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3 Changes in soil microbial communities in post 4 mine ecological restoration: implications for 5 monitoring using high throughput DNA 6 sequencing

7 van der Heyde, M.[1,2], Bunce, M.[2,3], Dixon, K.[1], Wardell-Johnson, G.[1], White, N.E.[2], Nevill,
8 P.[1,2]

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10 [1]ARC Centre for Mine Site Restoration, School of Molecular and Life Sciences, Curtin
11 University, Bentley, GPO Box U1987, Perth, Western Australia, 6845
12 [2]Trace and Environmental DNA Laboratory, School of Life and Molecular Sciences, Curtin
13 University, GPO Box U1987, Perth, Western Australia, 6845
14 [3]Environmental Protection Authority, 215 Lambton Quay, Wellington 6011, New Zealand.
15 Corresponding author email:
16 mieke.vanderheyde@.curtin.edu.au

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20

21 **Abstract**

22 The ecological restoration of ecosystem services and biodiversity is a key intervention
23 used to reverse the impacts of anthropogenic activities such as mining. Assessment of the
24 performance of restoration against completion criteria relies on biodiversity monitoring.
25 However, monitoring usually overlooks soil microbial communities (SMC), despite increased
26 awareness of their pivotal role in many ecological functions. Recent advances in cost,
27 scalability and technology has led to DNA sequencing being considered as a cost-effective
28 biological monitoring tool, particularly for otherwise difficult to survey groups such as
29 microbes. However, such approaches for monitoring complex restoration sites such as post-
30 mined landscapes have not yet been tested. Here we examine bacterial and fungal
31 communities across chronosequences of mine site restoration at three locations in Western
32 Australia to determine if there are consistent changes in SMC diversity, community
33 composition and functional capacity. Although we detected directional changes in
34 community composition indicative of microbial recovery, these were inconsistent between
35 locations and microbial taxa (bacteria or fungi). Assessing functional diversity provided
36 greater understanding of changes in site conditions and microbial recovery than could be
37 determined through assessment of community composition alone. These results demonstrate
38 that high-throughput amplicon sequencing of environmental DNA (eDNA) is an effective
39 approach for monitoring the complex changes in SMC following restoration. Future
40 monitoring of mine site restoration using eDNA should consider archiving samples to
41 provide improved understanding of changes in communities over time. Expansion to include
42 other biological groups (e.g. soil fauna) and substrates would also provide a more holistic
43 understanding of biodiversity recovery.

44 # 1. Introduction

45 The loss of biodiversity and ecosystem services due to land degradation is a global crisis
46 that undermines the wellbeing of 3.2 billion people, costing approximately 10% of annual
47 gross domestic product, and prompting the United Nations to declare a UN decade on
48 Ecosystem Restoration (2021-2030) (UN Environment Programme, 2020). Mining represents
49 an extreme form of land degradation, where the ecosystem of a site is removed and is
50 reinstated or rehabilitated often at large scales. For example, the footprint of land degraded
51 by mining in China alone is 3.7 million hectares, an area the size of the Netherlands (Li et al.
52 2006). The cost of landforming and restoring such large areas is high (Menz et al. 2013), with
53 a per hectare cost of up to AU$34,000 in Australia (Gardner et al. 2007). Understanding if a
54 restoration target has been reached (see Gann et al. 2019) requires accurate monitoring to
55 ensure the best return on investment is achieved, indicate when restoration has been
56 successful, and apply adaptive management principles to future restoration projects (Herrick
57 et al., 2006; Miller et al. 2017). There is increasing realization that more nuanced approaches
58 to restoration are needed that take into account the return of ecosystem services and the
59 interactions that occur between lifeforms, from microbes to mammals.

60 Soil microbial communities (SMC) represent emerging targets for restoration monitoring
61 (Harris, 2003; Nurulita et al., 2016; Gellie et al. 2017; Sun et al., 2017; Yan et al., 2018) as
62 they provide a functional basis for ecosystems and are key agents in the soil-root interface
63 involved in nutrient cycling and decomposition (Meena et al., 2017), plant performance and
64 community composition (Yang et al., 2018). They also respond rapidly to changes in the
65 environment and are easily affected by soil chemistry (Leff et al., 2015; Šmejkalová et al.,
66 2003), physical soil disturbance (Dong et al., 2017; Kabiri et al., 2016), and plant
67 communities (Burns et al., 2015). As a result, characterizing soil communities could provide

68 indicators of edaphic and biotic capabilities in restoration and act as early indicators of
69 problems or predict restoration trajectory (Muñoz-Rojas, 2018).

70 Soil bacteria are the most abundant form of soil microbes and have growth rates 10-fold
71 faster than fungi. As a result, they also tend to have higher variation over time (Sun et al.,
72 2017). With slower growth rates, fungi are often more disturbed by soil modifications as they
73 are suppressed by nutrient addition (Rajapaksha et al., 2004; Suzuki et al., 2009) and
74 disruptions to their hyphal networks (Dong et al., 2017; Frey et al., 1999). However, patterns
75 of SMC responses to changes are often inconsistent and difficult to predict (Dong et al.,
76 2017; Sipilä et al., 2012), likely due to the highly diverse and variable nature of these
77 communities.

78 Several studies have assessed aspects of SMC recovery in a restoration context; most
79 commonly reporting decreased biomass and activity in restoration, and different community
80 composition (Mummey et al., 2002a; Muñoz-Rojas et al., 2016; Yan et al., 2018). The
81 increased availability over the last 15 years of high-throughput sequencing has made
82 available an increasingly cost-effective way to monitor SMC community composition (Yan et
83 al., 2018). Several studies have found directional changes in community composition with
84 restoration age (Banning et al. 2011; Gellie et al., 2017; Sun et al., 2017; Yan et al., 2019,
85 2018). However, these patterns are typically complex. For example, Banning et al. (2011)
86 found that bacteria (and not fungal) communities showed directional changes, with older
87 restoration sites more similar to reference communities. In contrast, Sun et al. (2017) found
88 that fungal communities showed more distinct differences between restoration ages than
89 bacteria. Few if any studies have looked at SMC of restoration at multiple locations. Most
90 studies are limited to either a single restoration and reference site (e.g. Mummey et al.,
91 2002b; Muñoz-Rojas et al., 2016) or one chronosequence of restoration sites (e.g. Gellie et
92 al., 2017; Sun et al., 2017; Yan et al., 2019, 2018). Recent studies using high throughput

93 sequencing of SMC for monitoring highlight the need to firstly test for consistency across
94 locations, and secondly, to define the functional significance of the measured SMC diversity
95 (Gellie et al., 2017; Yan et al., 2018). To accurately assess consistency, it is important to use
96 the same methodological and analytical framework to account for any biases.

97 By integrating measures of SMC diversity, community composition, and microbial
98 functionality, we test the hypotheses that restoration of mine sites will lead to the recovery of
99 SMC. We used high throughput (amplicon) sequencing of fungi (ITS2) and bacteria (16S) to
100 examine changes in SMC across mine site restoration chronosequences at three locations. We
101 aim to improve the application of high-throughput amplicon sequencing to restoration
102 monitoring by addressing the following questions:

103 1) Are soil bacterial and fungal communities in older restoration sites more similar to
104 reference communities than those at younger restoration sites?

105 2) Which functional groups are indicators of the different stages of restoration?

106 3) Are soil chemical properties (moisture, potassium, carbon, etc.) associated with
107 restoration age, and/or change in bacterial and fungal community composition?

108 4) Are consistent patterns observed across the three study locations?

109 The aim of this work is to provide recommendations for future implementation of high
110 throughput sequencing as a more holistic monitoring tool for restoration.

111

112 2. Material and Methods

113 *2.1 Study Sites*

114 The term 'chronosequence' describes a set of ecological sites that share similar attributes
115 but represent different ages. Traditionally these have been used to describe sites with the

116 same parent material with different periods of soil formation (Stevens and Walker, 1970), but
117 it has also been used to refer to sites with different ages of restoration (Banning et al., 2011;
118 Harris, 2003). Three chronosequences of mine site restoration were studied from three
119 locations in Western Australia; Swan Coastal Plain (SCP), Jarrah Forest (JF) and hot desert
120 Pilbara (PB). Each showed consistency in restoration approaches, soil type, climate and site
121 aspect within the location. All three locations used topsoil in their restoration, and these were
122 stripped to consistent depth within each location and homogenized before application. At
123 each chronosequence, sites of different restoration age were sampled as well as two spatially
124 separated reference sites (see Appendix Figure 1). Reference sites were selected for their
125 proximity to restoration sites and similarity to ecosystems mining companies were attempting
126 to restore. To our knowledge, none of the reference sites were recently impacted by
127 disturbances such as overgrazing or fire. At all three locations, we sampled at least two sites
128 less than 9 years old (Young), and at least two sites older than 9 years (Older).

129 The Banksia Woodland of the Coastal Plain (SCP) has a warm-summer Mediterranean
130 climate with mild cool wet winters; temperature has a mean minimum of 12.8°C, mean
131 maximum of 24.7°C, with 757 mm mean annual rainfall (Australian Bureau of Meteorology).
132 This location occurs within the Southwest Australian Globally Biodiversity Hotspot (Myers
133 et al., 2007). The mine is located on the silicaceous Bassendean dunes, which are
134 characterized by low nutrient, leached podzols, with high acidity and low water-holding
135 capacity (Dodd and Heddle, 1989; McArthur, 1991). The dominant tree species are *Banksia*
136 *attenuata* and *B. menziesii*, with less dominant *Eucalyptus todtiana* and *Nuytsia floribunda.*
137 The understory consists of woody species of Myrtaceae, Fabaceae, Proteaceae, and
138 Ericaceae, and non-woody species in Anthericaceae, Stylidiaceae, Cyperaceae, and
139 Haemodoraceae (Trudgen, 1977). In October 2018, we sampled seven sites at a Hanson
140 Construction Materials sand quarry in Lexia (-31.76°, 115.95°); two reference sites and

141 restoration sites 1, 3, 7, 14, 22 years old. Restoration sites were previously sites of open pit
142 mining. The sites have been restored with the aim of returning mined areas to the
143 surrounding native Banksia woodlands. All restoration was done by Hanson and previous
144 mine owners and included direct transfer of fresh topsoil, ripping, and seeding with native
145 species. A previous study found that species richness and density tended to be higher in
146 restoration than reference sites, and that percent cover increases with restoration age and is
147 highest in reference sites (Benigno et al., 2013). This study also found that restored sites have
148 more basic soils with less organic matter than reference sites (Benigno et al., 2013).

149 The second chronosequence located in the Jarrah (*E. marginata*) forest is also within the
150 Southwest Australian Biodiversity hotspot (Myers et al., 2007) and has a similar hot-summer
151 Mediterranean climate; temperatures have an mean min. of 8.6°C, mean max. of 23.7°C, and
152 668.9 mm annual mean rainfall (Australian Bureau of Meteorology). The lateritic soils are
153 nutrient poor and high in gravel with surfaces rich in iron and aluminum (McArthur, 1991).
154 The overstorey vegetation is primarily *E. marginata,* with *E. patens,* and *E. wandoo* also
155 present. The understory is sclerophyllous and dominated by taxa from numerous families,
156 including Fabaceae, Asteraceae, Proteaceae, Dasypogonaceae, and Myrtaceae (Havel, 1975).
157 We sampled six sites from the bauxite mine South32 (-32.96°, 116.48°) in October 2018; two
158 reference sites and restoration sites 2, 6, 11, 20 years old. Restoration sites were previously
159 sites of strip mining. All restoration was undertaken by South32 or the previous mine owners.
160 Post mining the landscape is shaped using waste material and gravel is returned. Topsoil is a
161 homogenized mix of stockpiled topsoil and topsoil that is directly transferred from newly
162 mined areas. The sites are then ripped, seeded with over 100 native species, recalcitrant
163 plants (mostly grasses) are planted, and a one-time treatment of superphosphate is applied.
164 Reference and restoration sites are dominated by Myrtaceae and Fabaceae species. Total
165 cover increases with age of restoration, eventually achieving similar cover percentages to

166 reference sites. Organic carbon increases slowly with age while soil nitrogen increases at a
167 faster rate and soil pH decreases with rehabilitation age (Banning et al., 2008).

168 The third chronosequence is located in the Pilbara in northwestern Western Australia.
169 The Pilbara has a hot, arid climate with most rainfall occurring in the summer along with
170 cyclonic activity (McKenzie et al., 2009). Temperatures have a mean min. of 15°C and mean
171 max of 30.6 °C, with 263.8 mm mean rainfall (Australian Bureau of Meteorology). Soils are
172 acidic stony loams with low fertility, which support open woodlands of snappy gum (*E.*
173 *leucophloia*) over hummock grasses (*Triodia wiseana, T. basedowii, T. lanigera*) and low
174 Acacia shrubs. (McKenzie et al. 2009). The Pilbara is a significant mining region and
175 accounts for 39% of global iron ore production (Government of Western Australia 2019). We
176 sampled 6 sites at a BHP iron ore mine (-22.84°, 118.95°) in September 2018, 2 reference
177 sites and restoration sites 4, 7, 11, and 15 years old. The restoration sites were primarily
178 borrow pits as these provided the longest, flat chronosequence. Restoration was conducted by
179 the mine owners; landscapes were reformed and stockpiled topsoil (average age 10 years)
180 was applied and then ripped. Restoration areas tended to have higher coverage of woody
181 shrubs (*Acacia*), while reference sites and older restoration areas have more hummock
182 grasses (*Triodia*). Vegetation cover was low in reference sites (~30-40%). Restoration areas
183 also had invasive species such as buffel grass (*Cenchrus ciliaris*) and kapok bush (*Aerva*
184 *javanica*) which were absent in reference sites (Data from BHP).

185 *2.2 Sample Collection*

186 Soil samples were collected from 5 points at each restoration/reference site for a total of
187 95 samples (35 SCP, 30 JF, 30 PB). For each sample, 8 sub-samples were taken randomly in
188 a 10 x10 m plot using a 15 cm soil probe; these were then manually homogenized in a large
189 sample bag and a portion was collected in a 50 mL falcon tube for microbial analyses, while
190 the rest of the sample was kept for soil chemical analyses. The soil probe was cleaned with

191 bleach between each sample and gloves were changed between each sample point. Samples
192 were collected at each location within 2-3 days to minimize variation in environmental
193 conditions caused by weather. Soils were frozen as soon as possible in a mobile freezer and
194 taken to Perth, where they were stored at -20°C until they were processed.

195 *2.3 Soil chemical properties*

196 Soils to be used for chemical analyses were dried at 50°C for 48 hours and sieved with 2
197 mm mesh. Soil moisture was determined gravimetrically by measuring a known quantity of
198 soil before and after drying. Further soil chemical analyses were conducted by the CBSP Soil
199 and Plant Analysis Laboratory in Perth. Phosphorus and Potassium were determined using
200 the Colwell method (Colwell, 1965), plant available Sulfur with the Blair/Lefroy Extractable
201 Sulfur method using a 0.25M solution of potassium chloride solution to extract the soil and
202 analyzing it using inductively couple plasma spectroscopy (Blair et al., 1991). Organic
203 carbon was measured using the Walkley Black method (Walkley and Black, 1934). Soil
204 nitrate and ammonium were extracted using a 2M potassium chloride solution and measured
205 colourimetrically after dilution. For pH and conductivity, soils were extracted in deionized
206 water with a 1:5 ratio and then measured with a pH meter and a conductivity electrode. Trace
207 elements (Copper, Zinc, Manganese, Iron) were measured by extracting the soil in a
208 diethylene-triamine-penta-acetic acid (DTPA) solution (ratio of 1:2) measuring with atomic
209 absorption spectroscopy.

210 *2.4 Soil Microbial Analysis*

211 For DNA extraction, we first used a TissueLyser (Qiagen) to homogenize the soils for 1
212 min at 30/s in 50 mL falcon tubes. DNA was extracted from 250 mg soil using the DNeasy
213 PowerLyzer PowerSoil kit (Qiagen) on the QiaCube Connect automated platform (Qiagen).
214 The final elution volume was 100 µL, and extraction controls (blanks) were carried out for

215 every set of extractions. Quantitative PCR (qPCR) was run on neat extracts and a 1/10
216 dilution to see if samples exhibited inhibition, and to determine the optimal DNA input for
217 PCR for each sample to maximise input relative to any inhibitors (Murray et al., 2015). The
218 qPCR assays were run with two primer assays one targeting the V4 location of the 16S rRNA
219 for Bacteria (16SBact515F -Turner et al., 1999/ 16SBact806R -Caporaso et al., 2011) and the
220 Internal Transcribed Spacer ITS2 for fungi (ITS7F-Ihrmark et al., 2012/ ITS4R-White et al.,
221 1990). These are common regions to target for bacterial and fungal sequencing and are
222 standard for the Earth Microbiome Project (Thompson et al., 2017).

223 The qPCRs were run on a StepOne Plus (Applied BioSystems) real-time qPCR
224 instrument with the following conditions: 5 min at 95°C, 40 cycles of 95°C for 30s, 30s at the
225 annealing temperature (50°C for Bacteria, 54°C for Fungi) and 45s at 72°C, a melt curve
226 stage of 15s at 95°C 1 min at 60°C and 15s at 95°C, ending with 10 min elongation at 72°C.
227 The PCR mix for quantitation contained: 2.5 mM MgCl2 (Applied Biosystems, USA), 1×
228 PCR Gold buffer (Applied Biosystems), 0.25 mM dNTPs (Astral Scientific, Australia), 0.4
229 mg/ml bovine serum albumin (Fisher Biotec, Australia), 0.4 µmol/L forward and reverse
230 primer, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 0.6 µl of a 1:10,000
231 solution of SYBR Green dye (Life Technologies, USA). Extraction control and non-template
232 controls were included in qPCR assays.

233 After optimal DNA input was determined by qPCR (most soil extract required a 10x
234 dilution), each sample was assigned a unique combination of multiplex identifier (MID) tags
235 for each primer assay. These MID tags were incorporated into fusion tagged primers, and
236 none of the primer-MID tag combinations had been used previously in the lab to prevent
237 cross contamination. Fusion PCRs were done in duplicate and to minimize PCR stochasticity,
238 the mixes were prepared in a dedicated clean room before DNA was added. The PCRs were
239 carried out under the same conditions as the standard qPCRs described above. Samples were

240 then pooled into approximately equimolar concentrations to produce a PCR amplicon library
241 that was size-selected to remove any primer-dimer that may have accumulated during fusion
242 PCR. Size selection was performed (150-500bp Bacteria, 250-600bp Fungi) using a
243 PippinPrep 2% ethidium bromide cassette (Sage Science, Beverly, MA, U.S.A). Libraries
244 were cleaned using a QIAquick PCR Purification Kit (Qiagen, Germany) and quantified
245 using Qubit Fluorometric Quantitation (Thermo Fisher Scientific). Sequencing was
246 performed on the Illumina MiSeq platform using the 300 cycle V2 (Bacteria), or the 500
247 cycle V2 (Fungi) as per manufacturer's instructions.

248 *2.4 Sequencing analysis*

249 Sequences were demultiplexed using OBITools (Boyer et al., 2016) for the Bacterial
250 library and a demultiplex function in the "insect" package (Wilkinson et al., 2018) on the R
251 3.5.1 platform (R Core Team, 2018). Further sequence processing was performed in R using
252 the "DADA2" package (Callahan et al., 2016) where sequences were quality filtered, the
253 error rates were estimated, and the sequences were dereplicated. The error rates are then used
254 in the sample inference stage to remove sequences likely to be errors and leave Amplicon
255 Sequence Variants (ASV). These ASVs are equivalent to zero radius operational taxonomic
256 units (ZOTUs) in usearch (Edgar, 2016). The sequences are then merged (Fungi only) and the
257 sequence table is constructed and chimeras removed. Taxonomy was assigned with DADA2
258 using the naive Bayesian classifier method of Wang et al. (2007). The databases used were
259 Greengenes (DeSantis et al., 2006) for Bacteria and UNITE (Nilsson et al., 2019) for Fungi.

260 *2.5 Statistics*

261 All statistics were run using R 3.5.1 (R Core Team 2018). Sequence variants that were
262 present in the extraction controls were removed from the dataset, then sequencing depth was
263 rarefied to the minimum in the 'phyloseq' package (McMurdie and Holmes, 2013). Alpha

264 diversity was calculated using the 'phyloseq' package (McMurdie & Holmes 2013) and
265 tested using a two-way analysis of variance (anova) with location and restoration as factors.
266 This was followed by a Tukey HSD test from the 'agricolae' package (Mendiburu, 2019).
267 Community composition was visualized using Non metric multidimensional scaling
268 (NMDS), based on the log transformed ASV table and with Bray-Curtis dissimilarity.
269 Differences between restoration ages were tested using permutational multivariate analysis of
270 variance (PERMANOVA). However, as there was significant spatial autocorrelation between
271 SMC in the Jarrah and Pilbara replicates, we also pooled replicates together and calculated
272 the similarity of each restoration age to reference communities. We also ran regression
273 analyses on these separately for each location. When pooled, there was no spatial
274 autocorrelation, although there was a loss in power. We also looked at the rarefied read
275 abundance of the top 10 most abundant phyla and tested whether that differed across
276 restoration using permuted anovas. We adjusted the *P*-values for multiple tests using the
277 "BH" method (Benjamini and Hochberg, 2007).

278 Soil chemical variables were tested for homogeneity of variance and log transformed as
279 needed before using one-way anovas to test differences between restoration age and reference
280 sites within each location. Distance Based Redundancy Analysis (dbRDA) was used to
281 determine the relationship between soil chemical variables and the community composition
282 of the soil. Soil chemical variables were normalized using the *decostand* function in the
283 'vegan' package (Oksanen et al., 2019) and then used in a dbRDA with a Bray-Curtis
284 dissimilarity matrix of the log-transformed ASV table. Variables with high "vif" or variable
285 inflation factors were removed as they are likely collinear with other variables. All dbRDAs
286 were run separately for each location.

287 To assess functional differences across restoration ages, we first assigned functionality
288 using FUNGuild (Nguyen et al., 2016) for fungal sequences and METAGENassist (Arndt et

289 al., 2012) for bacterial sequences. FUNGuild uses third party annotation to assign
290 functionality, such as trophic mode, based on taxonomy. METAGENassist uses phenotype
291 information of bacterial species listed on the NCBI database to add information such as
292 metabolism and energy source based on taxonomy (Genus level). For the fungi, we used
293 multipattern analysis from the R package 'indicspecies' (De Cáceres and Legendre, 2009)
294 and then tested the differences in the number of ASVs in each trophic mode across
295 restoration using a chisquare test for associations. For bacterial functionality we looked at the
296 normalized number of reads assigned to each metabolism category, and tested the difference
297 between sites using a two-way PERMANOVA with location and age as the grouping
298 variables. Again, P-values were adjusted for multiple comparison using the 'BH' method
299 (Benjamini and Hochberg, 2007).

300 3. Results

301 In total, we generated 4,836,541 quality-filtered bacterial sequences from 93 samples
302 and 4,331,020 quality-filtered fungal sequences from 95 samples. These were rarefied to
303 23,305 seqs/sample for Bacteria and 11,784 seqs/sample for Fungi

304 *3.1 SMC diversity, community composition and similarity to reference sites*

305 Bacterial richness (alpha diversity at the local scale) and Shannon diversity responses to
306 restoration were dependent on location (Richness: $F_{3,74}$=4.59, p=0.005; Shannon: $F_{3,74}$=7.19,
307 p<0.001). Tukey HSD results (Figure 2) show that reference sites and older restoration sites
308 were not more diverse than younger restoration sites. Similarly, fungal richness responded
309 differently depending on the location ($F_{3,76}$=6.88, p<0.001) but fungal diversity did not
310 ($F_{3,76}$= 2.351, p=0.079). Bacterial diversity varied more in the Pilbara and Coastal Plain sites,
311 while fungal diversity varied more between Jarrah sites (Figure 2). In general, there are few
312 clear directional changes in richness and diversity, with the possible exception of fungi in the

313 Jarrah sites where younger sites tend to have the lowest richness and diversity compared to
314 older restoration and reference sites (Figure 2).

315 Community composition was significantly different across locations for both bacteria
316 and fungi ($p<0.001$, Appendix Table 1). Location and restoration age were also highly
317 significant for both bacteria ($p<0.001$) and fungi ($p<0.001$). However, there was also
318 significant spatial autocorrelation in the Jarrah ($p<0.03$) and Pilbara ($p<0.001$)
319 chronosequences, although the Coastal Plain had no spatial autocorrelation (Appendix Table
320 2). Because of the loss in power from merging replicates, we use a more conservative
321 significance level of 0.1 for the linear models predicting similarity to reference sites. For both
322 the Jarrah and the Pilbara chronosequences, Bacterial communities in older restoration are
323 more similar to reference communities than younger restoration sites (Pilbara $p=0.05$, Adj
324 R2=0.84; Jarrah $p=0.06$, Adj R2=0.83). In the Coastal Plain, the youngest and oldest sites
325 have the greatest similarity to reference, while the intermediate aged sites are more
326 dissimilar. This relationship follows a quadratic linear model ($p=0.09$, Adj R2=0.81). For the
327 fungal data, the Jarrah chronosequence was the only one with a significant relationship
328 between restoration age and community similarity to reference ($p<0.001$, Adj R2=0.99).
329 However, for the Coastal Plain and the Pilbara the community similarity between restoration
330 and reference sites approached the community similarity between the two reference sites
331 (PB=0.19, SCP=0.49, JF=0.57)

332 There were 10 dominant bacterial phyla and 5 dominant fungal phyla (>2% relative
333 abundance), 93% (10 bacteria, 2 fungal) of which showed significant differences in rarefied
334 abundance between restoration ages and reference sites in at least one location (Table 1).
335 However, phyla that showed significant differences in rarefied abundance were not
336 significant at all locations. For example, Ascomycota only showed a significant decrease in
337 abundance in the Jarrah location. Gemmatimonadetes was the only phyla that consistently

338 decreased in abundance with restoration age (Table 1). Whether rarefied read abundance
339 increased or decreased with restoration age depended on the phyla and the location, and the
340 same phylum (e.g. Chloroflexi, Proteobacteria) could have opposite results in the different
341 locations.

342 *3.2 Functional groups*

343 Bacterial functional differences in metabolism were heavily driven by location, which
344 accounted for 45.7% of the variation. For example, Dinitrogen fixers were associated with
345 the Coastal Plain sites, sulfide oxidizers with the Pilbara, and bacteria that store
346 polyhydroxybutyrate were characteristic of the Jarrah sites (Appendix Figure 2). Restoration
347 sites tended to have more bacteria that degrade aromatic hydrocarbons (Table 4), particularly
348 at the Jarrah location. At both Jarrah and Coastal Plain locations restoration was also
349 associated with Napthalene degrading bacteria and sulfide oxidizers. Reference sites at the
350 Jarrah and Coastal Plain locations were associated with chitin and xylan degradation,
351 dehalogenation, and nitrogen fixation (Table 4)

352 We were able to assign function to 1209 out of 1678 fungal ASVs, and of these, 492
353 were identified as having significant (alpha=0.05) associations to one or more groups. The
354 chi-square test for association revealed there were significant differences in the number of
355 indicator ASVs in each trophic mode at the Jarrah (x2=51.11, df=12, p<0.001), but not in the
356 Pilbara (x2=10.81 df=10, p=0.372), or the Coastal Plain (x2=15.42, df=12, p=0.219) (Table
357 3). The reference sites tended to have more symbiotrophic ASVs, the older restoration sites
358 and the reference sites were similar in their levels of saprotrophic ASVs, while the younger
359 restoration sites had less saprotrophic and symbiotrophic ASVs.

360 *3.3 Soil chemical properties*

361 Responses of soil chemical properties to restoration also varied across the locations.
362 Reference sites tended to be less basic and have higher organic matter (Table 2), but this
363 relationship was not significant at all locations. The distance-based Redundancy Analyses
364 show which soil variables were significant in explaining the variations in bacterial and fungal
365 communities (Figure 4). Soil pH was one of the few significant variables that was higher in
366 newly restored sites; most significant variables (e.g. organic matter, ammonium, magnesium)
367 were higher in reference soils. In the Pilbara, there were more variables that were higher in
368 restoration, such as calcium, magnesium, and soil moisture. Overall, soil variables explained
369 over 65% of the variation in bacteria (65.7% JF, 78.2% PB, 67.9% SCP) and over 48% of the
370 variation in fungal communities (63.7% JF, 58.7% PB, 48.7% SCP). Soil properties such as
371 Ammonium, pH, Sulfur, and organic carbon are drivers of variation in microbial
372 communities at the Jarrah forest, similar to the Coastal Plain. Ammonium was a significant
373 factor in all three chronosequences for both bacteria and fungi, while other soil properties like
374 pH were significant only in the Jarrah sites. Organic carbon was also identified as common
375 driver of microbial communities, with the exception of bacterial communities in the Coastal
376 Plain.

377 4. Discussion

378 In this study, we assessed SMC across three restoration chronosequences using a high-
379 throughput amplicon sequencing approach. We demonstrated changes in SMC at restored
380 sites, but found that patterns were complex. Understanding the responses of soil microbes to
381 restoration is important as they are increasingly popular targets for monitoring biodiversity
382 recovery.

383 *4.1 Are SMC in older restoration sites more similar to reference communities than*
384 *those at younger restoration sites?*

385 Overall, there were no consistent changes in microbial richness or diversity across the
386 three restoration chronosequences (Figure 2). These trends support previous work where the
387 greatest changes identified were in the shifts in community composition, rather than diversity
388 (Banning et al. 2011; Sun et al. 2017; Yan et al. 2018). Our results indicated strong
389 compositional differences within each of the chronosequences. However, these should be
390 interpreted with caution, as there was also significant spatial autocorrelation between
391 replicates at two out of three locations (Jarrah and Pilbara). Spatial autocorrelation is an
392 important consideration when using SMC for monitoring (Yan et al. 2019), especially in
393 mine site restoration where site locations are dependent on presence of resources, rather than
394 the ecology of the surrounding environment. While spatial scale is a strong driver of
395 microbial diversity (Nunan et al., 2002; O'Brien et al., 2016), especially in reconstructed
396 soils (Mummey et al. 2002), soil chemical factors and plant associations account for more
397 variation in SMC (Burns et al., 2015; Nunan et al., 2002). Accordingly, spatial
398 autocorrelation does not mean the data are not informative for restoration. Instead, it indicates
399 the importance of multiple reference sites for comparison as done in this study where we
400 were able to include spatially separated reference sites located near the restoration sites. The
401 collection and archiving of soil samples throughout restoration might help generate time-
402 stamped data that is less impacted by autocorrelation as sampling sites can be more closely
403 controlled.

404 Several studies have found a trend of increasing similarity to reference bacterial
405 communities with restoration age (Banning et al. 2011, Yan et al 2019, Sun et al. 2017). We
406 found similar directional changes in bacterial communities at the Jarrah and Pilbara locations.
407 In contrast, at the Coastal Plain, the youngest and oldest sites were most similar to the
408 reference soils. The use of stored topsoil for restoration of study sites presents a potential
409 confounding factor as stockpiling topsoil under different conditions may cause variation in

410 SMC; the Coastal Plain was the only location in our study where direct transfer of topsoil
411 during the restoration process was conducted. During direct transfer, soil is stripped from an
412 area to be mined and transferred immediately to a site to be restored. This approach to
413 restoration preserves the integrity of the soil seed banks (Rokich et al. 2000) and may also
414 allow the bacterial communities to be maintained in the short term. However, following this
415 initial phase the dynamic interactions of edaphic and vegetation factors (e.g. absence of
416 mature trees) present in the re-growing restoration may cause shifts in the bacterial
417 communities in the intermediate aged sites. In contrast, the Jarrah and Pilbara sites stockpile
418 and store topsoil until required (from months to years) during which time there may be
419 reduction of microbial communities to those capable of surviving the biologically hostile
420 conditions within a stockpile (Birnbaum et al. 2017).

421 Higher orders of bacteria such as phyla are considered to share some general life history
422 strategies as a result of shared evolutionary pathways (Fierer et al., 2007; Philippot et al.,
423 2010). Therefore, despite the diversity within the phyla, they can be an indicator of
424 successional trajectories (Banning et al. 2011, Yan et al. 2019). We found that the response of
425 bacterial rarefied phyla abundance was very different between locations. For example, with
426 increasing restoration age, Proteobacteria were more abundant at the Jarrah location, less
427 abundant in the Pilbara, and their response was variable in the Coastal Plain. Other studies
428 have also found increases of Proteobacteria with restoration age (Yan et al. 2019, Banning et
429 al. 2011, Gellie et al. 2017), similar to our results in the Jarrah forest. However, none of these
430 studies were located in a hot arid climate, and the decrease of Proteobacteria in the Pilbara
431 may be due to the extreme climatic and soil conditions of the arid zone. Proteobacteria
432 abundance is often related to carbon availability (Fierer et al. 2007) and there were no
433 significant changes in organic carbon at the Pilbara chronosequence sites. However, there are
434 also examples such as the phylum Chloroflexi, which showed a pattern of decrease in the

435 Jarrah site, yet another study in the same ecosystem identified it as one of the phyla that
436 increases with restoration (Banning et al. 2011).

437 Bacteria are highly variable, and because of their high growth rates communities can
438 change rapidly in composition within a year (Lauber et al., 2013; Sun et al., 2017). It is
439 common in microbial studies to find distinct communities between disturbed and undisturbed
440 sites, but the taxa driving those differences are often inconsistent between studies (Lauber et
441 al. 2013). Study-specific soil and site conditions likely drive this variability in taxa, which is
442 why using only certain taxa as indicators is questionable. It also emphasizes the importance
443 of reference sites near restoration sites sampled concurrently, to account for variability over
444 time and space to provide an indication of general trajectory due to climatic variables.

445 Unlike bacteria, fungal communities showed progressive recovery towards the reference
446 communities only at the Jarrah location, with no clear trends at the other two sites. Similarly,
447 while there was a trend of increasing diversity with age for fungi in the Jarrah location, there
448 were no such trends for bacteria. For both the Pilbara and Coastal Plain locations, there are
449 two possible explanations for why fungal communities are not becoming more similar to
450 reference communities with increasing age. Firstly, fungal communities may resist
451 restoration as they show no trajectory towards reference communities with increasing
452 restoration age. This was a key finding in a previous study at the Coastal Plain location (Hart
453 et al. 2019). Secondly, the similarity between restoration and reference sites was
454 approximately the same as the similarity between the two reference sites, implying that the
455 communities may have achieved maximum similarity to reference communities early in the
456 restoration process. The use of topsoil in restoration may have adequately preserved the
457 fungal communities in these systems. The latter is supported by the fact that fungal phyla also
458 showed few significant differences in rarefied abundance in the Pilbara or Coastal Plain,
459 whereas phyla in the Jarrah chronosequence showed clear differences in phyla (Ascomycota

460 and Basidiomycota) that agreed with previous studies (Yan et al. 2018). As has been
461 previously emphasized (Lauber et al. 2013) community composition alone may not be as
462 important as the presence of particular functional groups which may also vary as site
463 conditions mature from a disturbance event.

464 *4.2 Which functional groups are indicators of the different stages of restoration?*

465 The functional capacity of SMC is an appealing target for monitoring restoration
466 because there is considerable functional redundancy and it is less variable than community
467 composition over small spatial and time scales (Kumarasan et al. 2017). Despite this, there
468 are few examples where high-throughput sequencing has been used to explore change in
469 functional groups across a restoration chronosequence (Yan et al. 2019). This information is
470 important because SMC underpin many ecological and physiological functions (e.g., organic
471 matter decomposition, regulation of mineral nutrient availability) (Meena et al., 2017; Yang
472 et al., 2018) that are essential to building ecological resilience. We observed a higher
473 incidence of bacteria involved in organic matter decomposition (chitin degradation, lignin
474 degradation, xylan degradation) in reference sites at the Jarrah and Coastal Plain locations.
475 Chitin is the structural element of organisms such as fungi and insects (Merzendorfer, 2006;
476 Roncero, 2002), while lignin and xylan are biopolymers in plant cell walls (Ochoa-Villarreal
477 et al. 2012). All of these are more abundant in reference ecosystems, resulting in a higher
478 prevalence of organic matter degrading bacteria in those sites. Reference sites were also
479 associated with nitrogen fixing bacteria, providing plants with an important limiting nutrient
480 (Vitousek et al., 2002). Conversely, restoration sites were associated with bacteria that
481 degrade aromatic hydrocarbons and naphthalene at Jarrah and Coastal Plain (Table 4). These
482 organisms are likely responding to a major shift in chemical composition of the soil as a
483 result of topsoil stripping and storage. This may shift microbe abundance to reflect the
484 disequilibrium of very altered substrates that are not present in the reference sites.

485 Including the fungal functionality analysis improves the interpretation of the community
486 composition results by showing there were also no differences in fungal trophic modes
487 between the sites at those locations. However, in the Jarrah forest, where there was a
488 trajectory in fungal community composition, we also found significant differences in trophic
489 modes between younger restoration and reference sites. Reference sites tended to have more
490 saprotrophs, necessary for decomposing accumulated leaf litter. They also had more
491 symbiotrophs, which are fungi that exchange nutrients with host cells (Nguyen et al. 2016)
492 such as mycorrhizae, providing nitrogen and phosphorus to their plant partners in exchange
493 for carbohydrates (Glen et al., 2008). These mycorrhizal networks underpin forest growth and
494 health as found in the jarrah forest ecosystem (Glen et al., 2008). In contrast, Yan et al.
495 (2018) working in a coastal revegetation system found little difference between the number
496 of indicator taxa in each trophic mode. The differences between the Jarrah forest location and
497 the Coastal Plain and Pilbara locations reflect the higher biomass in Jarrah, and higher
498 proportion of root biomass attributed to mycorrhizal species.

499 *4.3 Are soil chemical properties associated with restoration age, and/or change in*
500 *SMC composition?*

501 The effect of restoration age on some soil chemical variables was consistent across
502 sites and the patterns similar to those found in previous studies on restoration
503 chronosequences (Banning et al. 2008; Munoz-Rojas 2016; Yan et al. 2018). For example,
504 there was an increase in organic carbon and decrease in pH at older sites, and the direction of
505 this change was towards the values found at reference sites. However, we found trends in
506 other soil chemical variables (e.g. calcium, magnesium) that tended to be different in the
507 Pilbara compared to the Jarrah and Coastal Plain, likely reflecting differences in climate and
508 vegetation between these locations. Similarly, the soil variables that are significant in
509 explaining the variation in SMC changed between locations although there were some

510 common trends (e.g. organic carbon driving communities closer to reference). Soil abiotic
511 variables are known drivers of microbial community composition (Burns et al. 2015; Yan et
512 al. 2018), although the mechanisms behind many of these relationships is not fully
513 understood.

514 ## 5. Conclusion

515 Our findings show that ecological restoration of mine sites can lead to the development
516 of soil microbial communities, which over time become increasingly similar in composition
517 to those of natural reference sites. However, the trajectory response of SMC was location
518 and organism (fungal vs bacterial) specific and affected by topsoil application. Thus, high
519 throughput monitoring of SMC changes should be treated with caution and applied to
520 appropriate ecosystems (i.e. monitoring fungi in ecosystems more reliant on fungal
521 symbioses). Further studies are needed that include sites located in different climate zones,
522 on different soil types or with different plant communities. Also needed are studies of older
523 restoration sites, studies with multiple time points and across different seasons, to enable
524 understanding of background levels of variability. Archiving of samples is suggested, to
525 enable better understanding of how SMC communities change over time. Our results also
526 emphasize the importance of multiple reference sites to account for the variability over space
527 that is common in soil microbial communities.

528 Including functional analyses of microbial data improved our understanding of the
529 microbial responses to restoration. Currently, the tools to examine functionality from high-
530 throughput sequencing data are available, and will continue to develop in the future
531 especially as microbial analysis is increasingly employing metagenomic (i.e. shotgun)
532 approaches (Kumaresan et al. 2017). We advocate that restoration studies involving SMC
533 should explore functionality as well as composition, but that measurements of richness are

534 less informative. In addition, assessing functionality using non sequencing based methods
535 such as microbial respiration (Haney et al. 2008; Munoz-Rojas et al. 2016) and plant
536 bioassays will be important in validating high-throughput sequencing results.

537 eDNA studies could also be extended to include other biological groups such as soil
538 fauna (Eaton et al. 2017) or to other sources of DNA (Fernandes et al. 2019; Heyde et al.
539 2020), enabling a more holistic understanding of biodiversity recovery. Many companies in
540 the resources sector strive towards 'best practice' restoration, although what constitutes best
541 practice is not always clear. The approaches herein and in other published studies show great
542 promise in our capacity to incorporate a wider microbial lens on the issue (Gellie et al. 2017;
543 Yan et al. 2018). With further refinement to experimental design and better ways to study
544 microbial function, these approaches may help guide future restoration efforts and
545 interventions (i.e. microbial inoculation) and expand past mining to agricultural land and
546 contaminated sites.

547 Declaration of competing interest

548 The authors declare no conflict of interest.

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557 CRediT Author Statement

558 **Mieke van der Heyde**: Conceptualization, Investigation, Formal Analysis, Visualization
559 Writing-Original draft preparation. **Michael Bunce**: Conceptualization, Writing-Review and
560 Editing. **Nicole E White**: Conceptualization, Writing-Review and Editing. **Grant Wardell-**
561 **Johnson**: Conceptualization, Writing-Review and Editing. **Kingsley Dixon**: Writing-Review
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563 ## Data Accessibility Statement

564 All sequencing data and DADA2 scripts can be found online at
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838 # Tables and Figures

839

840 Table 1: Effects of restoration on the rarefied abundance of certain phyla at three Western
841 Australian locations. Only phyla making >2% total abundance were included. JF-Jarrah
842 Forest, PB-Pilbara, SCP-Swan Coastal Plain
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	JF		*PB*		*SCP*	
Phylum	**P val**	**direction**	**P val**	**direction**	**P val**	**direction**

Acidobacteria	0.785		**0.002**	Decreasing	**0.002**	Variable
Actinobacteria	**0.035**	Variable	0.627		**0.002**	Increasing
Bacteroidetes	0.627		**0.011**	Variable	0.627	
Chloroflexi	**0.002**	Decreasing	**0.002**	Increasing	**0.002**	Decreasing
Firmicutes	**0.002**	Decreasing	**0.005**	Variable	**0.002**	Variable
Gemmatimonadetes	**0.002**	Decreasing	**0.002**	Decreasing	**0.002**	Decreasing
Planctomycetes	**0.007**	Decreasing	0.080		**0.002**	Decreasing
Proteobacteria	**0.002**	Increasing	**0.002**	Decreasing	**0.002**	Variable
Thaumarchaeota	0.367		**0.009**	Decreasing	**0.002**	Decreasing
Verrucomicrobia	**0.011**	Variable	**0.026**	Variable	**0.031**	
Ascomycota	**0.015**	Decreasing	0.330		0.4	
Basidiomycota	**0.040**	Increasing	**0.015**	Variable	0.573	
Glomeromycota	0.602		0.540		NA	
Mortierellomycota	0.625		0.330		0.573	

Table 2: The effect of restoration on soil chemical variables at three Western Australian locations. Numbers show the mean in each group with the standard error in parantheses. Variables with significant differences (alpha<0.05) are bold, and the letters indicate Tukey HSD test results. JF-Jarrah Forest, PB-Pilbara, SCP-Swan Coastal Plain

	JF			*PB*			*SCP*		
Age	**Young**	**Old**	**REF**	**Young**	**Old**	**REF**	**Young**	**Old**	**REF**
Ammonium (mg/kg)	**8.1 (±0.5) b**	**13.6 (±0.7) a**	**15.6 (±1.2) a**	2.1 (±0.3)	2.8 (±0.4)	2.1 (±0.2)	**1.8 (±0.3) b**	**1.8 (±0.2) ab**	**2.7 (±0.2) a**
Nitrate (mg/kg)	**1.2 (±0.2)ab**	**1.0 (±0.0) b**	**1.5 (±0.2) a**	2.0 (±0.4)	1.4 (±0.3)	1.1 (±0.4)	0.5 (±0.0)	0.5 (±0.0)	0.5 (±0.0)
Phosphorus (mg/kg)	6.9 (±3.5)	3.0 (±0.4)	4.2 (±0.4)	3.3 (±0.8)	5.3 (±0.6)	4.4 (±0.5)	1.1 (±0.1)	1.0 (±0.0)	1.0 (±0.0)
Potassium (mg/kg)	59.2 (±5.0)	77.5 (±7.8)	81.7 (±6.8)	290.4 (±27.3)	290.8 (±21.6)	244.5 (±27.5)	11.3 (±0.7)	13.5 (±1.5)	12.8 (±1.0)
Sulfur (mg/kg)	7.3 (±1.1)	7.1 (±0.2)	12.1 (±0.9)	6.6 (±3.9)	2.5 (±0.3)	2.3 (±0.4)	1.5 (±0.1)	1.9 (±0.2)	1.4 (±0.1)
Organic Carbon (%)	**2.41 (±0.15)c**	**3.41 (±0.09)b**	**4.66 (±0.09) a**	0.41 (±0.04)	0.49 (±0.05)	0.54 (±0.08)	**0.91 (±0.09)ab**	**0.77 (±0.06) b**	**1.14 (±0.07) a**
Conductivity (dS/m)	**0.041 b (±0.004)**	**0.063 b (±0.005)**	**0.077 a (±0.006)**	0.030 (±0.006)	0.024 (±0.004)	0.017 (±0.002)	0.017 (±0.003)	0.019 (±0.002)	0.015 (±0.001)

pH	**6.4 (±0.0)a**	**6.0 (±0.0)b**	**6.0 (±0.0)b**	**7.1 (±0.2) a**	**6.8 (±0.2)ab**	**6.5 (±0.1) b**	6.4 (±0.2)	6.5 (±0.1)	5.9 (±0.1)
Copper (mg/kg)	**1.61 (±0.39)a**	**0.90 (±0.12)ab**	**0.61 (±0.07)b**	1.01 (±0.13)	1.18 (±0.08)	1.38 (±0.14)	0.32 (±0.03)	0.28 (±0.07)	0.25 (±0.04)
Iron (mg/kg)	**40.6 (±3.5)b**	**57.9 (±6.3)a**	**59.8 (±5.0)a**	**9.7 (±0.3)b**	**12.4 (±0.5)a**	**12.6 (±0.7)a**	**13.5 (±0.6)ab**	**10.9 (±1.2)b**	**14.2 (±1.0)a**
Manganese (mg/kg)	**6.88 (±0.49)c**	**13.54 (±1.30)b**	**30.73 (±1.41)a**	**19.28 (±1.90)b**	**34.31 (±4.11)a**	**33.02 (±2.76)a**	**0.89 (±0.11)b**	**0.79 (±0.09)b**	**1.39 (±0.1)a**
Zinc (mg/kg)	0.82 (±0.27)	0.53 (±0.06)	0.30 (±0.03)	0.29 (±0.01)	0.38 (±0.08)	0.32 (±0.04)	0.31 (±0.02)	0.37 (±0.05)	0.40 (±0.13)
Aluminium (meq/100g)	**0.05 (±0.01)c**	**0.13 (±0.02)b**	**0.19 (±0.02)a**	0.13 (±0.02)	0.14 (±0.01)	0.11 (±0.02)	**0.04 (±0.00)ab**	**0.03 (±0.00)b**	**0.05 (±0.00)a**
Calcium (meq/100g)	**4.72 (±0.44)b**	**5.54 (±0.31)b**	**8.88 (±0.65)a**	**5.81 (±0.90)a**	**4.39 (±0.88)ab**	**2.52 (±0.26)b**	2.04 (±0.24)	1.78 (±0.34)	1.56 (±0.11)
Magnesium (meq/100g)	**1.17 (±0.10)b**	**1.42 (±0.06)b**	**2.54 (±0.23)a**	**3.20 (±0.61)a**	**1.25 (±0.18)b**	**1.61 (±0.31)b**	**0.27 (±0.03)a**	**0.16 (±.01)b**	**0.28 (0.02)a**
Soil Moisture	**6.9 (±0.3)c**	**8.2 (±0.3)b**	**10.2 (±0.5)a**	**2.9 (±0.1)a**	**2.8 (±0.1)a**	**2.1 (±0.2)b**	1.5 (±0.1)	2.0 (±0.1)	2.0 (±0.3)

850
851 Table 3: Number of fungal indicator taxa in each trophic mode at the restoration and
852 reference sites. JF-Jarrah Forest, PB-Pilbara, SCP-Swan Coastal Plain
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	JF			*PB*			*SCP*		
Trophic Mode	**Young**	**Old**	**REF**	**Young**	**Old**	**REF**	**Young**	**Old**	**REF**
Pathotroph	6	3	3	8	12	5	3	6	7
Pathotroph-Saprotroph	20	54	42	8	17	5	35	34	27
Pathotroph-Saprotroph-Symbiotroph	16	7	2	11	10	9	12	10	9
Pathotroph-Symbiotroph	2	16	22	0	0	0	2	1	2
Saprotroph	20	43	41	22	21	8	38	25	29
Saprotroph-Symbiotroph	6	8	4	8	5	4	5	3	1
Symbiotroph	5	12	20	13	7	9	3	4	12

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865 Table 4: Multipattern Analysis showing the bacterial metabolic pathways that are
866 significantly associated with each restoration category at the three locations. Only those with
867 significant (alpha<0.05) were included. Numbers indicate adjusted *P*-values where there were
868 significant associations.
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	JF			***PB***	***SCP***	
Metabolism	**Young**	**Old**	**REF**	**Young**	**Old**	**REF**
Ammonia.oxidizer						0.002
Atrazine.metabolism					0.002	
Carbon.fixation					0.002	
Chitin.degradation			0.011			0.002
Chlorophenol.degrading					0.002	
Degrades.aromatic.hydrocarbons	0.011			0.019	0.002	
Dehalogenation			0.045			0.002
Lignin.degrader			0.010			
Naphthalene.degrading	0.010				0.004	
Nitrogen.fixation			0.011			0.002
Streptomycin.producer		0.045				
Sulfate.reducer						0.002
Sulfide.oxidizer	0.013				0.002	
Sulfur.metabolizing						0.002
Sulfur.oxidizer					0.005	
Xylan.degrader						0.002

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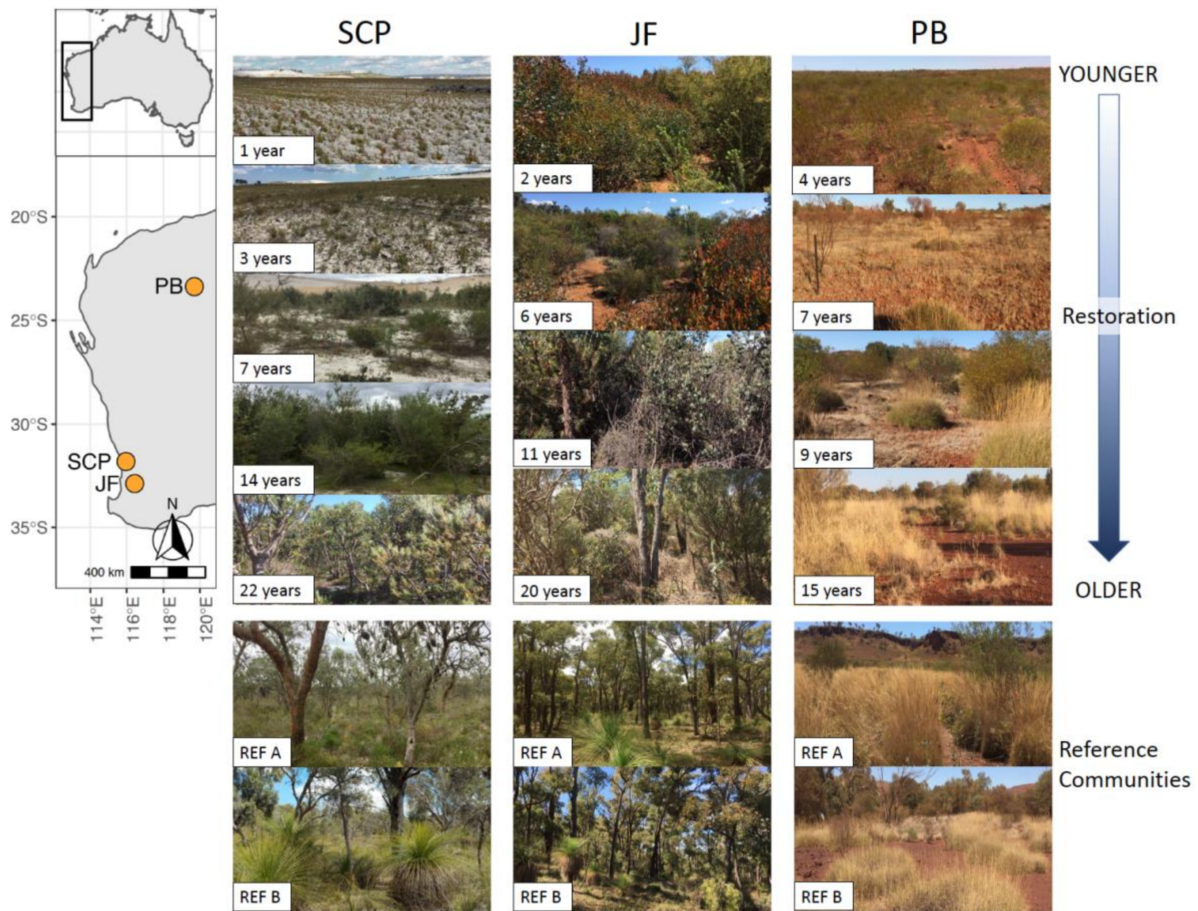

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875 Figure 1: Chronosequences of mining restoration where soil samples were collected.
876 Restoration sites shown with the number of years restoration from 1 to 22 years. Reference
877 sites shown below. JF-Jarrah Forest, PB-Pilbara, SCP-Swan Coastal Plain
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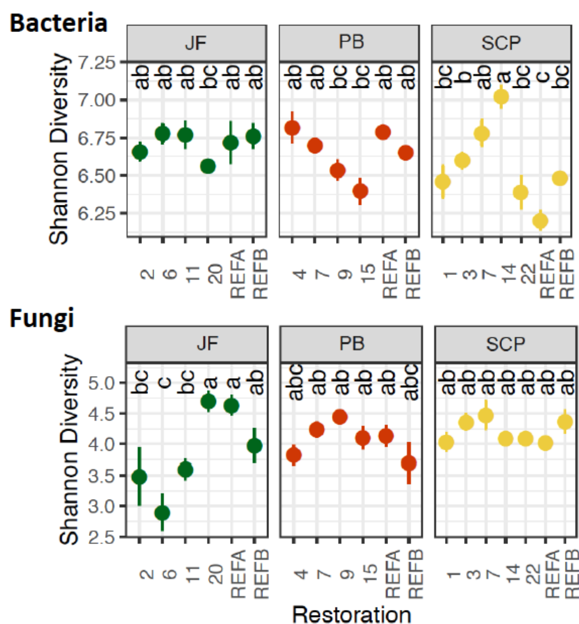

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882 Figure 2: Alpha diversity of bacterial (above) and fungal (below) communities at restoration
883 sites. Letters indicate results of the Tukey HSD test. Richness showed similar patterns to
884 Shannon diversity.
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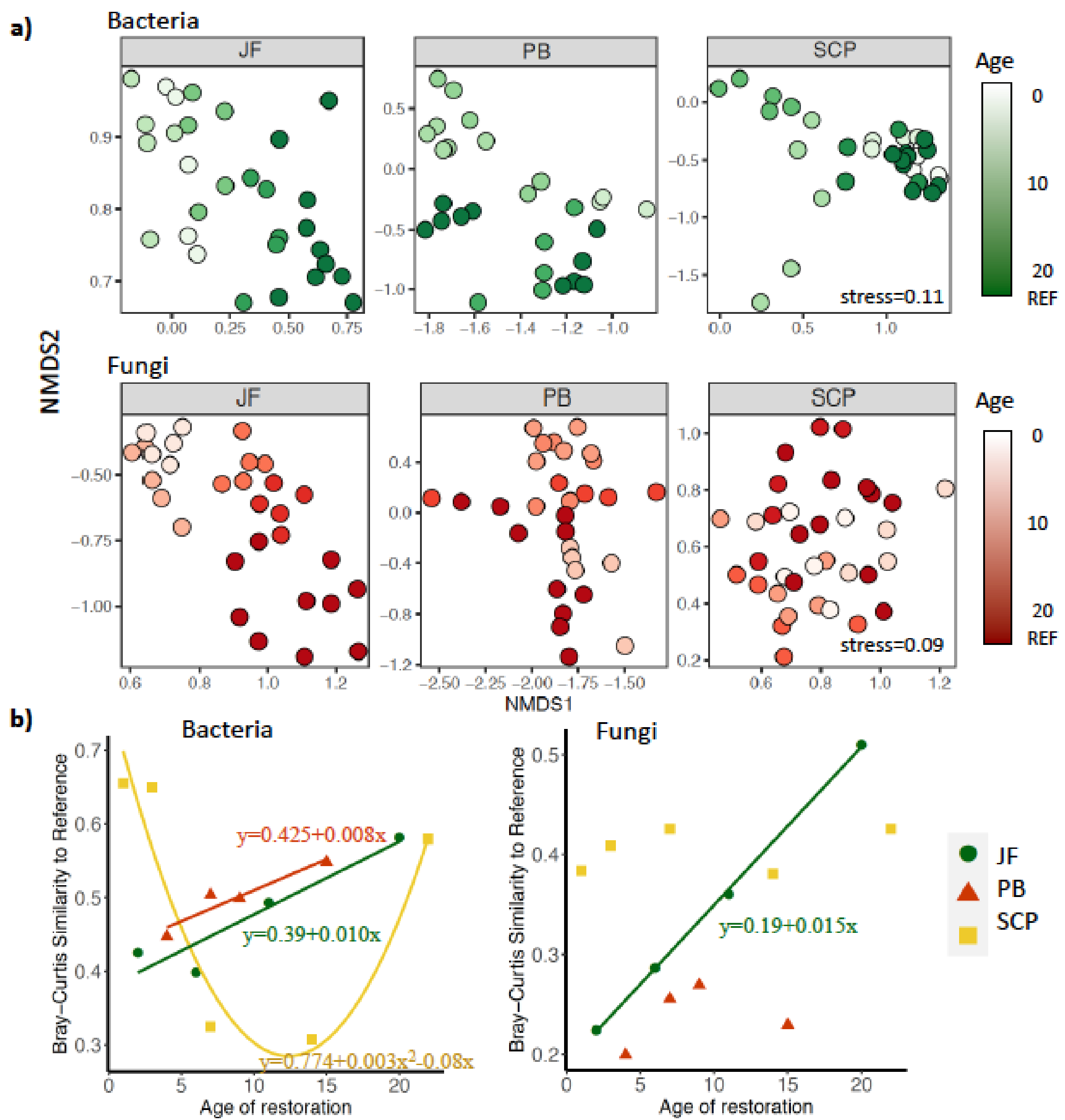

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890 Figure 3: Community composition of soils in restoration and reference sites at three
891 chronosequences of mine site restoration. NMDS Ordinations (a) of bacterial (above) and
892 fungal (below) community composition (similarity=bray curtis). The bray-curtis similarity
893 (b) between each site and the most similar reference site. Lines are included for linear models
894 that were significant (alpha=0.1). JF-Jarrah Forest, PB-Pilbara, SCP-Swan Coastal Plain
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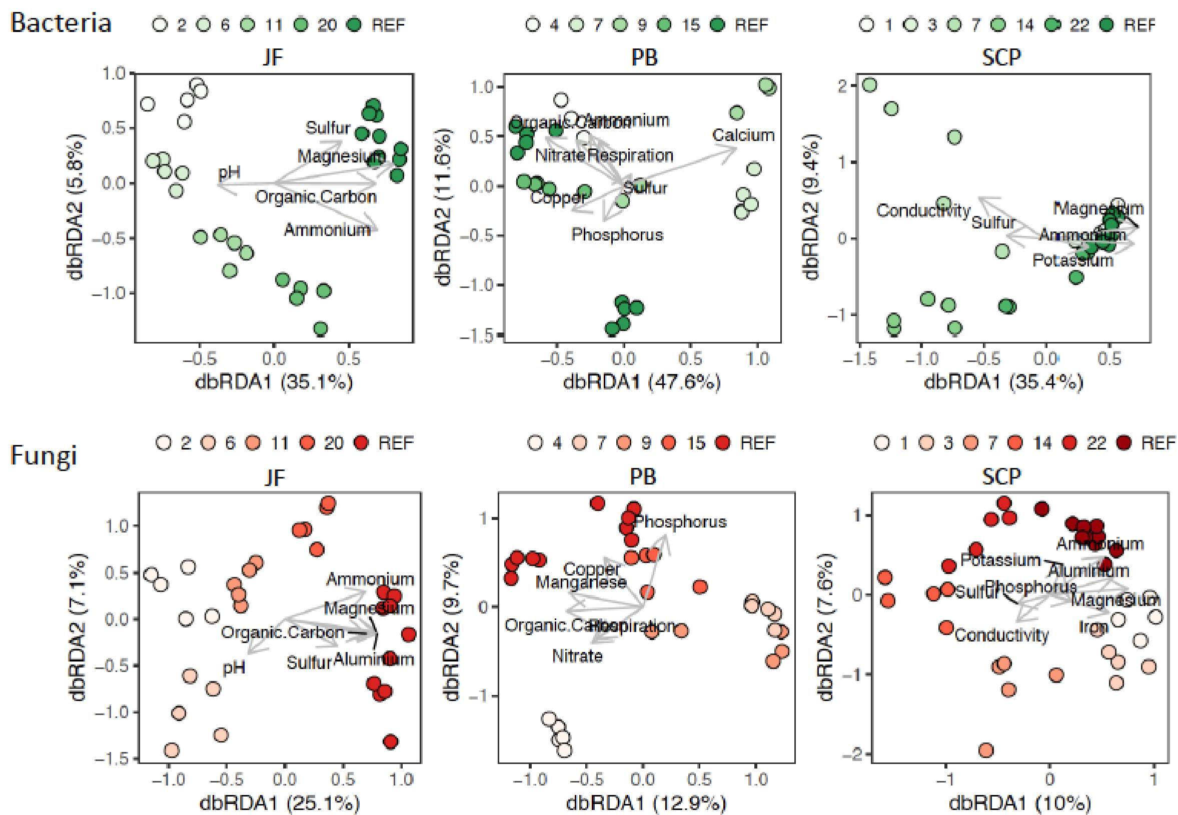

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900 Figure 4: Results of distance-based Redundancy Analyses for bacterial communities above
901 (green) and fungal communities below (red). Significant soil terms (alpha=0.05) are shown
902 using arrows and labels. JF-Jarrah Forest, PB-Pilbara, SCP-Swan Coastal Plain
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