Research Article

Soil DNA chronosequence analysis shows bacterial community re-assembly following post-mining forest rehabilitation

Running headline: **Post-mining changes to soil bacterial communities**

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IMPLICATIONS FOR PRACTICE

- Considering soil microbiota in mine site rehabilitation and restoration is important for returning functional, self-sustaining biodiverse ecosystems and improving restoration practices.
- Bacterial community variation can be high among reference sites which highlights the need for appropriate sampling design in assessing soil microbial recovery trajectories.
- Monitoring of changes in bacterial communities through time can now routinely provide insights into the recovery of soil microbiota towards restoration targets.

ABSTRACT

Mining activities modify both above- and below-ground ecological communities, presenting substantial challenges for restoration. The soil microbiome is one of these impacted communities and performs important ecosystem functions but receives limited focus in restoration. Sequencing soil DNA enables accurate and cost-effective assessment of soil microbiota, allowing for comparisons across land use, environmental, and temporal gradients. We used amplicon sequencing of the bacterial 16s rRNA gene extracted from soil samples across a 28-year post-mining rehabilitation chronosequence to assess soil bacterial composition and diversity following rehabilitation at a bauxite mine in Western Australia's jarrah forest. We show that while bacterial alpha diversity did not differ between reference and rehabilitated sites, bacterial community composition changed dramatically across the chronosequence, suggesting strong impacts by mining and rehabilitation activities. Bacterial communities generally became increasingly similar to unmined reference sites with time since rehabilitation. Soil from sites rehabilitated as recently as 14 years ago did not have significantly different communities to reference sites. Overall, our study provides evidence indicating the recovery of soil bacterial communities towards reference states following rehabilitation. Including several ecological reference sites revealed substantial natural variability in bacterial communities from within a single mine site. We urge future rehabilitation chronosequence studies to sample reference sites that geographically envelope the rehabilitation sites and/or are spatially paired with rehabilitated sites to ensure this variability is captured and to improve any inferences on recovery.

INTRODUCTION

The global mining sector is reliant on access to mineral deposits and expansions into intact biodiverse ecosystems (Stevens & Dixon 2017). In Australia, it is estimated that mining has impacted approximately 10 million hectares of land (Grant 2009). Mining activities extensively modify landscapes, directly impacting on both above- (e.g., animal, plant) and below-ground ecological communities (e.g., soil microbiota) (Banning et al. 2011; Stevens & Dixon 2017; Kneller et al. 2018). These often-severe ecosystem impacts present challenges in restoring or rehabilitating biodiverse and functional ecosystems (Doley et al. 2012; Tibbett 2015). Indeed, as the ecological impacts of mining continue to grow, so does the need for improved understanding of how best to repair the damage done.

Restoration projects have tended to focus on recreating aboveground plant communities, often overlooking soil biodiversity (Heneghan et al. 2008; Farrell et al. 2020). However, there is increasing attention paid to soil biodiversity and plant-soil-biota relationships, which has largely been enabled by DNA sequencing technologies (Breed et al. 2019). The important role of soil in ecological restoration has long been known, especially regarding physical and chemical processes such as nutrient cycling and soil formation (Heneghan et al. 2008; Kardol & Wardle 2010). However, soil microbiota (i.e., communities of bacteria, archaea, eukaryotes) and their interactions within the soil system and with aboveground biota have received less attention (Harris 2009; Eisenhauer et al. 2017; Mendes et al. 2019). This presents a problem since soil microbiomes are highly diverse and functionally important ecosystem components and therefore understanding their ecology and

responses to both impacts and restoration or rehabilitation is integral to ecosystem restoration (Cameron et al. 2018; Delgado-Baquerizo et al. 2020).

Open-cut mining results in strong and long-lasting impacts on soil biotic and abiotic properties, including decreases in soil microbial activity and organic matter content, and changes in pH and salinity levels (George et al. 2010; Lewis et al. 2010; Sheoran et al. 2010; Banning et al. 2011). These impacts can be driven by the removal and stockpiling of topsoils for extended periods of time, which can expose soils to high temperatures and subsequent drying (Golos & Dixon 2014). Although best practice in the mining industry for open-cut rehabilitation is to stockpile topsoil for the shortest time possible (Lewis et al. 2010), in reality, topsoils are still routinely stockpiled for extended periods before being used to restore mine sites (Golos & Dixon 2014; Ngugi et al. 2018). The degradation of topsoils that occurs with their storage impacts on soil biodiversity and microbially-driven processes, such as organic matter decomposition, energy transfer, and aggregate formation (Harris 2009). Best practice should mitigate the impact of storage on soil properties by implementing 'direct return', where topsoil is removed from an active mining area and transferred directly – in as short a time possible – to a previously mined area ready for rehabilitation (Rokich et al. 2000; Tibbett 2010). While the intent of this 'direct return' process is to limit the impact of the mining process on soil properties, how the biological properties of soil respond following direct return of topsoil and subsequent rehabilitation is still unclear.

While the potential use of soil microbiota as an ecosystem indicator is beginning to be explored as part of an interrelated matrix of biotic and abiotic ecosystem

components (Muñoz-Rojas 2018; Tibbett et al. 2019), cause and effect relationships regarding the response of soil microbiota post-rehabilitation remains a notable knowledge gap. A pragmatic approach to begin to understand changes in microbiota with rehabilitation has been to use space as a proxy for time using a rehabilitation chronosequence design (i.e., sampling across a series of similar sites with different times since rehabilitation), and there are examples of this type of study in a post-mining context (Ngugi et al. 2018; Schmid et al. 2020; van der Heyde et al. 2020). Chronosequence studies provide an efficient approach to study the effect of time as an alternative to long-term longitudinal sampling (Walker et al. 2010). However, spatial and temporal confounding factors (e.g., spatial and/or temporal variation in soil, climate and rehabilitation methods), can impact inferences made from chronosequence studies (Pickett 1989; Fleming 1999). With variation of soil microbial communities being so scale dependant (Martiny et al. 2011; Fierer 2017), how spatial variability among reference sites impacts these chronosequence studies, and especially in regard to rehabilitation targets and completion criteria (Manero et al. 2021), needs to be assessed.

Recent advances in DNA sequencing technologies have enabled improved assessments of whole communities of soil microbiota compared to historical culture-dependent methods (Thompson et al. 2017; Breed et al. 2019; Berg et al. 2020; Nkongolo & Narendrula-Kotha 2020). One such method is to use high-throughput sequencing to generate amplicon datasets, which can be used to compare the diversity and composition of targeted microbial groups (e.g., bacteria) across different environmental conditions, locations, land uses, rehabilitation interventions, or chronosequences to determine how soil microbial diversity and community

composition may be impacted (Fierer et al. 2012; Thompson et al. 2017; Breed et al. 2019; Tedersoo et al. 2019). Here, we used sequencing of the bacterial 16s rRNA gene from soils collected across a 28-year rehabilitation chronosequence to observe how soil bacterial communities vary with time since rehabilitation at a bauxite mine site in Western Australia's northern jarrah (*Eucalyptus marginata*) forest. We addressed the following research questions:

1. Does soil bacterial diversity and community composition differ between rehabilitated sites and non-mined reference sites?
2. Does the soil bacterial community change through the chronosequence with communities in older rehabilitated sites becoming more like those found in reference sites?
3. Does the soil bacterial community vary among reference sites?
4. Does the soil bacterial community correlate with changes in soil abiotic properties?
5. Does geographic distance between samples associate with soil bacterial properties?

Our study improves our understanding of how soil bacterial communities can change with time following mine site rehabilitation and helps to enable rehabilitation practitioners to better consider soil bacteria in their interventions. Further, we highlight the variation of bacterial communities across our six reference sites pointing to the need to account for spatially dependent factors through appropriate reference site selection in chronosequence studies.

METHODS

Study site and soil sampling

This study was conducted at the Worsley Alumina mine in southwest Western Australia (Fig. 1) where bauxite has been mined since 1984. Mining and rehabilitation work are ongoing, with approximately 5900 hectares of land cleared for mining and 3200 hectares rehabilitated to date. The mine is located in the northern jarrah (*Eucalyptus marginata*) forest within the Southwest Australian Floristic Region, an international biodiversity hotspot (Myers et al. 2000). The northern jarrah forest is a dry sclerophyllous open forest or woodland dominated by jarrah (*E. marginata*) and marri (*Corymbia calophylla*) trees with an understory dominated by species from the Fabaceae, Asteraceae, Proteaceae, and Myrtaceae families (Koch & Samsa 2007). The mine site has a Mediterranean-type climate with dry hot summers and cool wet winters, a mean annual rainfall of 505 mm (Australian Bureau of Meteorology, 2021) and lateritic, nutrient poor soils.

The mining process at this site first involves removal of all vegetation and topsoil, then overburden is stripped away to access the bauxite ore. Long term topsoil storage for rehabilitation is limited where possible. Instead, the preferred practice is for 'direct return' of topsoil from donor locations (e.g., newly mined areas) to a previously mined area. Following bauxite extraction, mined areas are first contoured to reflect surrounding topography using non-ore and gravel material, and then topsoil is spread to a minimum depth of 10 cm before being furrowed and seeded with a mix of local native plant species. This plant species mix has increased from 40 species in 1994 to over 200 by 2015.

For our study, soil sampling occurred between October and December 2019 as part of the Australian Microbiome (AM) Initiative, following the protocols of the Biomes of Australian Soil Environments (Bissett et al. 2016). Sample sites were chosen, as far as practicable, to provide an even distribution of sampling locations to cover the spatial extent of mining activities and sites of varying rehabilitation age (Fig. 1). We were also conscious of the need to evenly distribute sampling locations to limit the effect of spatial autocorrelations. Six uncleared reference sites that were largely embedded within the mine were selected to compare with the rehabilitated sites. Restored sites included: two from 1991, four from 1996, two from 1999, two from 2002, two from 2005, one from 2007, three from 2011, and three from 2017 (n = 25 sites in total). Sites rehabilitated in 2017 were rare within the main mine area, forcing samples to be taken from two sites rehabilitated in 2017 and an adjacent reference site, from a spatially separate area approximately 4km away from the main sampling sites.

In each site, soil was sampled from two depths (0-10 cm and 20-30 cm), where each sample represented a composite from nine subsamples (chosen to represent site heterogeneity) within 25 x 25 m plots. The nine subsamples from each depth were pooled into a sterile plastic bag, and then homogenised. From each pooled sample, a 500 g subsample of soil was taken for physicochemical analysis and a 50 mL subsample for DNA extraction. Soil chemical analyses were performed at CSBP Laboratories (Perth, Western Australia) to quantify soil organic carbon, ammonium, potassium, sulphur, calcium, pH, nitrate, and phosphorous. The 50 mL sample was frozen on-site and sent packed on dry ice to the Australian Genome Research

Facility (AGRF) in Adelaide, South Australia for DNA extraction (described below). Each replicate had GPS coordinates and a panoramic photograph taken (Fig. S1).

DNA extraction, sequencing, and bioinformatics

DNA was extracted from each sample in triplicate using the Qiagen DNeasy Powerlyzer Powersoil Kit following manufacturer's instructions and quantified fluorometricly. Soil bacterial 16S rRNA was amplified using the 27F (Lane 1991) and 519R (Lane et al. 1985) primer set before sequencing (300bp PE) on the Illumina MiSeq platform. Sequence data used for this work was generated by the Australian Microbiome using their amplicon analysis workflow (Bissett et al. 2016) (https://www.australianmicrobiome.com/protocols/16sanalysisworkflow/) and were downloaded as amplicon sequence variant abundance tables from the AM portal (12 Aug. 2020) (https://www.australianmicrobiome.com/; samples 102.100.100/138358-138407). Paired end reads were merged using Flash2 (Magoč & Salzberg 2011), merged sequences were then further screened to remove those with ambiguities, long homopolymer runs, or too short/long using Mothur screen.seqs (Schloss et al. 2009). Reads passing filter were dereplicated and denoised to zero radius operational taxonomic units (ZOTU) using the UNOISE3 algorithm (Edgar 2016) in USEARCH (Edgar 2010). All reads were then mapped to ZOTUs to construct a ZOTU by read count table. ZOTUs were assigned taxonomy with the RDP Bayesian classifier (Wang et al. 2007) and the SILVA v132 rRNA database (Quast et al. 2013; Yilmaz et al. 2014; Glöckner et al. 2017). ZOTUs not classified as "Bacteria" or classified as "Bacteria_unclassified" at the phylum level were discarded, along with those classified "Mitochondria" or "Chloroplast". ZOTUs which did not occur in at least two samples were also discarded to avoid unrepresentative taxa.

Data analyses

R version 4.0.2 (R Core Team, 2020) was used for all downstream statistical analyses. Rarefaction curves were generated comparing observed ZOTU richness against sample sequence read depth to assess if sample diversity was adequately represented by read depth, as well as to determine an appropriate read depth for rarefaction (Fig.S2). Two samples (one 20-30 cm deep reference site and one 20-30 cm deep 2017 site) were found to have low sequence read depths (80 and 28,854 reads respectively) and were removed from analysis. The remaining samples were rarefied to the lowest remaining sample read depth (n = 54,840 reads) using the *rarefy_even_depth* function in *Phyloseq* (McMurdie & Holmes 2013) to ensure unbiased comparisons across samples. ZOTUs that were not present in at least two samples were discarded to avoid non-representative taxa.

We calculated observed bacterial ZOTUs richness, and estimated Chao1 richness, Gini-Simpson (Simpson), and Shannon-Weiner (Shannon) diversity indices using *phyloseq* to assess sample (*alpha*) diversity. These diversity data were compared across soil depths and year of rehabilitation using permuted analysis of variance with the *aovp* function in *lmperm* v2.1.0 (Wheeler et al. 2016) with 5000 permutations.

Variation in bacterial community composition (beta diversity) across depth and year of rehabilitation was visualised with non-metric multi-dimensional scaling (NMDS) ordinations of Bray-Curtis distances from the rarefied ZOTU abundances using *ordinate* in *phyloseq*. Differences in bacterial community composition across depth

and year of rehabilitation were assessed using permuted multivariate analysis of variance (PERMANOVA) implemented with the *adonis* method in *vegan* (Oksanen et al. 2013). Homogeneity of group dispersions was tested with the *betadisper* function in *vegan*.

Bray-Curtis distances were also used to assess the 'similarity to reference' for each sample. This involved calculating similarity values (i.e., 100%*(1 – distance)), for each sample to all reference samples, including each reference sample to all other references. The distribution of similarity to reference values across the different years of rehabilitation were then displayed as a series of boxplots. A Kruskal-Wallis multiple comparison test was used to determine whether the similarity to reference of samples changed with year of rehabilitation, and any significant differences between rehabilitation years were identified using post-hoc Dunn tests with Bonferroni correction.

Associations between bacterial community composition and soil chemical variables were assessed with PERMANOVA using the *adonis* method. Nitrate and phosphorous variables were not included in analysis as they returned below-threshold measurements for multiple samples.

We investigated the association between bacterial community composition (using Bray-Curtis ecological distances) and geographic distances between replicates to test for the presence of spatial autocorrelation. Here, we used Haversine distance matrices for each depth using the *distm* function in *geosphere* (Hijmans et al. 2017), which calculates the distance between every sample based on a spherical land

surface from GPS coordinates. The relationship between the Haversine distance matrix and Bray-Curtis distance matrix was examined via a Mantel test in *vegan* using the *spearman* method with 9,999 permutations.

RESULTS

A total of 4,192,984 bacterial 16s rRNA reads were generated across the 50 samples, which spanned the two soil depths across the 28year rehabilitation chronosequence. There were 70,199 unique bacterial ZOTUs identified with a mean of 83,859 ±19,546 SD sequence reads per sample (Table 1). Following quality filtering and rarefaction to the lowest sample read depth of 54,840 reads, 65,098 unique ZOTUs remained for analysis across the remaining 48 samples.

Bacterial diversity and community composition

Soil depth and year since rehabilitation had no effect on observed ZOTU richness, Chao1 estimated, Simpson, or Shannon diversity metrics (permuted ANOVA: p > 0.05 in each case; Table 2, Fig. S3). Bacterial community composition varied significantly by soil depth and year of rehabilitation (Fig. 2; PERMANOVA: depth F=7.16, p=0.001; year F=2.02, p=0.001). Community composition in rehabilitated sites became increasingly similar to reference sites with time since rehabilitation (Fig. 3). Bray-Curtis similarity to reference values showed significant variation (Kruskal-Wallis: 0-10 cm p<0.001, 20-30 cm p<0.001) and post-hoc Dunn tests indicated younger rehabilitation sites were significantly different to reference sites, while older rehabilitation sites were not significantly different to reference sites at both the 0-10 cm and 20-30 cm depths (Fig. 3).

Soil chemical associations

At the 0-10 cm depth, the bacterial community composition showed significant associations with both pH (PERMANOVA F=0.056, *p*=0.006) which decreased with age and potassium (PERMANOVA F=0.052, *p*=0.016) which increased with age (Table 3). Although not significantly associated with bacterial communities, organic carbon, potassium, calcium, sulphur, and ammonium all saw increases with age in the 0-10 cm soil profile (Table 3). No significant associations (PERMANOVA *p*>0.05) were observed between any physicochemical variable and bacterial community composition at the 20-30 cm depth (Table 3). Potassium was the only physicochemical variable to exhibit a clear increase with age, all other variables did not follow a clear trajectory (Table 3).

Spatial autocorrelation

Analysis of Bray-Curtis ecological distances, representing bacterial community composition, and the geographic distances between samples showed a significant, though weak, spatial autocorrelation (Mantel test: r=0.23, p=0.011; Fig. 4a). To explore if this spatial autocorrelation was being driven by three sites that were geographically separate from all other sites (Fig. 1), we removed these and reran the Mantel test which resulted in no significant correlation (Mantel test: r=0.08, p=0.162; Fig. 4b).

DISCUSSION

Here we quantified variation in soil bacterial communities across a 28-year rehabilitation chronosequence following rehabilitation of a bauxite mine site in

Western Australian jarrah forest. There was a clear association of time since rehabilitation with bacterial community composition, where older sites were more similar to reference sites than younger sites, with sites as young as 14 years since rehabilitation having no significant difference in similarity to reference compared with reference-to-reference similarities. These patterns reflect a successional transition in the structure of bacterial communities, where communities in rehabilitated sites increasingly resembled those from unmined reference sites with time since rehabilitation. Although community composition was associated with rehabilitation age, we observed no effect of rehabilitation age or soil depth on bacterial alpha diversity. We observed a weak, but significant, association between bacterial community composition and spatial proximity of sample sites. However, this association was mostly driven by three sites that were geographically separate from all others. When we removed these sites from our spatial analysis there was no longer a significant association, reinforcing our inference that we are observing an effect of time as opposed to space on community composition across the chronosequence. Together, these results indicate that ecologically important soil bacterial communities are on a trajectory towards recovery following rehabilitation techniques applied at the Worsley Alumina bauxite mine.

Our findings show that while the mining process impacts bacterial communities even with direct return of topsoil, these communities can respond rapidly to environmental changes following rehabilitation. This relatively rapid change can provide an early indication of ecosystem recovery trajectories moving toward the reference ecosystem (Banning et al. 2011; Yan et al. 2019). Bauxite mining is known to impact on soil biotic and abiotic properties. Microbial biomass, diversity, and functionality

have been shown to reduce following bauxite mining, and chemical properties like soil organic carbon, pH, phosphorus, or calcium are altered (Lewis et al. 2010; Banning et al. 2011; Lin et al. 2011; van der Heyde et al. 2020).

In natural soil systems, succession in bacterial communities is thought to be initially stochastic before becoming increasingly deterministic (Dini-Andreote et al. 2015), where soil properties (particularly pH, availability of soil carbon, and nitrogen) and plant-soil feedbacks drive succession (Fierer 2017). Succession in soil bacterial communities in human-altered systems, such as in response to rehabilitation interventions following mining and agriculture, are less understood with only a handful of recent soil genomic studies showing patterns of compositional differences in bacterial communities following rehabilitation (e.g. Barber et al. 2017; Gellie et al. 2017; Ngugi et al. 2018; Yan et al. 2018; Schmid et al. 2020; van der Heyde et al. 2020). None of these previous studies however address the degree of variation in bacterial communities among reference sites, or how this potential variation can impact what we determine to be an appropriate rehabilitation target to which we should be comparing rehabilitated sites against.

While our results clearly indicate bacterial communities in older rehabilitated sites were as similar to communities in reference sites as the reference sites are to each other, this similarity also highlights the considerable variation of bacterial communities among reference sites. Our reference-to-reference comparison showed a median similarity of 40% at the ZOTU level. While this degree of similarity is influenced by the analysis methods (i.e., ecological distance measures, sequence grouping or clustering approaches, multiplexing, denoising), we do not aim to

establish here if ZOTU levels of resolution provide the most appropriate indication of progress towards the reference target. We conduct a comprehensive methodological investigation using soil bacterial community data in chronosequence studies that explore these points in detail in Liddicoat et al. (2021).

Here, we sampled six reference sites embedded within both the mine and our rehabilitated sites, which should provide an indication of the variation present in the bacterial community among reference sites in general. This among-reference site variation is not surprising considering the known associations between soil bacterial communities and soil physical and chemical characteristics, and how both these factors can vary spatially (Green & Bohannan 2006; Neupane et al. 2019). Previous studies using a chronosequence design to explore changes in soil microbial communities following mine site rehabilitation have only sampled limited numbers of reference sites (e.g., 1-3 sites) (Ngugi et al. 2018; Schmid et al. 2020; van der Heyde et al. 2020) and none have reported on the variation present among reference sites. With spatial variation of bacterial communities being so scale dependant (Fierer 2017), the degree of variation among reference sites will likely impact interpretations of communities being used as the target. We recommend future studies that assess recovery trajectories of soil microbiota with a chronosequence design capture spatial variation among reference sites by sampling many reference sites that are geographically incorporated into the study site. We also note that the robustness of future studies would be improved through increasing the number of rehabilitation and reference samples taken.

While our study design included an even spatial distribution of our rehabilitation sites with reference sites throughout the mine area, we observed a significant positive correlation between bacterial community dissimilarity and geographic distance. This association was largely driven by three sample sites, and when these sites were removed there was no longer a significant association, supporting our conclusion of an effect of time, rather than space, on bacterial communities across the chronosequence. This spatial effect on bacterial community composition is likely to be driven not only by our spatial outliers but also by associations between soil abiotic properties (e.g., pH, potassium or other unsampled soil parameters) and microbial community composition (Martiny et al. 2011). As geographic distance between samples increased, so did changes in soil properties. This environmentally driven spatial variation of soil microbial communities highlights the need for appropriate experimental designs that limit spatial confounders where practicable or address their ecological implications. Furthermore, to experimentally test cause-effect relationships in a rehabilitation context, either experiments need to be embedded into rehabilitation sites (Gellie et al. 2018) or longitudinal studies need to be done to conclusively ascertain temporal changes in soil microbiota.

We observed significant associations between soil pH and potassium with bacterial community structure at the 0-10 cm depth but no association of any measured soil abiotic property and bacterial community composition at the 20-30 cm depth. Soil chemical properties have large effects on the composition and diversity of soil bacterial communities (Fierer 2017; Bahram et al. 2018; Delgado-Baquerizo & Eldridge 2019). Soil pH is among the strongest drivers of soil bacterial community composition at local and broad spatial scales (Fierer 2017). For example, Banning et

al. (2011) showed a decrease in soil pH with time since rehabilitation and a significant association between pH and bacterial community composition in rehabilitated bauxite mine sites in jarrah forest. Our surface soil results support this trend, with soil pH decreasing with time since rehabilitation and a significant association between bacterial community composition and changes in soil pH over time at the 0-10 cm depth. However, we found no associations between changes in bacterial structure and soil abiotic properties at the 20-30 cm depth. In the soils, pH also decreased with time since rehabilitation, but was trending away from the pH of reference sites. This negative association between pH with time since rehabilitation at this soil depth could be impacting on deeper soil bacterial community composition and may be a barrier to future bacterial community recovery. This highlights the importance of targeting site specific ideal soil pH levels for microbiota in mine rehabilitation and may be an avenue to investigate the potential to shorten recovery time frames by optimising soil pH earlier in the rehabilitation process.

Although depth explained more variation in bacterial community composition than time since rehabilitation, the recovery trajectory with time is similar across both depths with bacterial communities in rehabilitated sites becoming increasingly similar to reference sites with time. Even with the homogenisation that occurs with direct return or storage of topsoils, our youngest sites still developed depth profiles in as short as two years following rehabilitation. This stratification of bacterial composition across soil depth is thought to result from differential availability of macronutrients and organic carbon and/or differing environmental gradients across soil depths (Allison et al. 2007), and both these trends are supported by our results that show lower nutrient levels in the deeper soils. While these results indicate a recovery

trajectory of bacterial communities returning to their pre-disturbance condition with time since rehabilitation, ascertaining whether soil edaphic variables, aboveground plant communities, or other factors are specifically driving this recovery is still unclear. Our results support recent soil genomic research that show variation in bacterial community composition between soil depths as well as directional trends of community composition with time since rehabilitation (Gellie et al. 2017; Yan et al. 2019).

While we found no difference in bacterial alpha diversity across the chronosequence, previous studies have shown a variety of bacterial diversity changes with rehabilitation, including higher diversity in younger sites before peaking in moderately aged sites and then diversity reductions towards reference sites (Barber et al. 2017; Sun et al. 2017). These previously published diversity patterns were explained as resulting from an initial disturbance, followed by rapid expansion of generalist and opportunistic taxa, before niche specific taxa begin to establish as the vegetation community re-establishes (Kardol & Wardle 2010; Liddicoat et al. 2019). However, these trends are by no means universal, and similar to our results, other studies have shown no change or significant differences in soil bacterial alpha diversity attributable to age across a chronosequence (Gellie et al. 2017; Yan et al. 2019; Schmid et al. 2020). These discrepancies in the response of soil bacterial alpha diversity to rehabilitation makes predicting a response of soil bacterial diversity *a priori* difficult. Soil microbial diversity has been shown to have links to aboveground biodiversity and ecosystem services and functions (Fierer et al. 2012; Bardgett & Van Der Putten 2014; Prober et al. 2015; Bender et al. 2016). However, higher diversity does not necessarily reflect greater ecological integrity than lower diversity,

and neither does it imply greater or improved functionality (Shade 2017). The initial topsoil disturbance in mining and any prolonged topsoil storage can negatively impact on soil microbial diversity, potentially reducing functionality. In this case however, the mine's direct return approach has potentially limited the impact of degrading processes on soil microbiota reducing impacts on soil diversity.

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TABLES AND FIGURES

Table 1. Mean (±SD) amplicon sequence variant (ZOTU) abundance by year of rehabilitation and depth. *Standard deviation was not calculated for 2007 with only one sample from each depth.

Year of rehabilitation	Samples (n)	Depth (cm)	Mean ZOTU abundance (±SD)
Reference	6	0-10	84,660 ±14,314.35
1991	2	0-10	59,787 ±6,843.38
1996	3	0-10	89,332 ±12,136.41
1999	2	0-10	86,825 ±4,585.59
2002	2	0-10	78,984 ±7,860.91
2005	2	0-10	86,476 ±19,240.36
2007	1*	0-10	116,520 *
2011	3	0-10	82,815 ±6,373.19
2017	3	0-10	80,934 ±5,380.64
Reference	6	20-30	77,613 ±41,010.35
1991	2	20-30	77,149 ±5,621.5
1996	3	20-30	91,201 ±9,412.71
1999	2	20-30	90,964 ± 379.01
2002	2	20-30	99,775 ±33,844.25
2005	2	20-30	85,143 ±163.34
2007	1*	20-30	86,817 *
2011	3	20-30	87,982 ±4,723.58
2017	3	20-30	69,488 ±34,863.61

Table 2. Mean (±SD) amplicon sequence variant (ZOTU) richness and diversity of bacterial communities assessed with permuted analysis of variance at South 32's Worsley Bauxite mine, Western Australia. *2007 (n=1) was excluded from statistical analysis for both depths.

Year of rehabilitation	Samples (n)	Depth (cm)	ZOTU Richness (±SD)		Diversity (±SD)	
			Observed	*Chao 1*	*Shannon*	*Simpson*
Reference	6	0-10	12576.0 ±1771.1	19695.7 ±3754.2	8.34 ±0.25	0.998 ±0.0003
1991	2	0-10	13928.5 ±487.2	23335.8 ±510.1	8.51 ±0.09	0.999 ±0.0001
1996	3	0-10	13156.5 ±597.5	21527.9 ±969.9	8.40 ±0.10	0.998 ±0.0004
1999	2	0-10	15764.5 ±637.1	25034.2 ±207.7	8.82 ±0.17	0.999 ±0.0002
2002	2	0-10	12436.5 ±2448.7	18038.6 ±6015.8	8.52 ±0.08	0.999 ±0.0003
2005	2	0-10	10595.0 ±4736.2	14763.1 ±7808.9	8.20 ±0.70	0.998 ±0.0013
2007*	1*	0-10	14140.0 *	22922.9 *	8.58 *	0.999 *
2011	3	0-10	11744.0 ±2739.6	16349.4 ±4921.6	8.47 ±0.32	0.999 ±0.0003
2017	3	0-10	12825.0 ±514.1	19235.4 ±402.2	8.49 ±0.12	0.998 ±0.0004
Reference	6	20-30	12993.6 ±2390.4	18389.5 ±5026.0	8.39 ±0.19	0.999 ±0.0002
1991	2	20-30	15152.0 ±1630.6	23301.9 ±3041.1	8.46 ±0.13	0.999 ±<0.0001
1996	3	20-30	16331.2 ±2189.7	24477.7 ±4478.3	8.62 ±0.23	0.999 ±0.0004
1999	2	20-30	16193.5 ±392.4	23343.4 ±752.1	8.66 ±0.24	0.998 ±0.0008
2002	2	20-30	13864.5 ±7428.2	20227.7 ±14273.4	8.48 ±0.61	0.999 ±0.0002
2005	2	20-30	11238.0 ±1195.0	14129.2 ±3158.1	8.39 ±0.06	0.999 ±0.0001
2007*	1*	20-30	16000 *	23211.3 *	8.64 *	0.999 *
2011	3	20-30	14858.3 ±2812.4	20633.8 ±5448.7	8.66 ±0.27	0.999 ±0.0001
2017	3	20-30	13821.0 ±1548.6	19487.1 ±5208.2	8.55 ±0.03	0.999 ±<0.0001
***P* values**		**Year**	0.07	0.07	0.74	0.66
		Depth	1.00	0.98	0.84	0.44
		Year x Depth	0.98	1.00	0.97	0.85

Table 3. Soil characteristic values (mean ± SD) for restoration years across a 28-year rehabilitation chronosequence at South 32 Bauxite mine, Western Australia. *P* values indicate associations between soil bacterial community composition and soil physicochemical properties tested with PERMANOVA. Soil pH and potassium significantly associated (p <0.05) with bacterial community composition at the 0-10 cm depth and there were no associations at the 20-30 cm depth. *2007 was excluded from summary analysis with only one replicate at each depth.

Year of Rehabilitation	Depth (cm)	Replicates (n)	Potassium (mg/Kg)	Ammonium (mg/Kg)	Sulphur (mg/Kg)	Organic carbon (%)	Calcium (meq/100g)	pH ($CaCl_2$)
Reference	0-10	6	103.83 ± 39.72	5.67 ± 1.03	9.13 ± 4.09	4.24 ± 0.48	7.71 ± 3.67	4.92 ± 0.19
1991	0-10	2	73.00 ± 19.80	5.00 ± 0.00	7.55 ± 2.76	3.68 ± 0.18	5.31 ± 0.11	4.75 ± 0.07
1996	0-10	3	91.00 ± 9.90	5.75 ± 1.26	7.23 ± 2.91	3.52 ± 0.13	6.08 ± 0.11	5.03 ± 0.10
1999	0-10	2	98.50 ± 14.85	7.00 ± 1.41	13.50 ± 10.61	4.18 ± 0.62	7.80 ± 0.09	4.85 ± 0.35
2002	0-10	2	47.50 ± 13.43	3.50 ± 0.71	3.95 ± 0.78	2.37 ± 0.14	3.67 ± 1.50	4.95 ± 0.35
2005	0-10	2	40.00 ± 4.24	2.50 ± 0.71	4.85 ± 1.62	1.84 ± 0.04	2.97 ± 0.76	5.25 ± 0.35
2007	0-10	1*	38.00 *	2.00 *	5.20 *	1.33 *	2.40 *	5.10 *
2011	0-10	3	51.67 ± 8.73	2.00 ± 1.00	6.63 ± 2.66	1.60 ± 0.52	3.54 ± 1.23	5.27 ± 0.15
2017	0-10	3	27.33 ± 2.52	2.67 ± 0.58	5.23 ± 0.61	1.97 ± 0.30	3.26 ± 0.58	5.20 ± 0.00
***P* values**			**0.016**	0.559	0.108	0.767	0.557	**0.006**
Reference	20-30	6	70.67 ± 53.08	3.00 ± 0.89	8.05 ± 3.45	1.68 ± 0.40	3.68 ± 1.42	5.52 ± 0.22
1991	20-30	2	57.00 ± 9.90	3.00 ± 0.00	17.00 ± 19.97	1.75 ± 0.13	2.92 ± 0.88	4.95 ± 0.21
1996	20-30	3	53.50 ± 9.33	3.25 ± 0.96	18.98 ± 19.08	1.82 ± 0.26	3.22 ± 0.69	5.28 ± 0.21
1999	20-30	2	80.50 ± 31.82	5.00 ± 1.41	30.65 ± 36.27	2.49 ± 0.28	5.64 ± 2.67	4.95 ± 0.07
2002	20-30	2	36.00 ± 7.08	2.50 ± 0.71	4.15 ± 1.62	1.55 ± 0.84	2.63 ± 1.26	5.20 ± 0.14
2005	20-30	2	43.00 ± 14.14	2.00 ± 1.41	5.60 ± 0.99	0.82 ± 0.13	1.33 ± 0.11	5.40 ± 0.14
2007	20-30	1*	38.00 *	2.00 *	5.20 *	1.33 *	2.40 *	5.10 *
2011	20-30	3	44.33 ± 21.13	2.33 ± 1.15	9.60 ± 6.58	1.31 ± 0.45	2.63 ± 1.12	5.43 ± 0.12
2017	20-30	3	33.67 ± 6.02	2.00 ± 0.00	7.90 ± 4.11	1.47 ± 0.52	2.98 ± 1.37	5.53 ± 0.15
***P* values**			0.085	0.220	0.249	0.165	0.363	0.067

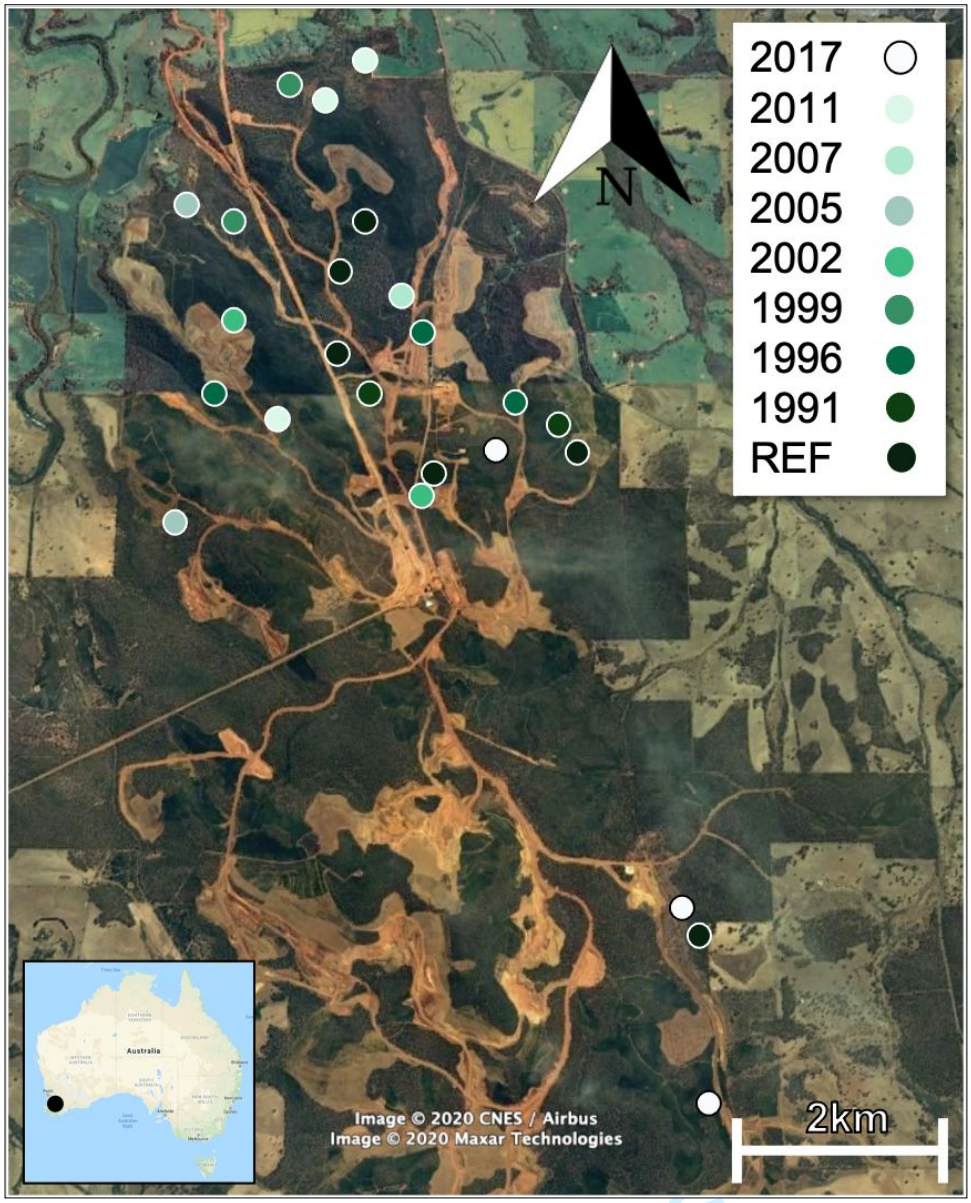

Figure 1. Map of South 32's Worsley bauxite mine in southwest Western Australia. Circles indicate sampling sites, with colour representing year of rehabilitation. Soil was sampled from two depths (0-10 cm and 20-30 cm) at each site.

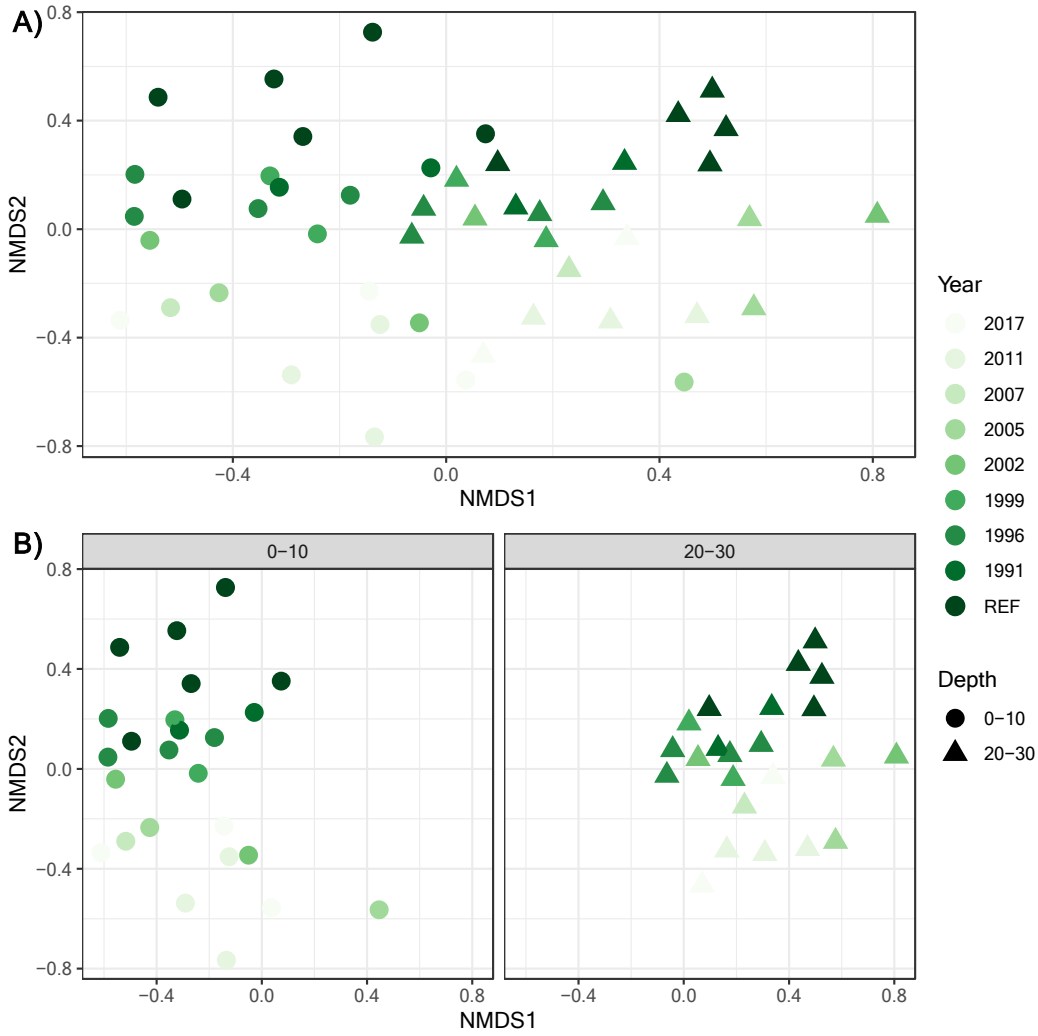

Figure 2. Non-metric multidimensional scaling (NMDS) ordinations of Bray-Curtis distance matrices indicating bacterial community composition across the rehabilitation chronosequence at Worsley Alumina, Western Australia. (A) ordination of samples from both 0-10 cm and 20-30 cm soil depths and (B) is ordinations for each depth separately.

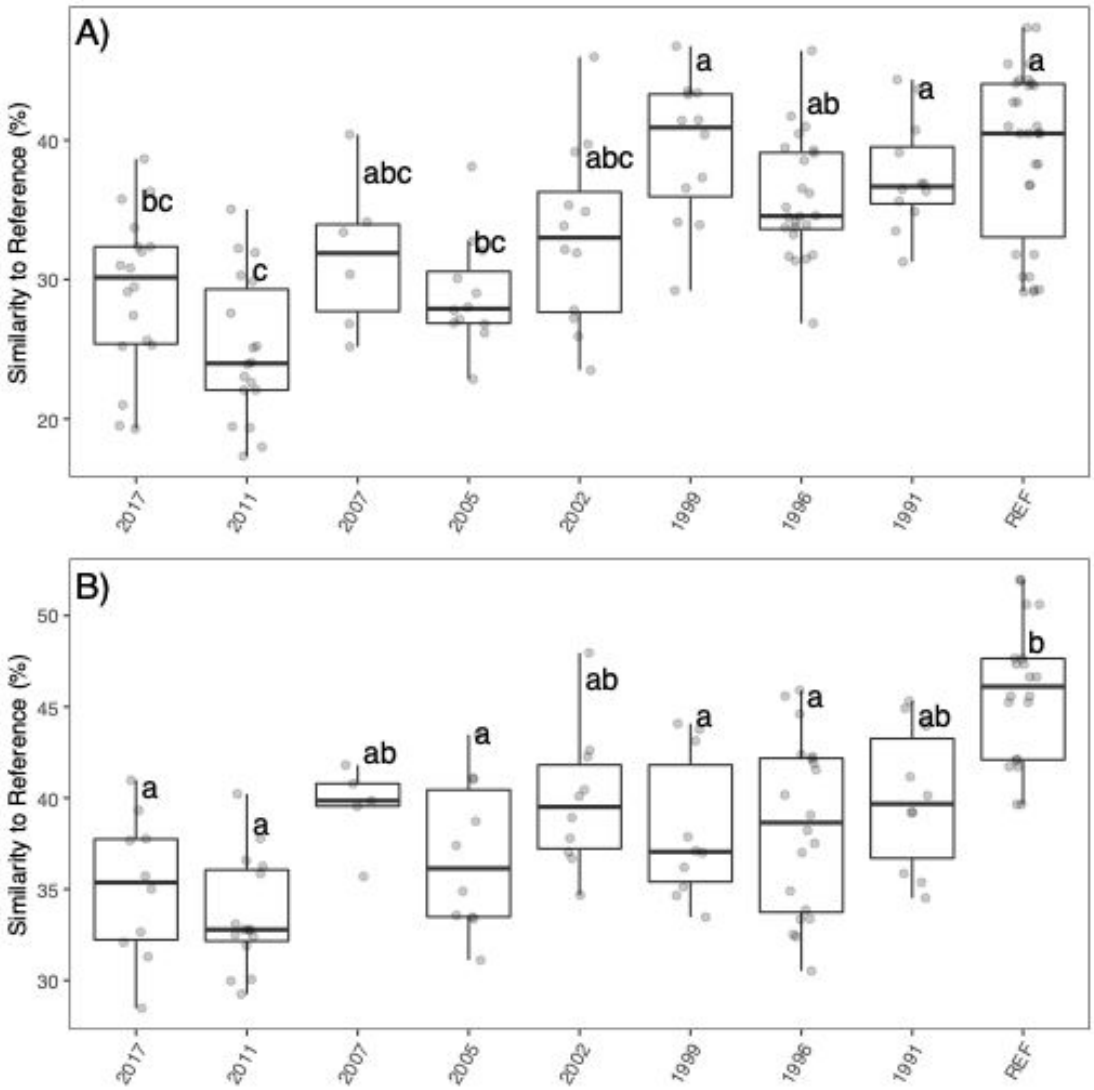

Figure 3. Boxplot indicating similarity to reference of Bray-Curtis distances for each sample at (A) 0-10 cm soil depth and (B) 20-30 cm soil depth. Horizontal lines indicate 25th, 50th (median), and 75th percentile of similarities to reference and vertical lines represent 95% confidence intervals. Kruskal-Wallis tests indicated significant differences (p<0.05) between years of rehabilitation at both depths and letters on plots are derived from Dunn post hoc tests indicating younger rehabilitated sites are different to references and older rehabilitated sites are comparable to references (groups not sharing a letter are significantly different.

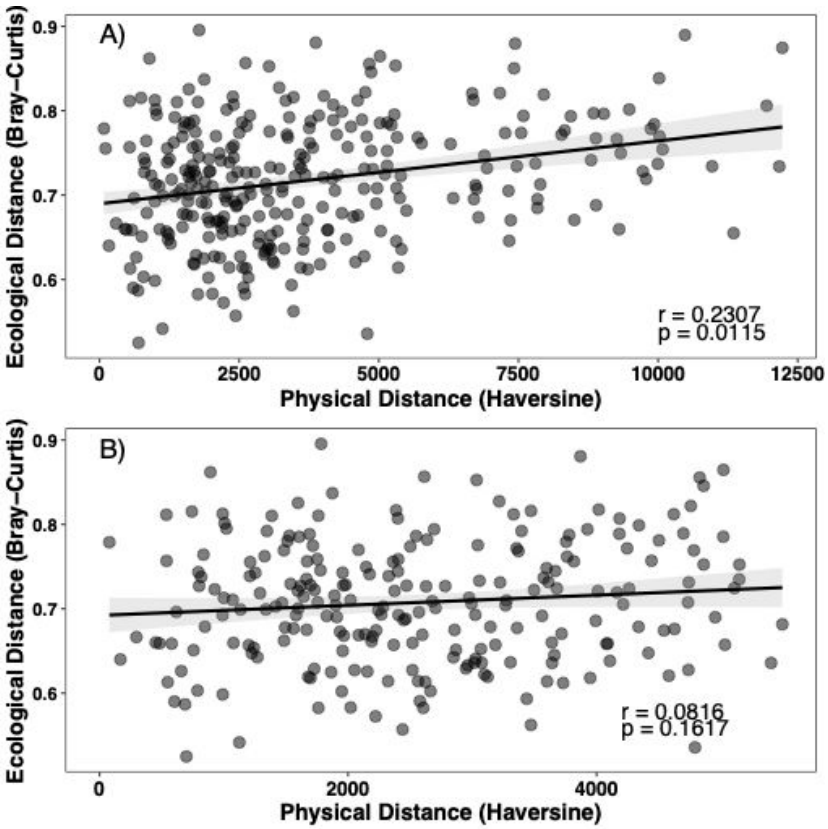

Figure 4. Scatterplot of the association between the distance between samples (Haversine distance matrix) and bacteria community composition (Bray-Curtis distance matrix), showing Mantel test statistics. (A) shows a significant correlation present with all sites included, and (B) shows no significant correlation with three geographically separate sites removed indicating these three sites are driving the spatial autocorrelation.

Supplementary Information for:

Soil DNA chronosequence analysis shows bacterial community re-assembly following post-mining forest rehabilitation

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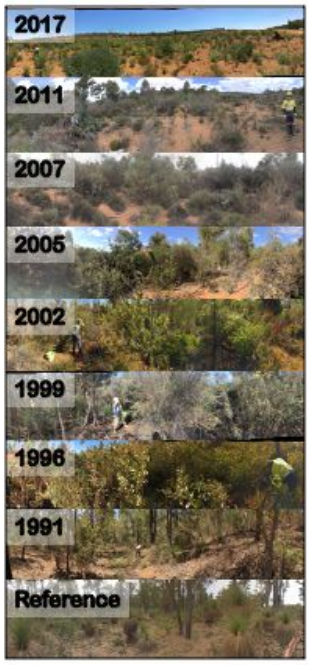

Figure S1: Panoramic photos taken at sampling sites showing the aboveground vegetation community across the 28year rehabilitation chronosequence at the South32 Worsley Alumina bauxite mine in Western Australia.

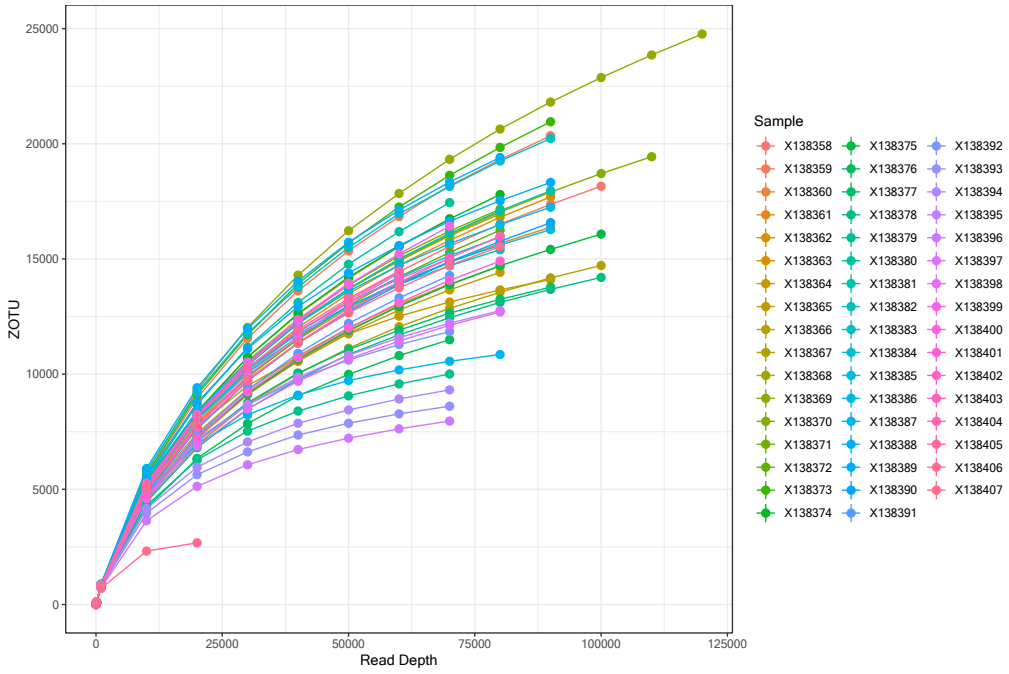

Figure S2: Rarefaction curve comparing observed zero radius amplicon sequence variants (ZOTU) and sequence read depth of each sample to assess if sample diversity was adequately represented by read depth. Two samples were removed because of low reads (80 and 28,845) and remaining samples were rarefied to the lowest remaining sample read depth (54,840) to ensure unbiased comparisons across samples.

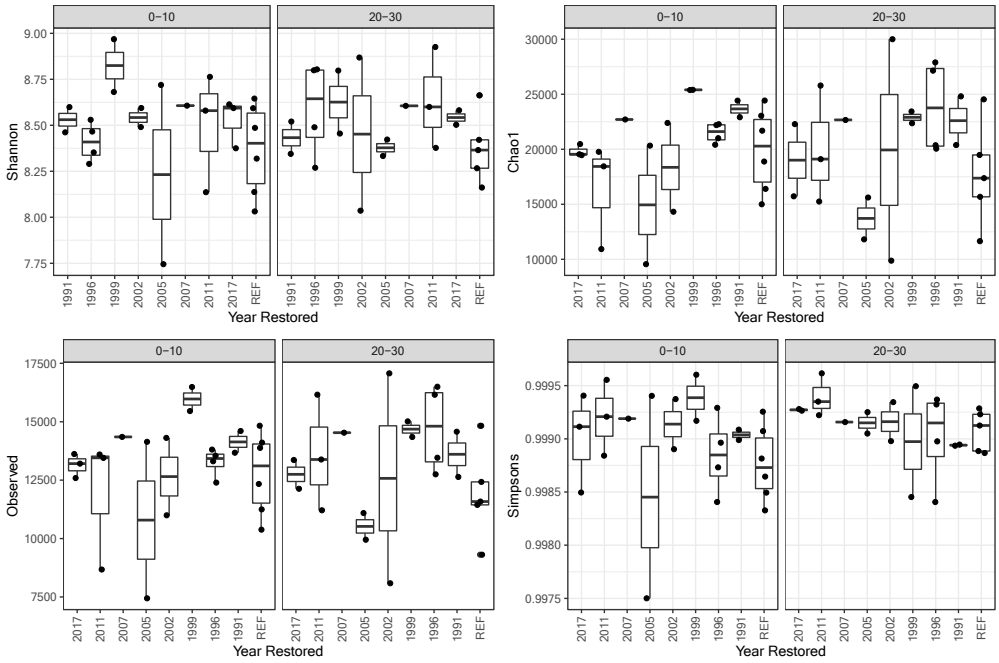

Figure S3: Boxplots of Shannon, Chao1 Observed, and Simpson soil bacterial alpha diversity metrics for both sampled depths (0-10cm and 20-30cm) across the 28year rehabilitation chronosequence at South32 Worsley Alumina bauxite mine in Western Australia. There were no significant differences (permuted anova, p >0.05 in all cases) in any alpha diversity metric across year of rehabilitation or soil depth.