RESEARCH ARTICLE

Topsoil Stockpiling in Restoration: Impact of Storage Time on Plant Growth and Symbiotic Soil Biota

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ABSTRACT

Addressing plant-soil relationships within restoration science may improve success and reduce costs. Here we assess the question of topsoil storage time: how does stockpile age impact plant biomass and soil microbial activity, particularly root symbionts such as rhizobia and arbuscular mycorrhizal fungi (AMF)? Working in Western Australia and in sandy soils, we grew a legume species *Acacia saligna* (Fabaceae) in one-, two-, three-, five- and ten-year-old stockpile soils under controlled glasshouse conditions. We assessed whether plant biomass, specific root length, and root diameter decreased with stockpile age. Further, we investigated how stockpile age affected the distribution and the number of effective nodules, nodule biomass and AMF colonization in roots. These above and belowground traits were chosen because they reflect the *A. saligna* response to growing in soils stockpiled for 1–10 years. We hypothesised that in older stockpiles there would be fewer rhizobial and AMF propagules which would constrain *A. saligna* growth, whereas in younger stockpiles there would be more rhizobial and AMF propagules, and *A. saligna* growth would be facilitated via these root symbionts. Using generalized linear mixed models, we found that total plant biomass was the lowest but AMF percent colonization was the highest when *A. saligna* was grown in ten-year-old soils, suggesting that AMF spores and hyphae are present in old stockpiles. Our results demonstrate that AMF communities may be initially disrupted by soil disturbance and storage, and then begin to re-establish between 5–10 years after stockpiling. However, other soil microbial communities, such as fungal pathogens that were not assessed in this study may have been responsible for decreased *A. saligna* biomass in older soils. Further research, particularly on other soil microbial communities, is needed to understand restoration success using stockpiled soil older than ten years.

Keywords: *Acacia saligna*, AMF, rhizobia, sandmining, Western Australia

Restoration Recap

- Understanding how long-term topsoil stockpiling affects beneficial root symbionts, which facilitate plant establishment and growth, is important for successful restoration outcomes using legumes.
- We examined how a legume, *Acacia saligna*, grew in post-mining topsoil stockpiles between one and ten years of age. We assessed whether soil age had an effect on beneficial rhizobia and mycorrhizal fungi, which assist legumes in acquiring limiting nutrients and water and are critical symbionts in nutrient impoverished soils.
- We found that ten-year-old stockpiles produced plants with the lowest biomass but highest mycorrhizal colonization compared with younger stockpiles.
- Our results suggest that mycorrhizal communities in stockpiled soils are re-establishing after between 5–10 years.
- Where feasible, open-air topsoil stockpile storage is recommended if long term (> 5 years) storage is necessary. Furthermore, natural or active revegetation of topsoil stockpiles is encouraged to facilitate re-establishment of belowground soil microbial communities.

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Plants depend heavily on soil microbes, especially root symbionts (e.g., rhizobia and mycorrhizal fungi), for survival and growth (Van der Heijden et al. 2008), especially in nutrient poor environments. However, above and

belowground linkages between plants and their associated soil microbial communities have received little attention in ecological restoration (Kardol and Wardle 2010). The soil microbial community has been reported to impact plant performance, plant community structure, and ecosystem function (Zak et al. 2003, Van der Heijden and Horton 2009). Thus, preserving and re-assembling the above and belowground linkages and networks between plants and their associated soil microbial communities will likely assist in improving restoration success.

Under circumstances in which a complete restoration is required (i.e., after mining), one restoration technique with some promise is topsoil transfer, which involves moving topsoil from intact natural areas to degraded restoration sites, thereby retaining stored propagules, other soil biota, and nutrients (Koch 2007a). Typically, this practice occurs when a donor site is being destroyed (mining and urban expansion, for example) and a nearby recipient site is identified. Globally, topsoil transfer has been successfully used to achieve biodiversity targets of restoration in Australia (Koch 2007a), South Africa (De Villiers et al. 2004), Spain (Tormo et al. 2007), and Norway (Skrindo and Pedersen 2004), among others. Topsoil transfer comprises three stages: stripping of topsoil from a donor site (typically ~ 10 cm depth constituting the A soil horizon), stockpiling and transferring, and spreading over donor site (typically 2–15 cm [Koch 2007b]). Duration of stockpile storage has an impact on restoration outcomes, with several studies suggesting minimal storage time or direct transfer whenever possible to maximize germination rates (Rokich et al. 2000, Parrotta and Knowles 2001, Koch 2007a). Typically, however, logistics require some period of storage, which may range from weeks to years (Strohmeyer 1999).

The process of removal and transport of topsoil physically disturbs the soil horizons. Subsequently, storage in stockpiles, especially over longer time periods, continuously modifies the physical, chemical, and biological conditions of the soil. These changes include reduction in aggregate stability and resistance to compaction (Abdul-Kareem and McRae 1984), reduction in soil water holding capacity (Miller and Cameron 1976), decrease in organic carbon with increasing storage time (Harris and Birch 1989), and changes in pH, nitrate levels, and available nutrients (Abdul-Kareem and McRae 1984). Microbial communities are also impacted by changes in soil physical and chemical conditions (Lauber et al. 2008). Harris et al. (1993) reported a considerable decrease in bacterial and fungal spore numbers in stockpiled soils compared with undisturbed soils, with effects larger with time and depth. Microbial communities, especially beneficial bacteria and mycorrhizal fungi, provide a number of positive services to plants, services that are especially relevant at early stages of plant growth and establishment, such as increasing plant growth (Guiñazú et al. 2010), improving resistance to abiotic stress (Selvakumar et al. 2012), and increasing photosynthetic capacity (Xie et al. 2009). Legumes in particular rely on soil mutualists (e.g., rhizobia and mycorrhizal fungi) for early establishment and growth (Pacovsky et al. 1986, Sprent and Parsons 2000). Thus, from an ecological restoration perspective, it is important to understand how long-term topsoil stockpiling may alter the soil chemical and microbial composition, which affects plant, especially legume, establishment and growth.

The aim of this study was to measure plant performance and soil microbial activity in relation to topsoil stockpile age. For this, we chose a study species commonly used in mine site restoration in Western Australia: the legume *Acacia saligna* (Fabaceae). Using stockpiles ranging in age from 1–10 years in sandy soils north of Perth, Australia we assessed plant performance (above and belowground biomass, specific root length, root diameter), colonization of roots by arbuscular mycorrhizal fungi (AMF) and nodulation rates and nodule biomass (*Acacia* forms N-fixing nodules in association with rhizobia). These above and belowground traits were chosen because they reflect the response of *A. saligna* to growing in topsoil stockpiled for 1–10 years. We hypothesised that soils in younger stockpiles would be more similar to reference soils in terms of their microbial composition (i.e., containing more rhizobia and AMF propagules), and thus we expected *A. saligna* to form symbioses with these organisms that would aid plant growth. Furthermore, we expected that soils in older stockpiles would be more degraded because of long-term storage, and over time the soil chemistry and microbial composition would have deteriorated; this would be reflected in the constrained growth in *A. saligna* because of absence of critical root symbionts. From a restoration perspective, the best outcome for revegetation using stockpiled topsoil would be successful establishment and growth of plants. In the context of the current experiment, this would be reflected by a higher biomass of *A. saligna* when grown in younger stockpiled soils as compared to *A. saligna* that were grown in soils that have been stockpiled for longer time periods (i.e., > 5 years). Specifically, we asked the following questions: 1) Does *A. saligna* performance, as measured by biomass, specific root length (SRL) and root diameter decrease with stockpile age? and, 2) How does stockpile age affect the distribution and the number of effective nodules, nodule biomass and mycorrhizal colonization in roots?

Methods

Study Site

Stockpiled soils were collected at the Tronox Ltd. (Tronox) Cooljarloo Mineral Sands Mine near Cataby, Western Australia, 170 km north of Perth (30.72° S, 115.42° E). The site is situated within the Swan Coastal Plain bioregion and experiences a Mediterranean climate with hot, dry summers and cool, wet winters (Jones et al. 2009). Long term

meteorological data (1962–2016) from the nearest Australian Bureau of Meteorology weather station (Badgingarra Research Station, 63.7 km north of Cataby) report mean monthly temperatures of 34.7°C in January and 7°C in August. Mean rainfall for January (summer) is 10.2 mm and for August (winter) is 84.1 mm (Bureau of Metereology 2016). The soils are composed mainly of acidic quartz sands (pH ~ 6, Bassendean sands) which are pale yellow to grey in color and overlaying clay or lateritic gravel at depths of 1–10 m. These sands are highly leached with very low quantities of clay or silt and low nutrient concentrations (Bettenay 1984, McArthur 1991).

Vegetation at the Coorjarloo site is a matrix consisting of five dominant vegetation types: dry heath, dry woodland, wet heath, wet woodland, and wetland (Bradshaw 2015). Dry woodlands are dominated by *Banksia* spp. (Proteaceae [e.g., *B. attenuata*, *B. ilicifolia*, *B. menziesii*, *B. prionotes*]), and heathland communities are dominated by *Melaleuca* spp. (Myrtaceae) and *Casuarina* spp. (Casuarinaceae; Bradshaw 2015]). Sampling in this study was restricted to woodland vegetation, which is by far the most extensive and speciose cover type in the area.

Before mining activity, topsoil stripping from remnant native vegetation is usually undertaken between April and October each year (Boonzaaler 2013). Stripping activities are avoided after recent rainfall to minimize the risk of spreading the dieback disease, *Phytophthora cinnamomi* (Boonzaaler 2013). Soil is stripped in two cuts with the first cut (upper 5 to 10 cm of topsoil) being stockpiled separately from the second cut (subsoil) to avoid mixing. Stockpiles are created as close as possible to the area from which they were stripped to minimize transportation effects and are usually 1–2 m in height (Boonzaaler 2013). In this study, only the soil from the first cut (topsoil) was collected for the glasshouse experiment.

Soil Collection

We limited our soil collection to the most common vegetation type, dry banksia woodland, which also afforded us the widest range of stockpile ages. Soil samples were collected from available stockpiles where we sampled one to three replicates at each of five ages: 1, 2, 3, 5, 10 years (Table 1). Each of these five ages had a corresponding reference site of undisturbed, intact woodland, giving us a total of n = 15 experimental units. Reference sites were selected based on mining company records and proximity of a) the closest representative but fully intact vegetation (i.e., unmined and with minimal weed presence), and b) proximity to the appropriate stockpiles (Bradshaw 2015). Importantly, the soil age distribution fit well with expectations and prior reports of rapid loss of seed viability with storage time (Golos and Dixon 2014).

Soil samples were collected in August 2014. For each stockpile and reference site, we collected five replicate samples yielding a total of 75 samples. Replicate collection points were spread over stockpiles and reference sites to capture inherent spatial variation in soils; for stockpiles, sampling spanned the length of the top of the stockpile, up to 10 cm deep after scraping away the surface layer and any litter, and for woodland, replicates were sampled from a 100-m² circular area at center and all cardinal directions. A total of 2 kg of soil was collected for each stockpile. Soil sampling and processing equipment was sterilized with 80% ethanol between piles. Soils were immediately transferred to a cooler in the field before being driven to laboratory facilities at Murdoch University on the same day and stored at 4°C. Soils were sieved through a 2-mm sieve in the laboratory to remove leaves and other coarse material and to homogenize samples.

Table 1. Year of stockpiling, stockpile age and number of replicates and reference sites for each topsoil stockpile soil used in glasshouse experiment collected from Tronox Ltd. Cooljarloo Mine, Cataby, Western Australia.

Year Stockpiled	Stockpile Age	Stockpile Replicates	Reference Site
2013	1	1	1
2012	2	2	1
2011	3	2	1
2009	5	3	1
2004	10	2	1
Total		10	5

Glasshouse Experiment

To investigate the microbial activity in stockpiled soils, we conducted a glasshouse experiment using *Acacia saligna* as a host species. *Acacias* have been used widely in mine restoration across Australia (Koch et al. 1996, Commander et al. 2013) because of their ability to fix nitrogen and their adaptation to nutrient-depleted soils (Langkamp et al. 1979). In this study, *A. saligna* was chosen because it is a fast growing native woody perennial legume, often used in post-mining rehabilitation, that is known to associate with both mycorrhizal fungi (Soliman et al. 2014) and nitrogen fixing bacteria (Sprent and Parsons 2000). Commercially sourced *A. saligna* seeds (PASES Pty Ltd., Perth, Australia) were placed in water just below boiling for one minute to promote germination (Clemens et al. 1977). All seeds were surface-sterilized in 6% bleach for 3 min and 80% ethanol for 1 min and sown on Petri dishes with Whatman filter paper. These were placed in growth cabinets for germination at a constant temperature of 18°C under dark conditions. Seedlings of uniform size were transplanted in October 2014 from Petri dishes into free-draining pots of 9 cm in diameter and 13 cm in depth, filled with 300 mL of field-collected soil from a given stockpile (five collected replicates per pile were bulked). A separate layer of 100 mL of pasteurized potting mix (70°C, 120 min cycle) was added to the bottom and the top of each pot, respectively. Five

Table 2. Classification of nodulation used to score the nodules from the glasshouse experiment. Reproduced with permission from Corbin et al. (1977). † Effectiveness judged on basis of nodule size and internal pigmentation; ineffective nodules not considered. ‡ Crown regarded as top 5 cm of root system.

	Distribution and number of effective nodules†	
Nodule score	Crown‡	Elsewhere
0	0	0
0.5	0	1–4
1	0	5–9
1.5	0	≥ 10
2	Few	0
2.5	Few	Few
3	Many	0
4	Many	Few
5	Many	Many

control pots were filled with 500 mL pasteurized potting mix. There were 80 pots in the glasshouse (ten stockpiles, five reference sites, five replicates of each). The location of pots within the glasshouse was randomized initially and fully re-randomized every week. Plants were watered daily with tap water and grown for 12 weeks. Glasshouse temperatures ranged between 18 and 25°C (night/day). At harvest, saplings were separated into shoots and roots. Shoot and root samples were oven dried at 80°C for 48 hours or until they reached a constant weight and then weighed to determine dry above and belowground biomass.

Belowground Traits

We used a variety of metrics to evaluate the condition of the soil biota, including specific root length (SRL [similar to leaf area, a measure of resource investment per unit area produced]), nodulation (a measure of nitrogen fixation and evidence of rhizobia present), and mycorrhizal colonization (evidence of AMF presence and a measure of phosphorus acquisition and uptake of water and other nutrients). A subsample of fresh root (~ 0.1 g) was collected to assess fine root traits (SRL, m/g); fine root length (m) per unit dry mass (g), and root diameter (mm), following standardized protocols (Cornelissen et al. 2003).

Nodulation Assessment

Roots were washed thoroughly with tap water and assessed for the distribution and number of effective nodules. Pink nodules indicate active N-fixing bacteria (i.e., rhizobia were present). Nodules were scored for each replicate according to Corbin et al. (1977) (Table 2). After scoring, nodules were removed from the roots and oven dried to a constant weight at 80°C (minimum of 48 hours) and then weighed.

Mycorrhizal Colonization

Small subsamples of fresh, fine roots (~ 0.5 g) were removed to assess status of AMF colonization among plants grown in different aged stockpiled soils. Fine roots for AMF colonization were stored in 60% ethanol until assessment commenced. Arbuscular mycorrhizal fungi colonization was assessed following modified protocols described in Vierheilig et al. (1998) and McGonigle et al. (1990). Preserved roots were cut into 1-cm long fragments and cleared by boiling in 10% potassium hydroxide solution for 10 min. Cleared roots where then rinsed in tap water for 2 min and boiled in 5% black ink (Parker Quink, France)-vinegar solution with pure household vinegar (5% acetic acid) for 3 min. Cleared roots where then rinsed in tap water for 20 min while being acidified with a few drops of vinegar. Nine randomly selected root fragments per replicate were mounted on one microscope slide and examined for AMF colonization giving a total of 36 and 180 intersections inspected for a replicate and a sample, respectively, which gave a total of 2880 intersections inspected (9 root fragments × 4 intersections × 5 replicates × 16 samples). Each root fragment was observed under a compound microscope (Leitz Diaplan, Germany) using a 40× objective and a 10× ocular lenses and checked for arbuscules, vesicles, and hyphae. Percent AMF colonization was determined using the magnified intersection method (McGonigle et al. 1990).

Soil Chemistry Analysis

We assessed soil chemistry to determine whether abiotic characteristics of soils varied between reference sites and stockpile sand with regard to stockpile age. A total of 15 samples (one per stockpile and reference site) were analysed for pH ($CaCl_2$ and H_2O), electrical conductivity, organic carbon (Walkley-Black), potassium (Colwell), sulphur (KCl 40), phosphorus (Colwell), ammonium ($NH4^+$) and nitrate ($NO3^-$). Soil samples were air-dried and analysed at the CSBP Soil and Plant Analysis Laboratory (Bibra Lake, Western Australia).

Statistical Analyses

To reveal the effects of topsoil stockpile age, we analysed a range of measures of seedling performance and condition, including mass (above and belowground, root:shoot ratio), root traits (SRL, root diameter), nodule score, and biomass as well as AMF colonisation. Our dataset was composed of multiple plants per stockpile and one to three stockpiles per age group. Given this nested data structure, we applied generalised linear mixed effect models where we assigned a random effect to stockpile and a fixed effect of age on each response variable. Each row of data corresponded to individual plants. The key objective was to examine the effect of stockpile age; therefore, analysis focused solely on stockpiles and with reference site data providing a benchmark comparison but not analysed. We followed

Table 3. Results of analysis of stockpile age effect on *Acacia saligna* seedlings biomass and root traits from soil collected from Tronox Ltd. Cooljarloo Mine, Cataby, Western Australia. * indicates a significant effect of stockpile age on plant biomass and root traits at $p < 0.05$. [a] p-value is 0.056. [†] Specific Root Length. [††]Arbuscular Mycorrhizal Fungi.

		Age			Intercept		
	Response	Estimate	SE	t-value	Estimate	SE	t-value
Biomass	Total Biomass	–0.19	0.08	–2.25*	3.68	0.41	8.87
	Aboveground Biomass	–0.12	0.06	–1.96*	2.48	0.30	8.20
	Belowground Biomass	–0.07	0.02	–2.93*	1.19	0.11	10.43
	Root: Shoot	–0.01	0.01	–1.96a	0.51	0.02	21.43
Root traits	SRL[†]	0.14	0.34	0.40	11.33	1.71	6.62
	Root Diameter	0.00	0.01	–0.54	0.33	0.03	12.05
	Nodule Score	–0.02	0.05	–0.36	3.92	0.23	17.23
	Nodule Biomass	–0.01	0.00	–1.89	0.10	0.01	6.93
	AMF[††]	5.85	1.72	3.41*	28.30	8.83	3.21

suggestions of Zuur et al. (2009) in preliminary data validation and model evaluation. During data validation, we discovered two extreme values of SRL (each > 2 standard deviations beyond the mean); the plants with these extreme observations were excluded from all data analyses. All analyses were performed in R (version 3.2.2, R Foundation, Vienna, Austria). The linear mixed models were conducted using the 'lme4' linear mixed-effects model package (Bates et al. 2015). Effects of stockpile soil age on soil chemical properties were assessed using one-way ANOVA, Tukey's HSD pairwise comparisons between stockpile groups were performed using "agricolae" package (Mendiburu 2015).

Results

Overall, our results revealed that stockpile age had a significantly negative effect on *A. saligna* total above and belowground biomass, with seedlings being smaller when grown in ten-year-old stockpiles (Table 3, Figure 1). Pairwise comparisons between stockpile age groups revealed that total biomass was significantly lower in ten-year-old than five-year-old stockpiles (effect size = –2.06, $p = 0.03$) and similar, though with a smaller effect size (effect size = –2.28, $p = 0.06$) in one versus ten-year-old stockpiles. Belowground biomass of *A. saligna* was significantly smaller in ten-year old stockpiles than in one- (effect size = –0.78, $p = 0.02$) and five- (effect size = –0.67, $p = 0.01$) year-old stockpiles (Figure 1). Root:shoot ratios also displayed a weak negative relationship with stockpile age (effect size = –0.01, $p = 0.056$) suggesting that root biomass as a portion of the plant decreased with stockpile age (Table 3).

Stockpile soil age had significant positive relationship with AMF percent colonization (Table 3, Figure 2). Colonization in five- (mean 69.1%) and ten- (mean 76.84 %) year-old stockpiles was significantly higher compared to one- (mean 25.00 %) and two- (mean 27.74%) year-old stockpiles

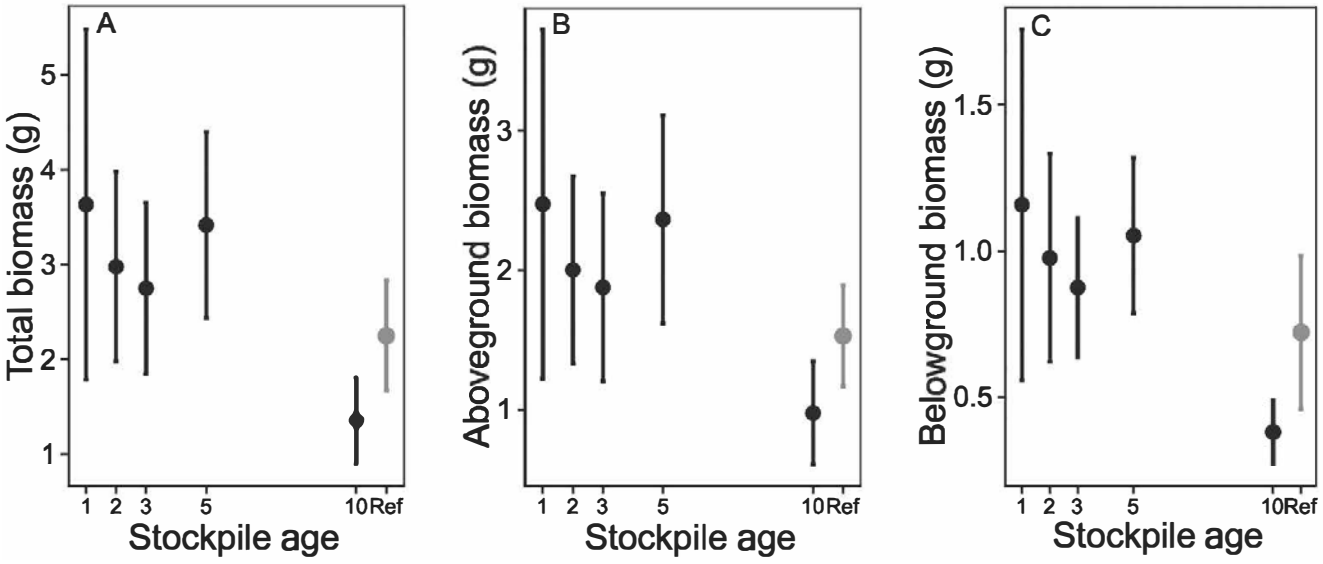

Figure 1. *Acacia saligna* A) total, B) aboveground, and C) belowground biomass after growing in topsoil from one-, two-, three-, five- and ten-year-old stockpiles from Tronox Ltd. Cooljarloo Mine in Cataby, Western Australia. Reference (Ref) sites are shown in gray. Values are means with 95% confidence intervals

Table 4. Soil chemical properties for one-, two-, three-, five- and ten-year-old stockpile topsoils and reference sites from Tronox Ltd. Cooljarloo Mine in Cataby, Western Australia, collected in August 2014. Values are means ± SE. Statistical analysis could not be conducted for one-year-old stockpiles due to low stockpile replicate numbers.

Soil Properties	Stockpile Age (years)					Reference Site
	1	2	3	5	10	
Conductivity (dS m^{-1})	0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.003	0.01 ± 0.004
N-$NH4^+$ (mg kg^{-1})	1.19	1.87 ± 0.40	2.08 ± 0.30	2.09 ± 0.21	0.68 ± 0.10	1.99 ± 0.47
N-$NO3^-$ (mg kg^{-1})	3.74	8.74 ± 1.67	8.37 ± 7.10	1.95 ± 0.49	3.84 ± 1.27	3.07 ± 0.99
Organic C (%)	1.31	0.84 ± 0.18	0.89 ± 0.14	0.72 ± 0.09	0.74 ± 0.01	0.91 ± 0.07
pH (H_20)	5.80	5.90 ± 0.10	6.00 ± 0.10	6.13 ± 0.14	5.95 ± 0.25	6.16 ± 0.09
pH ($CaCl_2$)	4.70	4.95 ± 0.05	4.95 ± 0.05	5.00 ± 0.32	5.00 ± 0.20	5.02 ± 0.09
Total K (mg kg^{-1})	6.65	10.56 ± 2.11	5.87 ± 0.45	5.39 ± 0.65	7.46 ± 1.17	9.03 ± 1.85
Total P (mg kg^{-1})	1.81	1.58 ± 0.19	2.11 ± 0.06	1.76 ± 1.19	2.31 ± 0.31	1.65 ± 0.07
Total S (mg kg^{-1})	1.10	2.40 ± 0.70	2.00 ± 0.80	1.33 ± 0.20	1.85 ± 0.15	1.60 ± 0.17

(five- versus one-year-old soils: effect size = 44.09, $p = 0.006$; five- versus two-year-old: effect size = 41.35, $p = 0.001$; ten- versus one-year-old: effect size = 51.84, $p = 0.005$; ten- versus two-year-old: effect size = 49.11, $p = 0.002$; Figure 2). Specific root length, root diameter, nodule score and nodule biomass were not affected by stockpile age (Table 3). Soil chemistry did not differ significantly between stockpiles and reference sites (Table 4).

Discussion

We found that topsoil age had a negative effect on *A. saligna* biomass, especially when it was grown in ten-year-old topsoils, suggesting that growth was impeded in these older stockpiles. *Acacia saligna* growth in older stockpiles may have been impeded for several reasons. For example, one explanation may be non-optimal pairings with its root endophytes, which resulted in high root colonization but yielded low growth benefits to the host (i.e., low to no nutrient supply; Gustafson and Casper 2006). Another mechanism explaining *A. saligna*'s low biomass in oldest soils may have resulted from microbial communities, e.g., fungal pathogens and antagonists that were not assessed in our study but may have affected host growth. Contrary to our predictions, AMF colonization was positively impacted by topsoil age, with *A. saligna* grown in five- and ten-year-old topsoils having the highest percent AMF root colonization.

Our findings support previous results by Jasper et al. (1987) who found that both disturbance (topsoil removal) and a period of storage contributed to the (initial) loss of AMF infectivity when plants, including *Acacia* spp., were grown in soils from four different mine sites, including Eneabba (100 km north of Cataby, Western Australia). However, these authors also reported that after five years of revegetation the number of AMF propagules was restored to a level similar to that of undisturbed soils. Several other studies have also shown negative effects of soil disturbance and storage on AMF colonization (i.e., Warner 1983). For example, some authors have shown that topsoil storage

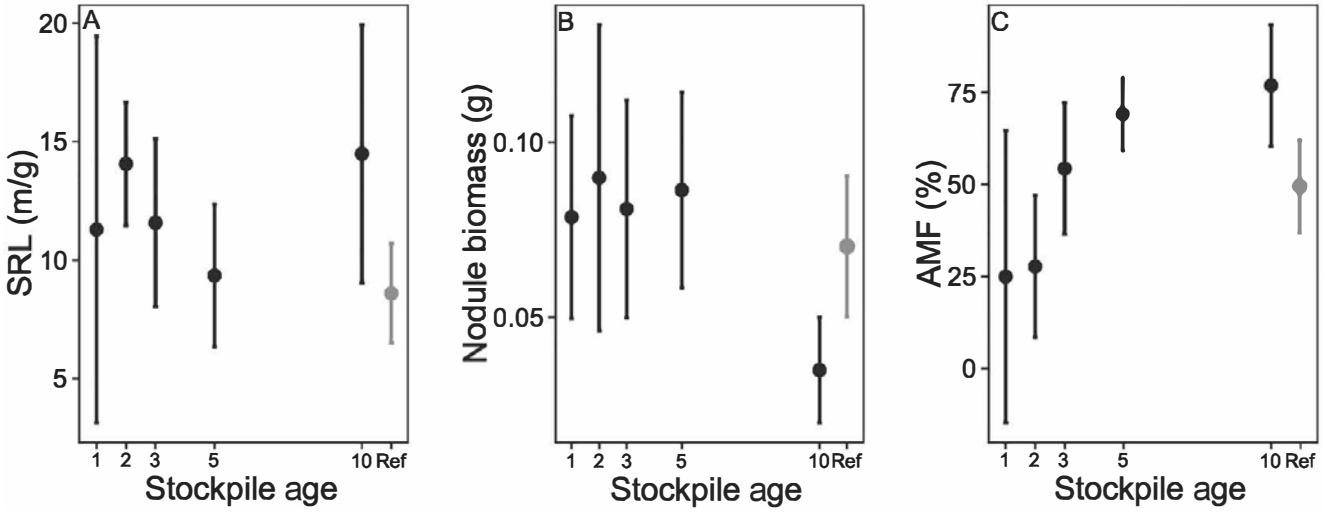

Figure 2. *Acacia saligna* A) specific root length (m/g), B) nodule biomass (g), and C) AMF percent colonization after growing in topsoil from one-, two-, three-, five- and ten-year-old stockpiles from Tronox Ltd. Cooljarloo Mine in Cataby, Western Australia. Reference (Ref) sites are shown in gray. Values are means with 95% confidence intervals.

for three years reduced AMF percent colonization of corn roots to 12%, compared with 85% in undisturbed soil (Gould and Liberta 1981). However, after four to five years of revegetation, the number of infective AMF propagules has been reported to be restored to a level that was similar to that of undisturbed soils (Jasper et al. 1987). We observed a similar increase in AMF % colonization in five and ten-year-old topsoils. Ross and Cairns (1981) found that microbial biomass in stockpiled soils was observed to be similar to that in soil from the surface of a ten-year-old stockpile and that of adjacent undisturbed soil. Similarly, a previous survey of root associated microbes at Cooljarloo mine showed that root biota had been re-established within all the rehabilitated sites seven to nine years after restoration (Dunstan et al. 2013). Thus, previous results, and ours, suggest that soil microbial communities, especially AMF, are initially disrupted by soil disturbance and storage and then begin to re-establish between five and ten years after stockpiling.

Presence and activity of soil microbial communities is likely to re-develop on older stockpiles. Microbes require carbon to survive, and older stockpiles are more likely to have dense vegetation cover that can provide them with necessary carbon. A recent unpublished study that assessed plant species richness in Cooljarloo mine in stockpiled soils as compared to extant vegetation found that highest soil seed bank similarity was between ten-year-old stockpiles and reference condition, (i.e., extant vegetation), suggesting that floristically, ten-year-old stockpiles begin to resemble the natural, surrounding vegetation (Bradshaw 2015). Visual inspection of the stockpiles also revealed heavy plant colonization starting from three- year-old stockpiles (pers. observation).

Although we found evidence for AMF presence in ten-year-old soils, *A. saligna* biomass, particularly belowground biomass, was lower in ten-year-old soils compared to other treatments. It is plausible that AMF in ten-year-old soils, despite being seemingly abundant, are less efficient in acquiring the phosphorus for *A. saligna* because of host-specificity towards particular AMF species. Recent studies have suggested, however, that *A. saligna* appears to associate with diverse fungal, as well as rhizobial, communities in Western Australia (Birnbaum et al. 2014, Birnbaum et al. 2016). Thus, it is unlikely that preference towards specific AMF would have caused lower P acquisition efficiency and reduced *A. saligna* biomass in ten-year-old topsoils. An alternative explanation for lower *A. saligna* biomass in ten-year-old topsoils may be higher susceptibility of saplings to plant pathogens. We did not describe the saprotrophic microbial composition of these soils, some of which may be competitors and antagonists to beneficial microbes and pathogens to seedlings (e.g., *Pythium* sp.; Dunstan et al. 2013). This warrants further investigation.

Soil abiotic conditions are often significant drivers of bacterial and fungal composition (Ettema and Wardle 2002, Lauber et al. 2008). However, it is likely that pedogenesis over ten years may restore the soil chemical properties in similar ways as have been found for microbial and plant communities. Indeed, Ross and Cairns (1981) found that mineral nitrogen and mineralizable nitrogen from the surface of a ten-year-old stockpile were similar to adjacent undisturbed soil. Notably, we did not detect any significant differences in soil chemical properties between different topsoil stockpiles and compared to references sites, suggesting that soil chemistry remains generally unchanged after stockpiling, at least in these kwongan ecosystems.

Our results showed that topsoil age had a significant negative effect on plant biomass and a positive effect on AMF percent colonization in ten-year-old stockpiles. These two observations are at odds with one another in relation to previously studied effects of AMF on plant biomass. Therefore, we suggest that there likely is an unaccounted-for effect of other biotic variables on observed results (i.e., small *A. saligna* biomass in ten-year-old soils), such as other soil microbial communities. This warrants further investigation using in-depth molecular tools (e.g., next-generation sequencing) that could elucidate the diversity of soil microbial communities more in-depth (e.g., soil pathogens). We suggest that future studies assessing the role of microbial communities in restoration in nutrient impoverished environments do the following: 1) describe the microbial communities from both stockpiled soils and from roots of bioassay plants, and 2) assess other abiotic conditions of stockpiles. For example, the water content of the different aged stockpiles may affect the survival of mycorrhizal fungal propagules (Miller et al. 1985).

Our results add to the body of restoration work showing that topsoil stockpiling is likely to initially alter the biotic conditions in the stockpiles, which contributes to changes in microbial communities. However, long-term stockpiling of topsoil, for longer than 5 years, facilitates development of micro ecosystems above and belowground, especially if stockpiles are left in the open air and are either actively revegetated or allowed to recolonize from adjacent native vegetation.

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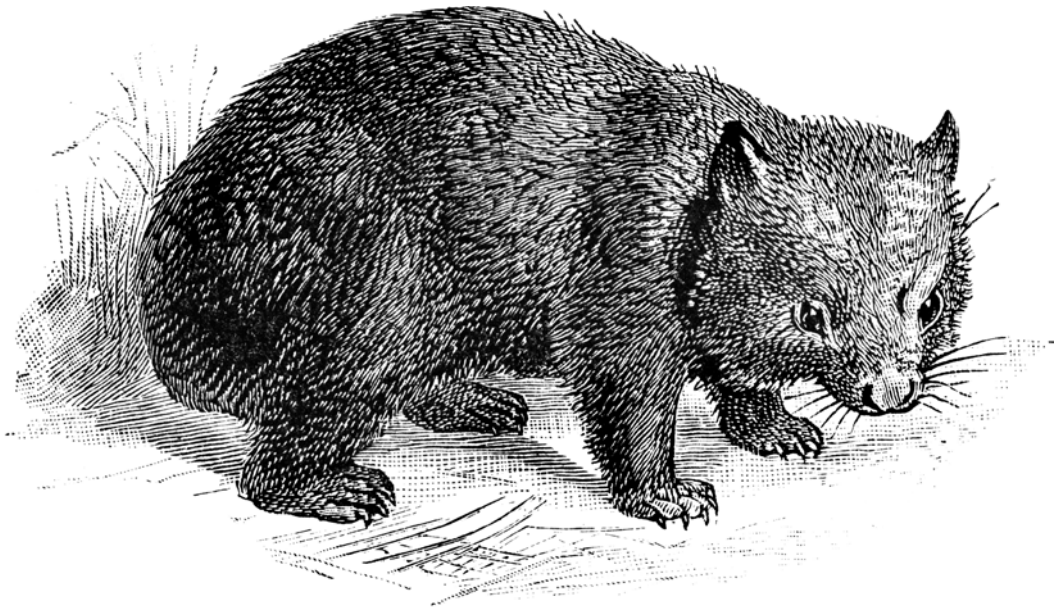

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