with NMDS axes (nitrogen and carbon were for a single axis), whereas sampling day, calcium, pH, nitrogen, carbon, C:N were associated with both the first and second axis, and FDA, potassium, and nitrate were significant for one axis. *Post hoc* comparisons (Table S6) indicate that fungal communities change readily across time, and that bacterial communities are stable over time initially, but toward the end of the growing season (Days 94 and 116), there are extensive differences in bacterial communities compared to the early growing season. Despite broad communities differing with treatment, only AV and glyphosate + Mow treatments had different fungal communities compared to the control plots (Table S6) and only aminopyralid and glyphosate + Mow treatments had different bacterial communities, but again, these treatments effects had very low $R^2$ values.

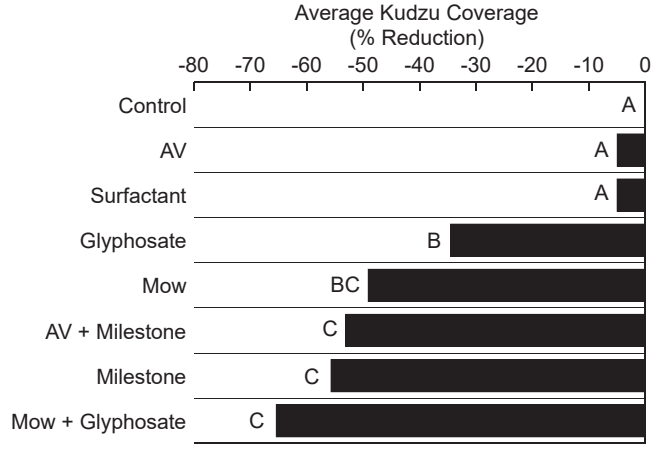


**Fig. 1.** Efficacy of kudzu control treatments options as compared to untouched control plots as an average percent reduction in kudzu coverage. Letters represent results from a Dunnett's Test comparing each treatment to the control, where letters are different from the control, significant reduction of kudzu was observed. AV is the biocide *Albifimbria verrucaria.*

Broad soil enzymatic activity (as measured by FDA hydrolysis) significantly differed across treatments ($F_{7,96}$ =32.385, $P < 0.001$), time ($F_{7,16}$ =19.601, $P < 0.001$, FDA increases over time), and treatment x time interactions ($F_{42,96}$ =3.601, $P < 0.001$). Dunnett's tests for FDA activity (against control) indicated that the treatments AV + Milestone (7.99% more FDA hydrolysis compared to control on average), surfactant control (10.86% more FDA), and AV (12.12% more FDA) had similar FDA activity to control plots, whereas Milestone (23.18% more FDA), glyphosate (27.10% more FDA), mowing (27.15% more FDA), and mow + glyphosate (30.42% more FDA) had increased soil enzyme activity compared to control (Table S4).

The LEfSe analyses identified fungal and bacterial biomarker OTUs that were overrepresented with treatment (class) whilst accounting for seasonality (subclass=time) (Table 4). Despite broad, albeit minor, community differences across treatments, there were only a few biomarker OTUs associated with a treatments suggesting community turnover was minimal. The LEfSe tests indicate few OTUs that are differentially abundant across treatments but only a single bacterial OTU was identified as a biomarker for a treatment. For fungi, representative OTUs associated with the AV treated plots include OTU 00054 (*Albifimbria* sp.), OTU 00128 (*Ramicandelaber taiwanensis*), OTU 00305 (*Mortierella* sp.), OTU 00151 (*Mortierella minutissima*), OTU 00497 (*Leucoagaricus* sp.), and OTU 01103 (*Lepiota* sp.). Only two other treatments had fungal biomarker taxa present: OTU 00033 (*Metarhizium marquandii*) for mowed treatments, and OTU 00069 (Ceratobasidiaceae sp.) was overrepresented for the combined mow and Glyphosate treatment. Among the bacterial OTUs, only OTU 000347 (*Burkholderia sp.*) was determined as a biomarker and was so for the mowed only treatments.

## 4. Discussion

In this study, we evaluated the impacts of different mechanical, chemical, and biological control strategies for kudzu on below ground microbial communities and soil functionality by comparing treated plots against two controls across a growing season. We detected statistically significant differences aboveground kudzu coverage (Fig. 1) and in soil enzymatic activity using FDA assays. FDA values in plots receiving glyphosate, mow, and mow + glyphosate treatments had enhanced ability to hydrolyze FDA, which is a measure of broad soil enzymatic capability, as compared to control treatments. Increased enzyme activities are likely linked to the increased carbon inputs (Sharma et al., 2020) associated with vegetation reduction with concomitant organic carbon inputs (as evidenced by reduced aboveground kudzu coverage see here; Table S5). Introduction of organic carbon into soils increases rates of liter decomposition, which leads to increased enzymatic activity

**Table 2**
Repeated measures ANOVA tests for fungal and bacteria relative OTU richness ($S_{obs}$), Diversity (1-D), and Evenness ($E_D$) across kudzu control treatments, sampling timepoint, and treatment x time interactions. F statistics are included with degrees of freedom based on Kenward-Roger first order approximations with Kacker-Harville correction, which allows for partial denominator degrees of freedom. Significant factors are bolded and italicized.

| Response | Treatment | Time | Treatment x Time |
|---|---|---|---|
| $S_{obs}$ (Fungi) | $F_{7,\ 15.832} = 2.63$, P = 0.051 | ***$F_{9,\ 138.142} = 2.44$, P = 0.013*** | $F_{63,\ 137.666} = 1.19$, P = 0.192 |
| 1-D (Fungi) | $F_{5,\ 15.968} = 2.00$, P = 0.118 | ***$F_{9,\ 138.145} = 2.65$, P = 0.007*** | ***$F_{63,\ 137.704} = 1.59$, P = 0.012*** |
| $E_D$ (Fungi) | $F_{7,\ 16.517} = 1.43$, P = 0.256 | $F_{9,\ 138.948} = 1.88$, P = 0.058 | $F_{63,\ 138.44} = 1.16$, P = 0.233 |
| $S_{obs}$ (Bacteria) | $F_{7,\ 33.476} = 0.43$, P = 0.873 | ***$F_{9,\ 134.834} = 4.76$, P < 0.0001*** | $F_{63,\ 133.102} = 1.21$, P = 0.171 |
| 1-D (Bacteria) | $F_{7,\ 27.319} = 0.89$, P = 0.526 | ***$F_{7,\ 135.419} = 2.76$, P = 0.005*** | $F_{63,\ 132.828} = 1.12$, P = 0.286 |
| $E_D$ (Bacteria) | $F_{7,\ 23.134} = 1.34$, P = 0.276 | $F_{9,\ 135.214} = 2.33$, P = 0.177 | $F_{63,\ 131.356} = 0.96$, P = 0.549 |

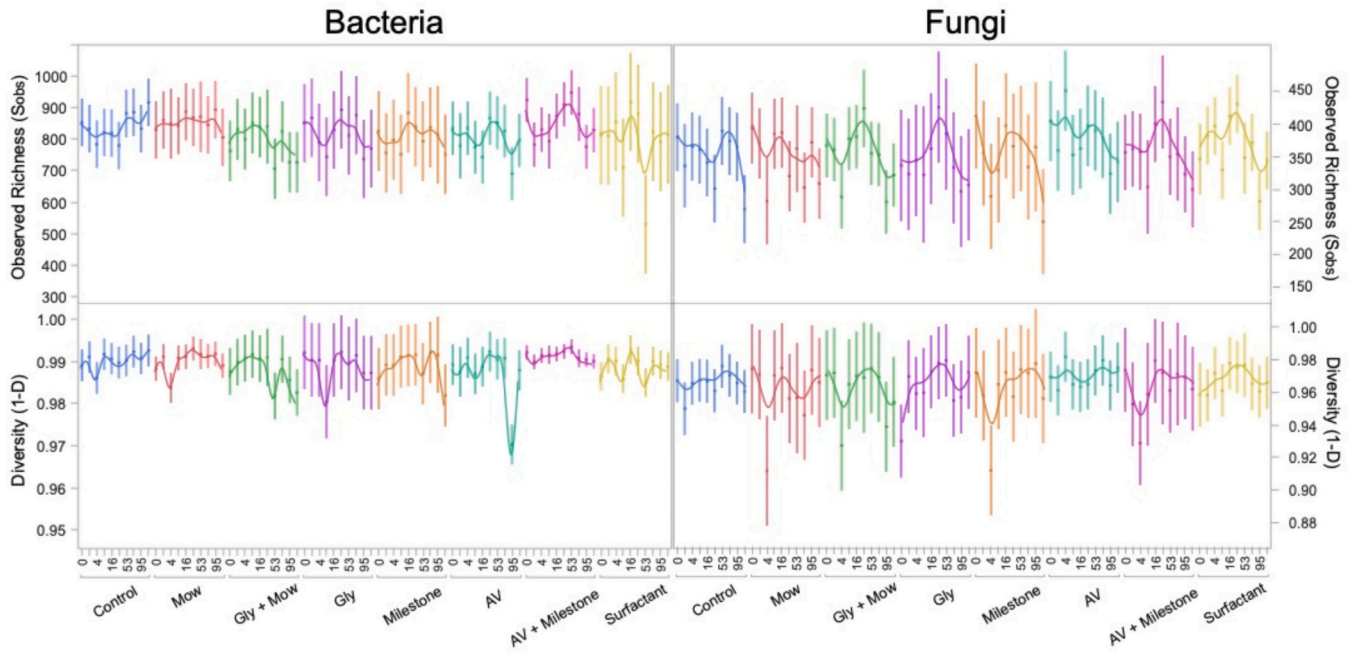


**Fig. 2.** Results of observed richness (top) and diversity (bottom) for Bacteria (left) and Fungi (right) over time and between kudzu control treatments. Presented are ranges of estimated diversity values with mean as a solid horizontal bar. Also presented are the results of locally weighed regression (LOESS) for these estimates. Note the change in scale between bacteria and fungi. Gly = glyphosate, AV = *Albifimbria verrucaria.*

**Table 3**
Results of PerMANOVA tests across treatments (Treatment), sampling dates (Time) and their interactions for fungi and bacteria. Presented are pseudo-F statistics, degrees of freedom, P-values and associated $R^2$ values. Significant results are in bold and in italics.

| Tests | Pseudo-$F_{df}$ | P-value | $R^2$ |
|---|---|---|---|
| **Fungi** | | | |
| ***Treatments*** | ***$F_{7,149} = 1.467$*** | ***0.001*** | ***0.0396*** |
| ***Time*** | ***$F_{9,149} = 4.287$*** | ***0.001*** | ***0.1487*** |
| Treatments x Time | $F_{63,149} = 0.977$ | 0.177 | 0.2373 |
| Residuals | | | 0.5742 |
| **Bacteria** | | | |
| ***Treatments*** | ***$F_{7,153} = 1.329$*** | ***0.001*** | ***0.0386*** |
| ***Time*** | ***$F_{9,\ 153} = 2.047$*** | ***0.001*** | ***0.0765*** |
| Treatments x Time | $F_{63,153} = 0.951$ | 0.323 | 0.2489 |
| Residuals | | | 0.6357 |

(Aneja et al., 2006; Blagodatskaya et al., 2009). However, our LEfSe analyses of did not identify many saprobic fungal biomarker taxa associated with treatments with reduced vegetation as would be expected of increased carbon inputs into the soil. While we are unable to fully resolve this discrepancy, it is likely that our FDA results are driven by bacterial enzyme production. FDA measures broad enzymatic capability and encompasses multiple lineages of organisms, including fungi and bacteria. It is likely that bacteria is more responsive to this organic carbon input, an idea that is partially supported the biplots in our NMDS ordinations (Fig. 3), which indicates that FDA is positively associated with both major NMDS axes of bacterial communities, but only one with the fungal ordination. More work is needed to confirm this direct carbon input link with FDA signals. We also identified similar shifts in metabarcoding derived fungal diversity estimates, which supports increased enzyme potential, however no difference in bacterial diversity was observed. Our findings have important implications for understanding the impact of kudzu management approaches on soil health, and it can provide the basis for future studies on sustainable kudzu control (Shahrtash and Brown, 2021).

All management strategies significantly reduced aboveground kudzu foliage compared to control except the biocontrol (AV). Contrary to previous reports (Weaver et al., 2009, 2016), *A. verrucaria* treatment had no significant effect on reducing kudzu growth here. While we are unsure why the AV treatments were not effective in this study, which is particularly surprising given previous reports of this strain of AV being useful for biocontrol purposes, we discuss a few potential reasons. It should be mentioned that conidial preparations were freshly generated from well controlled long-term frozen storage, grown on PDA. Cultures morphologically were identified as AV and after we noticed lack of kudzu suppression, were molecularly confirmed to be AV (Sanger sequencing of ITS1 region). Further, conidia viability is not likely to be an issue as these were prepared using common protocols. One reason may be that local environmental conditions were not optimal for

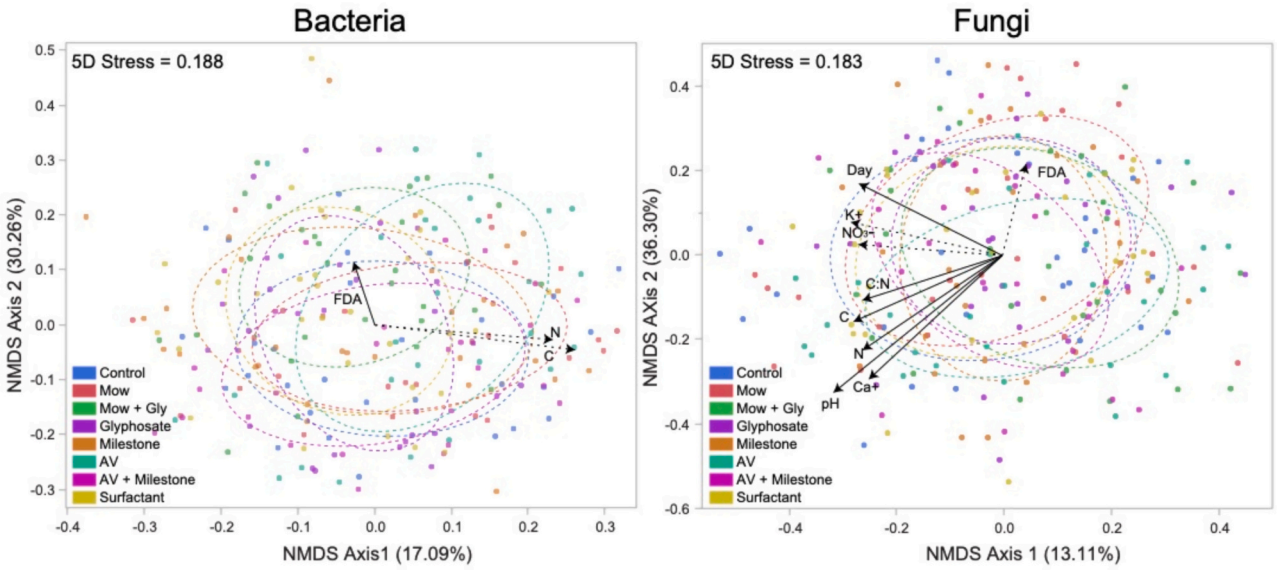


**Fig. 3.** NMDS ordinations based on Bray-Curtis dissimilarities across treatments. Biplot vectors were determined for all soil physiochemical data, FDA enzyme results, and sampling date (Day) based on Kendall Tau correlations. Only significant biplots are visualized with solid lines representing vectors that are significant associated with both NMDS axes and dashed lines representing where significant for only a single axis. Gly = glyphosate, AV = *Albifimbria verrucaria.*

**Table 4**
Differentially abundant OTU biomarkers crossed our treatments and higher taxonomic units across treatments (AV = utilization of the bioherbicide *Albifimbria verrucaria*), whilst accounting for temporal variation for only OTUs > 1000 sequence counts. Presented are Linear discriminant analyses (LDA) tests statistics, associated p-values, best taxonomic identities, and putative ecological role. Ecological roles, where unambiguously known, are determined for fungi using the FungalTraits database (Põlme et al., 2020) and for bacteria using primary literature.

| Biomarker OTU | Treatment | LDA | p-value | Taxonomic ID | Ecological Role |
|---|---|---|---|---|---|
| **Fungi** | | | | | |
| Otu00033 | Mow | 3.585 | 0.0050 | *Metarhizium marquandii* | Pathogen/Saprobe |
| Otu00054 | AV | 3.843 | < 0.001 | *Albifimbria* sp. | Plant pathogen |
| Otu00069 | Mow + Glyphosate | 3.588 | 0.0038 | Ceratobasidiaceae (unclassified) | Pathogen/Saprobe |
| Otu00128 | AV | 3.765 | 0.0437 | *Ramicandelaber taiwanensis* | Ectomycorrhizal |
| Otu00151 | AV | 2.788 | 0.0013 | *Mortierella minutissima* | Saprotroph |
| Otu00187 | AV | 2.930 | 0.0055 | *Ramicandelaber taiwanensis* | Saprotroph |
| Otu00305 | AV | 2.689 | 0.0285 | *Mortierella* sp. | Saprotroph |
| Otu00497 | AV | 2.714 | < 0.001 | *Leucoagaricus* sp. | Saprotroph |
| Otu01103 | AV | 2.024 | < 0.001 | *Lepiota* sp. | Saprotroph |
| **Bacteria** | | | | | |
| Otu000347 | Mow | 2.328 | 0.0022 | *Bulkholderia* sp. | Potential Phytopathogen |

conidial germination at the time of application (e.g. temperature or humidity), which has been demonstrated to affect the efficacy of biocontrols (Auld and Morin, 1995), but we find this to be unlikely, as there were multiple AV inoculation events and these conidia readily germinate (Weaver et al., 2016). Additionally, there may be unquantified associations between AV germination or colonization success and kudzu genetics (Gulizia and Downs, 2019) which controls host specificity to biocontrol agents (Pitt et al., 2012). Our previous work indicated that kudzu genotypes differ across the invaded range (Shahrtash and Brown, 2020), but invasive kudzu generally lacks spatial genetic variability (Bentley and Mauricio, 2016). It may be that the genotype of kudzu at our field site differs from previous kudzu genotypes that have demonstrated AV efficacy as a biocontrol. Perhaps this genotype is not as susceptible to infection from this AV strain, but we cannot confirm this with our current data.

We found relative stability in taxonomic profiles of soil under different treatments. Only a few taxa were identified as biomarkers, namely for AV and mow only treatments. Functional exploration of these OTUs using the FungalTraits database (Põlme et al., 2020) (Table 4) indicated that most of enriched OTUs are saprotrophs. Again, this could be due to accumulation of leaf material in soil after mechanical, biological and chemical treatments, which may lead to soil organic matter build-up and an increased saprotroph abundance involved with decomposition. While PerMANOVA tests indicated that communities differed with treatment for fungi and bacteria, these community shifts are slight with low $R^2$ values (Table 3), indicating that the community structure of treated plots were minimally different from the control plots. Consistent with our results are recent studies indicating minor changes in soil microbial community composition with herbicide treatments including by sulfosulfuron and chlorsulfuron (Medo et al., 2020), glyphosate (Bottrill et al., 2020), glufosinate, paraquat, and paraquat-diquat (Dennis et al., 2018), and triclopyr (James et al., 2022). This work adds to an expanding body of literature strongly suggesting that herbicidal control, when done at appropriate label rates, does not strongly impact soil communities and is unlikely to impact soil functionality, in the short term. Ecological damage from kudzu's continued presence and growth likely far outweighs minor and likely short-term shifts in soil communities and/or edaphic properties.

## 5. Conclusion

Our results emphasize the importance of integrated management practices as the most promising strategy to control kudzu. We found that mow only, AV, and mow + glyphosate treatments drove shifts in the

abundances of specific soil microbial taxa compared to untreated soils, however, the belowground community structure and functional potential were largely constant across treatments. Application of glyphosate or Milestone over a single season did not play a major role in structuring microbial communities or in altering soil functional capability within soil from a kudzu infested field. We conclude that when herbicides are applied with recommended field-application label rates, soils are only minimally impacted, at least across a single growing season. Further, there was little effect in general of kudzu control treatments on broad soil enzymatic activity, chemistry, and microbial communities. This suggests that land managers may be able to control kudzu in whichever way works best for their capacity, if they adhere recommended field-application label rates, without concern of negatively impacting soils, but additional work is required to confirm this recommendation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pedobi.2023.150897.

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