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RESEARCH ARTICLE

Site preparation impacts on soil biotic and abiotic properties, weed control, and native grass establishment

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In severely degraded systems active restoration is required to overcome legacies of past land use and to create conditions that promote the establishment of target plant communities. While our understanding of the importance of soil microbial communities in ecological restoration is growing, few studies have looked at the impacts different site preparation techniques have on these communities. We trialed four methods of site preparation: fire, top-soil removal (TSR; removal of top 50 mm of soil), slashing (vegetation cut to 30 mm, biomass removed), and carbon (C; as sugar and saw-dust) addition, and quantified resulting soil bacterial communities using DNA metabarcoding. We compared the effectiveness of these techniques to reduce weed biomass, improve native grass establishment, and induce changes in soil nutrient availability. TSR was the most effective technique, leading to a reduction in both available nutrients and competition from weeds. In comparison, the remaining methods had little or no effect on weed biomass, native grass establishment, or soil nutrient availability. Both TSR and C addition resulted in changes in the soil bacterial community. These changes have the potential to alter plant community assembly in many ways, such as via nutrient acquisition, pathogenic effects, nutrient cycling, and decomposition. We recommend TSR for ecological restoration of old-fields and suggest it is a much more effective technique than burning, slashing, or C addition. Restoration practitioners should consider how their management techniques may influence the soil biota and, in turn, affect restoration outcomes.

Key words: annual weed control, burning, carbon supplements, microbial nutrient immobilization, perennial grass establishment, slashing, soil microbes, top-soil removal

Implications for Practice

- Restoration practitioners should consider the influence management techniques can have on the soil biota considering both top-soil removal and carbon addition resulted in changes in the soil bacterial community.
- Top-soil removal was the most effective technique at reducing soil nutrients and competition from weeds, therefore we recommend this technique for old-field restoration where suitable.
- The lack of native seedling emergence in the top-soil removal treatment could be of concern and may need to be overcome in practice.

Introduction

Weed control is a significant challenge for ecological restoration and often has limited success in practice (Kettenring & Adams 2011). Site preparation before planting is perhaps the most important step in any restoration project to overcome this challenge (Hobbs 2007). During site preparation, structural properties and ecosystem processes must be manipulated to favor the desirable species and ensure replanted communities are resilient and self-sustaining (Hobbs 2007). This is particularly important in systems that are resistant to change and heavily degraded, such as previously cultivated landscapes (old-fields), which have experienced the replacement of perennial vegetation with exotic annual grasses (Corbin & D'Antonio 2004; Suding et al. 2004). While there is extensive

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literature on this topic, many studies simply look at the direct effects of different manipulations on weeds without considering indirect effects, such as soil abiotic and biotic characteristics, and manipulations often lack benefits for native plant communities (Kettenring & Adams 2011).

Soil biota can have strong influences on restoration outcomes because they can affect plant communities in many ways (e.g. nutrient acquisition, pathogenic effects, nutrient cycling, decomposition; Packer & Clay 2000; van der Putten et al. 2001; van der Heijden et al. 2006; Ayres et al. 2009). Therefore, it is important to understand how active restoration can impact these communities. It has been demonstrated that the soil microbial community at restoration sites begin to resemble that of reference sites (Araujo et al. 2014; Gellie et al. 2017), become more connected, and take up more carbon (Morriën et al. 2017) over time; however, this is not always the case or can take many decades to occur (Steenwerth et al. 2002; McKinley et al. 2005). Therefore, understanding the role restoration techniques play in transitioning the soil biota is important. Some techniques can have a greater impact than others. For example, the addition of a carbon (C) source (straw and wood), to stimulate microbial activity and facilitate microbial nutrient immobilization, had little effects on the soil biota in a Dutch grassland restoration trail; however, top-soil removal (TSR) reduced bacterial, fungal, and nematode biomass (Kardol et al. 2008). In a Montana ponderosa pine forest, only the additive effect of two restoration practices, thinning plus burning, had an effect on the soil biota whereby actinomycetes became more abundant compared with controls (Gundale et al. 2005). The presence of plants is also an important driver of microbial communities in restoration sites (Potthoff et al. 2006). Given that there are so few studies that incorporate the response of soil microbes to site preparation techniques, we thought it was important to revisit this topic using DNA-based techniques to characterize these communities in more detail.

In old-field restoration, reducing high soil nutrient content due to previous fertilizer use is usually a main aim to prevent dominance of fast-growing annual weeds (Standish et al. 2006). Addition of C to the soil has been demonstrated to reduce soil fertility, enhance native plant establishment, and reduce weed cover (Blumenthal et al. 2003; Prober et al. 2005; Morris & de Barse 2013). Where weed cover is extensive, techniques that remove aboveground biomass, such as grazing or burning, could be used in combination with C addition for faster reductions in available nutrients and competition from established plants. A more extreme technique, TSR removes around five to 10 centimeters of top soil and is often the most effective at eradicating weeds and minimizing post-seeding management (Corbin et al. 2004; Gibson-Roy et al. 2010a). However, this technique is expensive and may have adverse effects on other environmental factors such as soil structure, soil microbial communities, and water holding capacity (Kardol et al. 2008) as well as landscape effects or ongoing management if the soil is not removed from site. While the broad effects of these site-preparation techniques on restoration success are well studied, very few studies have looked at the effects they have on the microbial community in combination with native plant establishment, weed cover, and soil properties. This holistic approach is needed to improve our understanding of the potential impacts of these commonly used techniques in restoration and to guide restoration approaches.

Here, we report results from a field experiment where we compared the efficacy of four techniques of site preparation to help restore old-fields to native grasslands in southern temperate Australia. We applied treatments of: fire, TSR (removal of top 50 mm of soil), slashing (vegetation cut to 30 mm, biomass removed), and C addition (saw-dust and sugar mixed) to an old-field dominated by annual grasses. We measured several responses to these treatments: soil bacterial community, native plant and weed biomass, native seedling emergence, and mortality and soil abiotic properties after one growing season. Our aim was to determine which technique was the most effective at promoting native grass establishment while reducing weed competition. We hypothesize that there will be strong links between abiotic and biotic conditions, whereby site-preparation techniques that have the strongest influence on soil properties will also have a strong impact on the plant and microbial communities. To our knowledge, this is the most comprehensive short-term study comparing site-preparation techniques that utilizes DNA-based methods, gaining novel insight into the effects on soil microbial communities.

Methods

Study Site and Sampling Design

The study was undertaken in an old-field at Para Woodlands Reserve, South Australia (34.628°S, 138.785°E). The region has a Mediterranean-type climate with a mean rainfall of 450 mm/annum and a mean annual air temperature of 23.6°C (BOM 2017). The study site was a cereal and sheep farm that received regular fertilizer application until farming ceased in 2004. The soil is characterized as deep brown and gray cracking clays (Rosser 2013; but see results for physiochemical properties). All plant species present at the site were weed species, dominated by winter-growing annual grasses, in particular *Avena barbata* Pott ex Link, *Lolium rigidum* Gaud., and *Bromus* spp. (100%, 17%, and 17% cover, respectively; Fig. S1).

In May 2015 (austral autumn), an area relatively homogeneous in floristic composition and topography was fenced to exclude livestock and other grazers. Inside we established 24 plots (each plot measured 3 m × 3 m), separated by a 1 m buffer, and randomly assigned one of four weeding treatments (control, burn, slash, and TSR), resulting in six replicates per treatment. One burn plot was later excluded due to an error in set-up. Low-intensity fire was used to remove the litter layer and expose bare soil in the burn plots. The slash treatment cut vegetation to a height of 30 mm and litter was removed with a rake. The top 50 mm of soil, and all vegetation and litter above, was removed using a shovel in TSR plots. Two subplots (1 m × 2 m) were established in each plot, separated by a buffer of 0.5 m, and randomly assigned to receive either C addition or not (hereafter add-C and no-C). We used an equal part mixture

of sucrose (white sugar) and saw-dust applied to the soil surface at 0.42 kg C m^{-2} immediately after the application of the other manipulations (Blumenthal et al. 2003; Prober et al. 2005).

Each subplot was divided in two (1 m × 1 m) to test two planting materials of native grasses (seeded or planted; see Fig. S2 for full layout). *Rytidosperma caespitosum* Gaud. seed was applied to the seeded side at a rate of 1.5 g m^{-2} (approximately 1,480 seeds) and 1 L of water was added before and after sowing to reduce loss due to wind and to promote germination. On the opposite side of the subplot, *R. racemosum* R. Br. were planted as tubestock plants after being grown from seed in the previous winter at South Para Nursery (Kersbrook Landcare Group Inc., Kersbrook, South Australia). Two different species were utilized due to limited seed availability; however, these species are closely related and are both winter-growing perennial grasses, native to the region. This was considered a suitable compromise for the purposes of this experiment because no direct comparisons were made between seeded and planted sides (hereafter planting material). Simplifying the plantings to single species allowed us to record plant responses in greater detail than complex communities We upheld relevance for practice by selecting species that are common in Para Woodlands Reserve and surrounding areas and are often used for restoration.

Data Collection

Soil cores (10 cm diameter, 10 cm depth) were collected at the time of harvest (i.e. late australspring). Two subsamples were taken from both the seeded and planted sides at random points and homogenized. Care was taken to use sterilized equipment. For microbial genomic analysis, a representative 50 g sample of soil was collected from the samples on the seeded side, and stored on ice until frozen. Soil DNA extractions, PCR amplification, and sequencing were undertaken at the Australian Genome Research Facility (AGRF, Adelaide, Australia). Sequencing data were processed using the QIIME set of bioinformatics software, resulting in the identification and abundance of operational taxonomic units (OTUs). To account for differences in the sequencing depth we rarefied OTU abundance to the technical replicate with the lowest number of reads using the "single_rarefaction.py" function in QIIME (Weiss et al. 2017; see rarefaction curves in Fig. S3). Details of methods from extractions to bioinformatics can be found in Supplement S1. Soil physiochemical analysis was carried out at CSBP Limited (Bibra Lake, Western Australia) and included nitrate-N, ammonium-N, plant-available (Colwell method) phosphorus, potassium, sulfur, organic C, electrical conductivity (EC), and pH ($CaCl_2$).

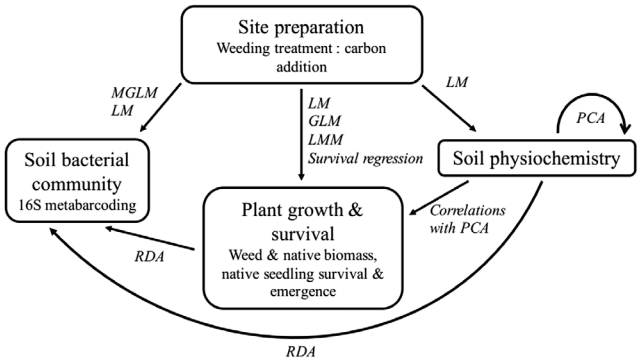

Figure 1. Diagram summary of treatments used (site prep.), data collected (bacteria, plant, and soil), and the analysis used to explain the results (italics). LM, linear model; GLM, generalized LM; MGLM, multispecies GLM; LMM, linear mixed model; PCA, principal component analysis; RDA, redundancy analysis. Arrows point from explanatory variables to dependent variables for each analysis type.

Emergence of native grass seedlings was recorded fortnightly in sampling quadrats (30 cm × 30 cm). Four quadrats were outlined in each seeded subplot, located 10 cm from the edge, and 20 cm from each other, and two were randomly chosen for seedling counts (Fig. S2). After peak emergence, 30 randomly selected individual seedlings within each of the quadrats were marked and monitored for survival over the growing season (note that one TSR quadrat only had 29 seedlings and two control quadrats had 25 and 15 seedlings). Mortality of tubestock plants was also recorded fortnightly in the planted subplots and dead plants were replaced. Aboveground biomass was harvested after peak plant growth (November 2015) and dried at 60°C for 24 hours before being weighed.

Statistical Analysis

The statistical methods are summarized in Figure 1 to demonstrate how they link the response variables but are separated here under two headings for simplicity. All statistics were performed in R version 3.1.1 (R Core Team 2017).

Statistical Analysis: Site Preparation and Plant Responses

In order to test for a relationship between soil physiochemical properties and plant growth we first summarized the variance in soil physiochemical properties across all sites using principal component analysis (PCA) using the *vegan* package in R (Oksanen et al. 2017). We checked for correlations between plant biomass (weeds and natives independently) and the first two principal components in order to assess the effects of soil properties and nutrient availability on plant growth. Pearson's product-moment correlation coefficient was used to test the strength of the correlations.

We then used linear models where response variables (soil properties and plant biomass) were analyzed as functions of weeding treatment (including control, burn, slash, and TSR), C addition (add-C and no-C), and planting material (seeded and planted; where appropriate), and their interactions. Where significant differences were detected with analysis of variance (ANOVA) tests, tests of pairwise comparisons (Tukey's contrast analysis) or general linear hypothesis tests (GLHT) were made using the R package *multcomp* (Hothorn et al. 2008). All models were graphically checked for their error distributions and homogeneity of variances and data were transformed using square root or log transformations where necessary to meet parametric assumptions.

We used three models to determine if site preparation (i.e. weeding treatment and C addition) affected the establishment of native grasses. First, the accumulated seedling emergence was tested against weeding treatment, C addition, and time (days since seeding) with a linear mixed model using the *lme4* package in R (Bates et al. 2014). An individual plot identification number was included as a random effect to account for the repeated measures taken over time. Second, seedling mortality was analyzed using parametric survival regression model with a Weibull distribution using the R package *Survival* (Therneau 2015). Lastly, final mortality (proportion of dead plants by day 141) was compared using a generalized linear model (GLM) where the response was binomial (0 = alive, 1 = dead) and tested as a function of weeding treatment and C addition.

Statistical Analysis: Site Preparation and Bacterial Community Responses

We carried out a redundancy analysis (RDA), using the *vegan* package in R (Oksanen et al. 2017), in order to assess how much of the variation in rarefied soil bacterial communities is explained (constrained) by the combination of soil properties, weed growth, and native seedling growth. Potassium and PBI were both removed prior to RDA as they correlate strongly with phosphorus. Forward selection was used to identify the variables that were sufficient explanatory variables (i.e. the addition of further variables did not provide any more explanatory power). Partial RDAs were also carried out to attribute the proportion of variance explained by each explanatory variable separately, after accounting for all other variables.

To support the RDA results we calculated OTU richness, that is, the number of unique OTUs in each sample, and Pielou's evenness (Pielou 1966), that is, the relative abundance of different OTUs in each sample. Pielou's evenness (J') was calculated as equation 1,

$$J' = \frac{H'}{\log(\text{richness})} \quad (1)$$

where H′ (known as Shannon–Wiener diversity) is calculated using equation 2.

$$H' = -\sum pi \ln pi \quad (2)$$

We then used linear models to associate OTU richness and Pielou's evenness with weeding treatment and C addition included as main effects. Model checking and posthoc testing were carried out with the same method as the plant biomass data above.

To test how the experimental factors shaped the bacterial community composition we used multispecies GLMs. GLMs explicitly model the mean–variance relationship characteristic of ecological counts, and are therefore recommended over distance-based methods such as ordination or PERMANOVA (Warton et al. 2012). Models were fitted using the *mvabund* package (Wang et al. 2012) with a negative binomial probability distribution. The explanatory variables weeding treatment and C addition were considered and significance tests were carried out using likelihood-ratio tests (ANOVA, pit-fall resampling, 300 bootstraps). This function also provided univariate tests for each OTU where *p*values were adjusted for multiple testing.

Results

Site Preparation and Plant Responses

Principal component analysis of soil physiochemical properties summarized 36.9% and 19.7% of the variance in the first two PCs. Measures of biomass for weeds and native tubestock and seedlings were then correlated against these first two PCs (described below). Positive values of principal component 1 reflect highly alkaline, low nutrient conditions, whereas more negative values reflect high nutrient, more acidic soil conditions. For principal component 2, negative values reflect higher ammonium content, and more positive values are found in soils with higher nutrient content and higher alkalinity (Fig. 2A). Results from linear models of each soil property as a function of weeding treatment, carbon addition, and planting material are presented in Table 1 and Table S1 (Table S2 has values of soil properties at the beginning of the experiment).

Weed biomass had a negative correlation with PC1 and a positive correlation with PC2 (Pearson's $r = -0.55$ and 0.33, respectively, $p < 0.01$), showing that weeds grew better in more nutrient-rich, acidic soils. The lowest biomass of weeds was found in the TSR plots, which generally had the most negative values of both PC1 and PC2 (Fig. 2A,B). This was supported by linear models of weed biomass as a function of weeding treatment, C addition, and planting material (and their interactions; see Table S3 for ANOVA results), whereby TSR had significantly less weed biomass than the controls (Tukey, $t = -21.1$, $p < 0.01$). Slashed plots also had less weed biomass than controls (Tukey, $t = -6.2$, $p < 0.01$), and weed biomass was lower in the add-C subplots than no-C subplots (ANOVA, $F = 18$, $p < 0.01$), and in planted subplots than in seeded subplots (ANOVA, $F = 32$, $p < 0.01$).

All correlations for the native plants contrasted with those for weed biomass, with positive correlations against PC1 and more negative correlations against PC2 (Fig. 2A,B). The correlations were highly significant against PC1 (Pearson's $r = 0.62$ and 0.63 for seedlings and tubestock, respectively, $p < 0.01$), suggesting that soil pH and nutrient content are strong predictors of native plant growth, where growth was greater in more alkaline, nutrient-poor soils. The correlations between biomass and PC2 were not significant for seedlings or tubestock (Pearson's $r = -0.09$ and -0.19, $p = 0.55$ and 0.2, respectively). The TSR plots resulted in the greatest biomass of native plants, while also generally having the most positive values of PC1 and most negative values of PC2. Again, this was supported by linear models of seedling and tubestock biomass against weeding treatment and C addition (and their interaction; see Table S4 for ANOVA results). For tubestock biomass, TSR alone resulted in higher biomass than all other weeding treatments (Tukey,

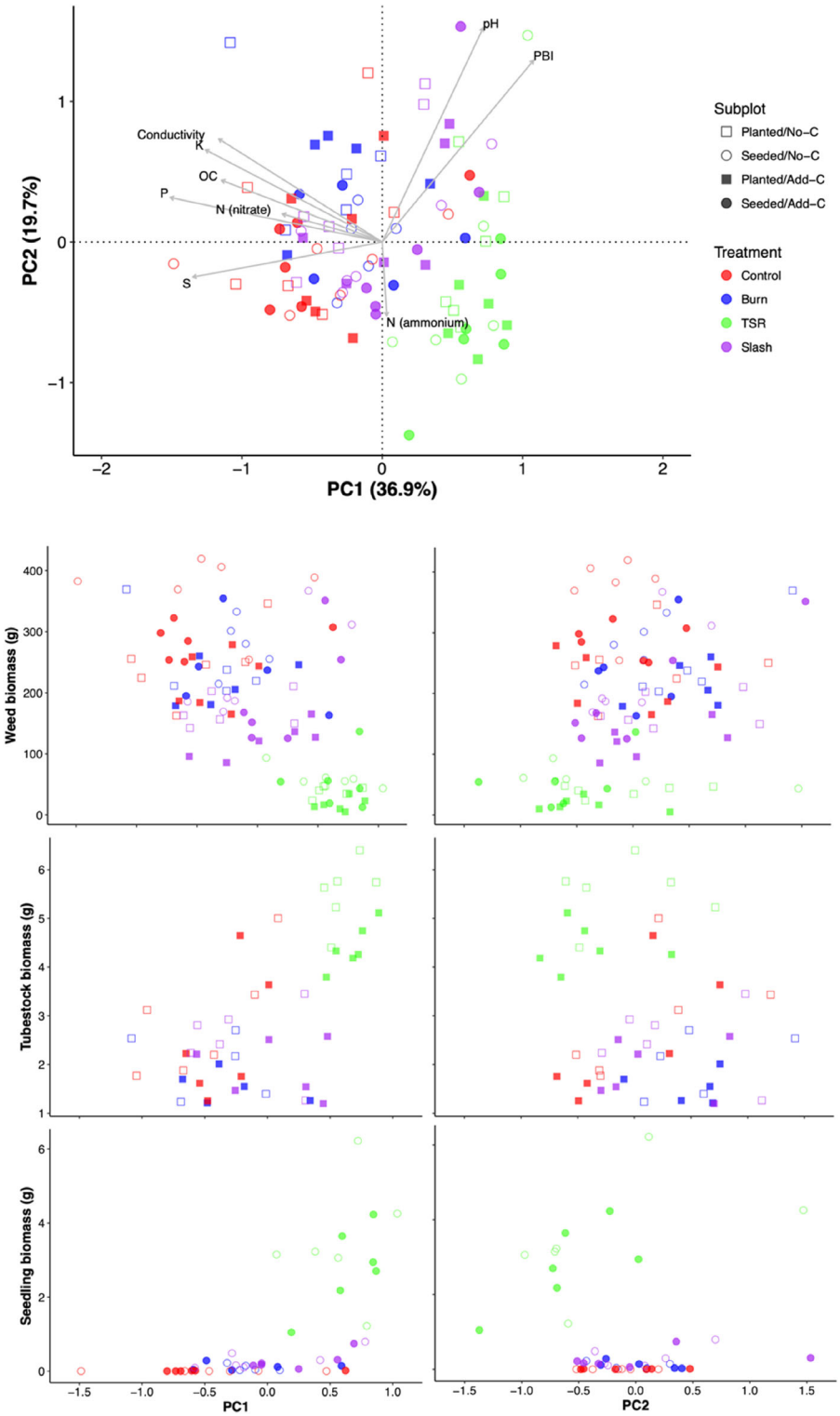

Figure 2. Relationships between soil physiochemical properties and weed and native plant biomass. (A) Principal component analysis (PCA) biplot of soil physiochemical properties of soil samples from 92 subplots; (B) correlations between plant biomass and the first two principal components from the PCA analysis of soil physiochemical properties shown in (A). Weed biomass (top plots) were measured in all 92 subplots, native tubestock (middle plots) and seedling (bottom plots) biomass were measured in 46 subplots each.

Table 1. Mean (± SE) of soil physicochemical properties for each treatment (n = 6 except for burn n = 5). Following ANOVA results (see Table S1) planned contrasts were made (where appropriate) using Tukey's honest significant difference tests. Significant differences ($p < 0.05$) are indicated by "*" for weeding treatments that differ to its corresponding control, "A–B" for differences between C (carbon) addition, and "y–z" for differences between planting material.

Weeding Treatment	*C Addition*	*Planting Material*	*NH_4^+–N (mg/kg)*	*NO_3^- – N (mg/kg)*	*P Cowell (mg/kg)*	*K (mg/kg)*	*Organic C (%)*	*S (mg/kg)*	*Conductivity (dS/m)*	*pH (1:5 $CaCl_2$)*	*PBI*
Control	No-C	Planted	6.2 ± 0.5	10.7 ± 1.1	34.7 ± 3.6	939 ± 36	$2.9 \pm 0.05^{A,y}$	19.8 ± 0.89^{A}	0.19 ± 0.01	6.3 ± 0.2	70.6 ± 5.9
Control	No-C	Seeded	5.8 ± 0.3	8.7 ± 2.0	31.2 ± 2.0	901 ± 14	$3.0 \pm 0.11^{A,z}$	16.4 ± 0.88^{A}	0.19 ± 0.03	6.0 ± 0.1	67.6 ± 4.5
Control	Add-C	Planted	7.3 ± 0.6	5.8 ± 0.7	30.5 ± 2.6	966 ± 30	$3.4 \pm 0.13^{B,y}$	18.2 ± 3.02^{B}	0.16 ± 0.00	6.1 ± 0.2	66.5 ± 3.9
Control	Add-C	Seeded	6.2 ± 0.5	5.5 ± 0.6	29.0 ± 2.9	900 ± 20	$3.5 \pm 0.09^{B,z}$	17.6 ± 2.32^{B}	0.22 ± 0.01	6.0 ± 0.1	65.8 ± 4.8
Burn	No-C	Planted	6.2 ± 0.2	8.6 ± 0.5	31.8 ± 2.5	$1{,}060 \pm 29^{*y}$	$3.0 \pm 0.14^{A,y}$	18.1 ± 1.61^{A}	0.18 ± 0.03	$6.6 \pm 0.1^{*}$	74.7 ± 3.5
Burn	No-C	Seeded	4.8 ± 0.4	8.6 ± 1.2	27.0 ± 2.1	873 ± 5^{z}	$3.0 \pm 0.09^{A,z}$	15.1 ± 1.36^{A}	0.17 ± 0.01	$6.3 \pm 0.1^{*}$	76.5 ± 4.3
Burn	Add-C	Planted	7.4 ± 0.2	7.4 ± 1.0	31.2 ± 3.9	996 ± 12	$3.2 \pm 0.07^{B,y}$	16.2 ± 0.95^{B}	0.19 ± 0.02	$6.5 \pm 0.2^{*}$	68.4 ± 2.9
Burn	Add-C	Seeded	6.4 ± 1.0	5.2 ± 1.3	27.6 ± 3.2	927 ± 15	$3.5 \pm 0.33^{B,z}$	12.7 ± 1.73^{B}	0.17 ± 0.02	$6.2 \pm 0.1^{*}$	67.8 ± 3.2
TSR	No-C	Planted	5.7 ± 0.3	$7.2 \pm 0.7^{*,A}$	$14.3 \pm 0.6^{*}$	$829 \pm 17^{*}$	$2.0 \pm 0.07^{*,A,y}$	$14.4 \pm 0.85^{*,A}$	$0.14 \pm 0.01^{*}$	6.4 ± 0.2	81.6 ± 4.2
TSR	No-C	Seeded	3.8 ± 0.5^{A}	9.5 ± 0.7^{B}	$13.7 \pm 1.2^{*}$	820 ± 23	$2.1 \pm 0.05^{*,A,z}$	$12.3 \pm 0.84^{*,A}$	$0.11 \pm 0.02^{*}$	6.4 ± 0.2	75.4 ± 2.7
TSR	Add-C	Planted	7.8 ± 0.3	$3.8 \pm 0.7^{*,A}$	$13.0 \pm 0.9^{*}$	$838 \pm 19^{*}$	$2.5 \pm 0.09^{*,B,y}$	$14.0 \pm 0.60^{*,B}$	$0.11 \pm 0.01^{*}$	6.2 ± 0.1	73.8 ± 9.3
TSR	Add-C	Seeded	$14.3 \pm 4.4^{*,B}$	6.7 ± 0.3^{B}	$14.7 \pm 1.2^{*}$	$765 \pm 10^{*}$	$2.2 \pm 0.12^{*,B,z}$	$13.8 \pm 1.12^{*,B}$	$0.13 \pm 0.02^{*}$	6.3 ± 0.1	74.3 ± 5.3
Slash	No-C	Planted	5.5 ± 0.2	8.7 ± 0.7	32.2 ± 2.2	$1{,}004 \pm 22^{y}$	$2.8 \pm 0.03^{A,y}$	15.5 ± 1.36^{A}	0.15 ± 0.01	$6.5 \pm 0.3^{*}$	73.6 ± 4.6
Slash	No-C	Seeded	5.8 ± 1.0	8.3 ± 0.9	27.8 ± 3.1	882 ± 31^{z}	$3.2 \pm 0.11^{A,z}$	14.6 ± 1.42^{A}	$0.14 \pm 0.01^{*}$	$6.3 \pm 0.2^{*}$	77.4 ± 4.3
Slash	Add-C	Planted	6.2 ± 0.6	4.7 ± 0.4	27.0 ± 2.5	936 ± 19	$3.0 \pm 0.15^{B,y}$	14.0 ± 1.03^{B}	0.15 ± 0.01	$6.4 \pm 0.2^{*}$	72.2 ± 5.1
Slash	Add-C	Seeded	5.3 ± 0.3	4.7 ± 0.8	26.3 ± 1.0	872 ± 15	$3.2 \pm 0.14^{B,z}$	12.2 ± 1.08^{B}	$0.14 \pm 0.01^{*}$	$6.4 \pm 0.2^{*}$	76.4 ± 8.4

$t = 6.8$, 9.1, and −8.2, $p < 0.01$ for all), and adding C resulted in lower biomass (ANOVA, $F = 11.3$, $p < 0.01$). For seedling biomass, on the other hand, all weeding treatments resulted in higher biomass than the control (Tukey, $t = 4.6$, 7.1, and 14.3, all $p < 0.01$), and C addition had no effect (ANOVA, $F = 0.01$, $p = 0.6$).

Burn and slash plots had higher seedling emergence than controls, particularly at day 111 when these treatments had nearly three times as many seedlings (GLHT, $z = -5$, and −5.2 for burn and slash, respectively, $p < 0.01$ for both; Fig. 3A). The difference between TSR and control plots was never statistically significant but became marginally significant (GLHT, $z = -2.7$, $p = 0.06$; Fig. 3A) at day 111 when TSR plots had nearly double the number of seedlings. Seedling mortality was greatest in control plots with only around 25% surviving by day 70 (Fig. 3B). Seedlings had the highest rate of survival in TSR plots and, while the rate of mortality increased rapidly after day 111 in burn and slash plots, survival remained higher in all treatments than controls (Fig. 3B; Tukey, $t = 9.7$, 13.5, and 13.2 for burn, TSR and slash respectively, $p < 0.01$ for all). See Tables S5 and S6 for ANOVA results.

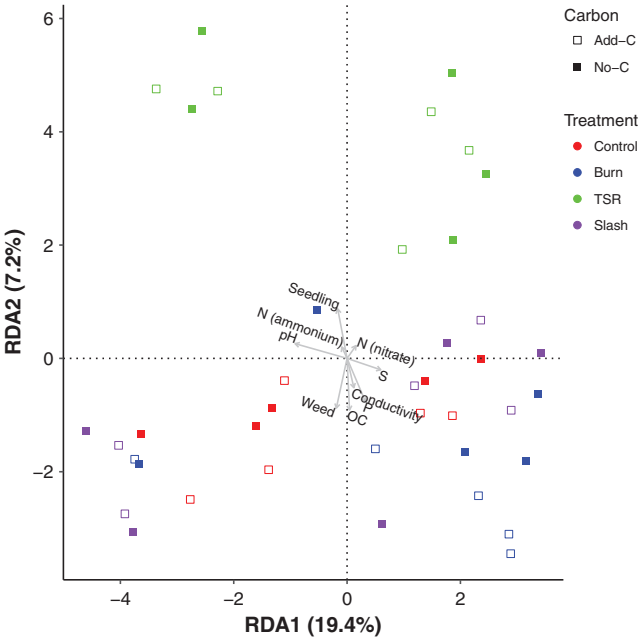

Figure 4. Redundancy analysis (RDA) biplot (axes 1 and 2) representing variation in the bacterial communities (colored symbols) in plots under different site preparation techniques. Arrows point in the direction of maximum variability explained by the respective soil physiochemical variables and measures of plant biomass (weeds and natives). Percentages in brackets show the percentage of variance explained by each axis.

Site Preparation and Bacterial Community Responses

In the full RDA, 42.7% of variance in the bacterial species was explained by soil properties and weed biomass ($F = 2.5$, $p < 0.01$, permutation test; Fig. 4). Forward selection identified pH, weed biomass, and ammonium content as sufficient explanatory variables suggesting that these variables had the strongest relationship with soil bacterial communities. The proportions of variance explained by each variable separately after conditioning on all other variables, as determined in the partial RDA, are given in Table 2, with pH (5.6%), weed biomass (4.1%), and ammonium content (2.6%) independently explaining the greatest proportions of variance.

The mean OTU richness (±SE) per sample ranged from 955 ± 23 to 1,131 ± 33 (see Table S7 for ANOVA results). The only difference with C addition was in TSR plots (Tukey, $t = -2.7$, $p = 0.05$) where there were fewer OTUs when C was

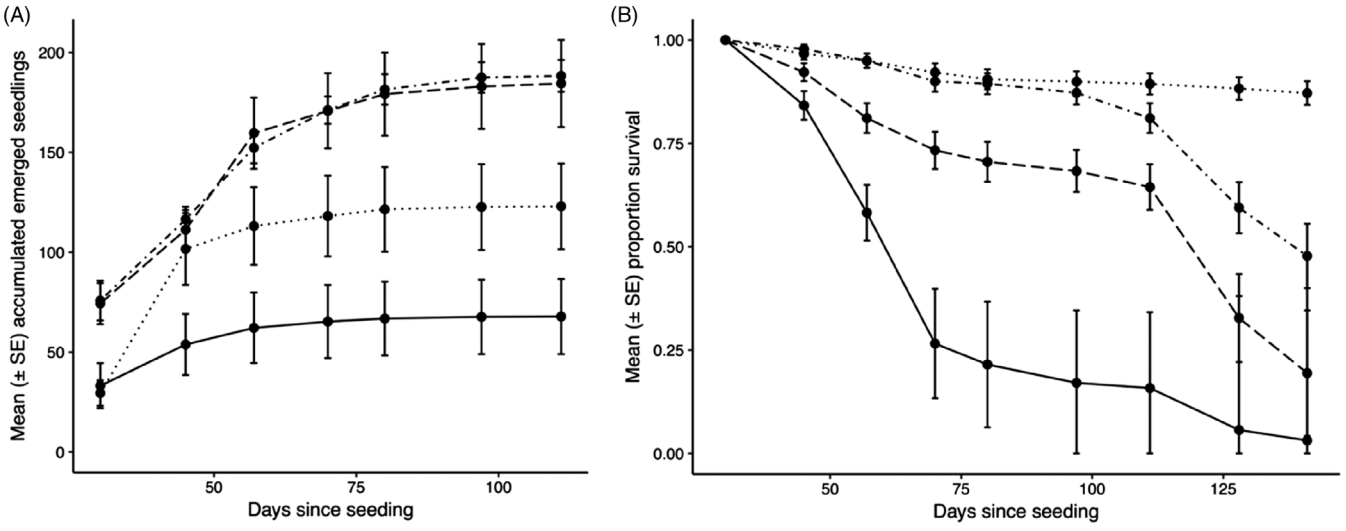

Figure 3. *Rytidosperma caespitosum* seedling (A) emergence ($N = 161$) and (B) survival ($N = 367$) rates during the experiment. Seeds were applied to plots on 2 June, 2015 (beginning of winter). Line type indicates the weeding treatment; solid, control; dashed, burn; dotted, TSR; and dot-dash, slash. Note points are mean values and error bars (±SE) do not appear below zero (see Tables S5 and S6 for ANOVA results).

Table 2. Proportions of variance explained by each variable separately, as determined in the partial RDA.

Variable	*Proportion of Variance Explained*
pH ($CaCl_2$)	0.057
Weeded	0.041
Ammonium	0.026
Nitrate	0.025
Conductivity	0.024
Phosphorus	0.022
OC	0.021
Sulfur	0.021
Seedling	0.020

added (955 ± 23 and 1,049 ± 31). In the add-C subplots, OTU richness was higher in slash (1,131 ± 33) than in other treatments: controls (1,021 ± 15, Tukey, $t = -3.1$, $p = 0.04$), burn (1,022 ± 25, Tukey, $t = -3.1$, $p = 0.04$), and TSR (955 ± 23, Tukey, $t = -4.9$, $p < 0.01$), but there were no differences between treatments in no-C subplots. Overall, there was not a substantial difference between Pielou's evenness (±SE) per sample (between 0.79 ± 0.02 and 0.83 ± 0.02) even though there were some statistically significant results (Table S7). The evenness of controls (0.83 ± 0.01) was higher than burn (0.81 ± 0.004, Tukey, $t = 2.9$, $p = 0.03$) and TSR (0.80 ± 0.01, Tukey, $t = 4.2$, $p < 0.01$) plots, and evenness in slashed plots (0.83 ± 0.01) was higher than in TSR plots (Tukey, $t = -3.6$, $p = 0.01$).

The multivariate GLM found that soil bacterial community composition was significantly different between add-C and no-C subplots, but this difference was only slight ($p = 0.05$), and there was a significant effect of weeding treatment ($p < 0.01$; Table S7). See Figure S4 for relative abundances of the bacterial phyla per treatment. Results were similar when using PERMANOVA except carbon addition was no longer significant (abundance data, $F = 2$, $p = 0.054$; presence/absence data $F = 1.3$, $p = 0.18$; Table S8, Fig. S5). Planned comparisons between weeding treatments found that the bacterial communities in TSR plots were significantly different to other treatments (p-adjusted = 0.026 for all comparisons), but there were no other differences. After controlling for multiple testing, the abundances of 35 OTUs could be explained by the main effects but only 12 were found in high abundance (> 1,000 reads; see Table S9 for details).

Discussion

Site preparation prior to restoration efforts is known to be a key step in the success of any restoration project (Hobbs 2007). When restoring old-fields back to native vegetation, site preparation techniques that reduce residual soil nutrients often have the most success in reducing weed competition and establishing sustainable target plant communities (Blumenthal et al. 2003; Corbin & D'Antonio 2004). However, the effects of site preparation on soil microbial communities and the role that these communities may play in achieving restoration success are seldom tested. Here, we demonstrate that preparation techniques that had the greatest impact on soil physiochemical properties resulted in the largest shift in soil bacterial communities, the greatest reduction in weed biomass, and the greatest production in restored native plants. This demonstrates clearly that the soil conditions in old-fields need to be greatly altered, away from high nutrient content and acidity and towards low nutrient content and alkaline, in order to favor native plant growth and establishment. Of the site preparation techniques included here, top-soil removal was the most effective at bringing about these changes, and therefore most suitable for restoring similar old-fields despite the low emergence of native seedlings. While the significance of the shifts in soil microbial community on native plant growth and establishment is less clear, we do clearly demonstrate that the microbial communities associated with successfully restored natives are considerably different from those where native restoration was less successful, suggesting that soil microbial communities could be an important factor in restoration success.

Bacterial Communities

Recent studies have shown that active restoration can have strong influences on the microbial community (Araujo et al. 2014; Gellie et al. 2017) but few have looked into how specific techniques can influence this change (Gundale et al. 2005; Kardol et al. 2008). The present study found evidence that TSR and C addition can change bacterial community structure, at least in the short term. This has important implications for restoration. For example, inoculation of mutualist soil biota is becoming more widely used in restoration (reviewed in Neuenkamp et al. 2019 for mycorrhizal inoculation) and the effectiveness of this technique may be compromised or enhanced by the site preparation techniques utilized. A restoration trial in the Netherlands found that TSR followed by inoculation improved restoration outcomes compared to inoculation without site manipulation (Wubs et al. 2016). While the reduction in nutrients surely was an important factor in that trial, perhaps the change in the microbial community brought about by TSR may have also been important. Future work is needed to explore this interaction between soil properties, soil biota (bacteria and fungi), and plant communities (but see Smith et al. 2018b).

Several studies suggest a change in the bacterial community can result in changes in plant performance (Packer & Clay 2000; Ayres et al. 2009), and we know from previous work at this field site that the microbial community in the old-field can induce negative effects, in terms of growth and survival, on native grasses (Smith et al. 2018a, 2018b). However, future work is needed to determine whether the changes recorded in this study could have affected plant performance, rather than being merely a result of plant performance. Of the OTUs whose abundances were affected by either C addition or TSR, we could not attribute their function to directly impact plant performance. However, the abundances of two possible cellulose metabolizers were reduced in TSR plots indicating possible indirect effects. Often, very little information on the function of OTUs was available or classification was too coarse. This highlights that, while genetic

tools show a lot of promise for expanding our knowledge on soil microorganisms, there is a need for better links between the description and function of microorganisms before they can be utilized to their full potential.

Plant Responses

The apparent success of TSR, that is, a two-to-five-fold reduction in weed biomass, growth of native plants doubled, and low native plant mortality, is supported by other trials in grasslands (Cole et al. 2005; Buisson et al. 2006; Gibson-Roy et al. 2010b), drained fens (Hedberg et al. 2014), and tropical areas (Bai et al. 2012). Even the somewhat negative result of fewer seedlings emerging on the seeded side was balanced by greater survival and growth of seedlings compared with the other treatments. For example, early in the season, seedling emergence was much greater in slash and burn treatments, and TSR did not differ much from the control, but this early benefit was not reflected in the biomass of the final harvest at the end of the experiment and was actually reversed, with TSR native seed biomass being significantly higher than the rest. The low seedling emergence in TSR plots suggests that this treatment can create a harsh environment (i.e. more exposed to the elements, possibly with less friable soil). Despite this, the larger seedlings and reduced competition from exotic plants in TSR plots would likely increase survival, compared with the other treatments (Lamont et al. 1993). Seedling survival could also be promoted by other methods, such as soil ripping, used in conjunction with these treatments (Commander et al. 2013). A longer-term study is certainly warranted to assess the survival of the seedlings through to the next growing season (austral winter).

The correlations between soil properties and plant biomass do indicate that a reduction in soil nutrients could be a contributing factor to the overall success of TSR. However, when C was added in an attempt to reduce nutrients, we found a reduction in biomass of both weeds and native plants even though soil nutrients became comparable to similar native grasslands (Prober et al. 2005; Cole et al. 2017). Therefore, it is likely other factors, or a combination of factors, result in TSR being successful. For example, a reduction in weed seed bank was probably a major contributor to reduced weed biomass in TSR plots (Verhagen et al. 2001) which could have knock-on effects for the native plant community.

Recommendations for Restoration

We found site-preparation to be a key step in restoring an old-field into a native grassland community, given the lack of seedling emergence or survival in control plots. Top soil removal resulted in the most severe reduction in weed biomass, soil fertility, and change in soil bacterial community demonstrating that a large shift away from the current soil conditions is required for the successful restoration of native species. It is important to note that TSR may not be suitable at all sites, particularly where soil depth is limiting; therefore, the mechanisms that explain its effectiveness should be considered to find suitable alternatives. In particular, a reduction in weed seed bank was probably a major contributor to reduced weed biomass in TSR plots and could be utilized to indiscriminately target weeds (Verhagen et al. 2001). Grazing and burning at the right time (i.e. before exotic species set seed) has been shown to reduce weed seed banks (Hastings & DiTomaso 1996; Stromberg & Kephart 1996); however, success may depend on the extent of weed seed banks and seed longevity and require repeated follow-up treatment, sometimes for several years (D'Antonio & Meyerson 2002). Given that burning and slashing had no restoration benefits in our study, even though we applied these treatments early in the season, we cannot recommend these for old-field restoration of degraded old-fields as single applications.

From this study, it is unclear whether the shifts in soil microbial community we saw in TSR plots compared to control and all other plots was a cause, consequence, or independent from the success of native plants in these plots. However, it has been shown previously that plant-microbe interactions are key to plant growth (van der Putten et al. 2001; van der Heijden et al. 2006). As such, restoration efforts that take into account soil microbial communities and act to shift the communities towards those that are associated with native plants via, e.g. inoculations, should deliver greater restoration outcomes. Future work should also include fungal communities as they can also influence plant communities (reviewed in van der Putten et al. 2001).

In conclusion, TSR was far superior to the other treatments in terms of nutrient and weed reduction and improved the growth of native species. The lack of native seedling emergence would need to be overcome if seed supply is scarce; however, this could possibly be achieved by cultivating before seeding to loosen the soil and reduce the loss of seeds due to wind or seed predators. Even though C addition was effective at reducing soil nutrients and weed biomass, the reduction in native biomass leaves us unable to recommend this technique for restoration practices of old-fields (but see Morris & Gibson-Roy 2017). Burning and slashing had little or no effect on weed biomass, native grass establishment, soil microbial communities, or soil nutrient availability and therefore are not suitable for restoration of this type of old-field, at least as a once-off application such as used here. In addition, TSR and C addition both prompted changes in the soil bacterial community composition, and given how important the plant–soil interactions have shown to be in shaping plant communities and their dynamics, further consideration is needed on how these may affect plant growth and community structure.

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Supporting Information

The following information may be found in the online version of this article:

Supplement S1. Bacterial data preparation

Table S1. *p*-values for ANOVA run separately for the soil properties as response variables (n = 6 except for burn n = 5)
Table S2. Mean (± SE) of soil physicochemical properties for each treatment (n = *6* except for burn n = 5)
Table S3. Results from the linear model for total aboveground biomass
Table S4. Results of linear models for biomass of *Rytidosperma racemosum* tubestock plants and *R. caespitosum* seedlings
Table S5. Results of linear model for accumulated seedling emergence
Table S6. Results of a parametric survival regression model with a Weibull distribution
Table S7. Soil bacterial community results from linear model
Table S8. PERMANOVA results on bacterial abundance and presence/absence (P/A) data
Table S9. Bacterial OTUs, with abundances greater than 1,000
Figure S1. Mean (± SE) percent cover of plant species during a pilot study at the field site
Figure S2. Example layout of four (out of 24) experimental plots which were randomly assigned as a control, burnt, slash or top-soil removed treatment
Figure S3. Rarefaction curves for bacterial OTU richness for each treatment combination
Figure S4. The relative abundance of the bacterial phyla and rare phyla (<1.5% reads) represented as "others"
Figure S5. Nonmetric multidimensional scaling (NMDS) ordination

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