Responses to Reviewer 1

We would like to thank Reviewer 1 for their recognition of the importance of this research. The suggestions provided by this thorough review has helped us improve the manuscript considerably. One of the difficulties in the preparation of the ms was the compilation of the work and the transformation of a mine report to a scientific paper. This explains a lot of generalisations especially in the discussion. I believe we have now addressed all your concerns in full. Please note ms has been fully revised and included at the end of this document. Comments and questions below:

R1: **Abstract** needs more specificity; alter first sentence with focus on degradation; remove sentence from lines 21, 22 as it is not relevant to this study.

Response: Abstract fully revised to include specific results and sentence removed.

R1: Additional up-to-date references required in introduction, in particular p2, L10, include additional cyanobacterial restoration references

Response: More recent references have been added to introduction (see edited ms below) and additional cyanobacterial restoration references have been added throughout.

R1: Use of word biofilm

Response: I would partially disagree with R1 regarding the use of biofilm being more a less restricted to aquatic and marine environments. For example: Rossi and De Philippis (2015) specifically refer to the role of EPS in the creation of a biofilm in arid environments as the first step in biocrust establishment. I have revised its use (and context) and referenced it appropriately. Sentence incorporating biofilm in introduction now reads: "Cyanobacterial biofilms provide initial stabilisation of disturbed surfaces that pave the way for diverse microbial communities, and form bioactive crust-like layers integrated into the soil surface (e.g. Büdel et al., 2009; Rossi et al., 2017; Bowker et al., 2014).

R1: define chlorophyll (type) measured (p2, L27) also in M&M section

Response: We have excluded ratio as not well explained so sentence now reads "EPS more than doubles the biocrusts compressive strength and increases cohesiveness by up to six times (Hu et al., 2002)."

Response: see M&M revisions – chlorophyll *a* has been defined in all areas.

In M&M main sentence now reads: "In order to define the cyanobacterial component of the biocrust, chlorophyll *a* pigmentation (unique to cyanobacteria) was measured following resurrection."

R1: p2, L32 change 'was to were'

Response: completed

R1: "Research into…is rare" be more specific.

Response: Refer to paragraph revised paragraph above that has included additional references and a more complete description of known research to date.

R1: p3, Line 22 change plant-available to biological-available

Response: completed

R1: Clarify focus of work

Response: We have removed site descriptions and background to start of methods and clarified the lead in paragraph to hypotheses to read as follows:

"This project is based on designing mining rehabilitation plans that will achieve improved long-term outcomes. The restoration of landscape function and the accompanying need for the restoration of the soil ecosystem that included biocrusts after high-levels of disturbance directed the development of this biocrust research project. As cyanobacterial communities often develop their species richness, abundance and structure in response to their environment (e.g. Aboal et al., 2016; Büdel et al., 2009; Williams et al., 2014; Williams and Büdel, 2012), it was important to examine the biocrust community structure and survival. Within this design a polyphasic approach considers the essential ecosystem services provided through the reestablishment of biocrusts. The conceptual design is based on the net ecosystem benefits that must be achieved through biocrust regeneration. It follows that cyanobacterial inoculum in the topsoil stockpiles would be central to early stabilisation of mobile surfaces subjected to the potential impacts of wind and rain splash erosion. We sought to determine whether shallow 'biobanks' of cyanobacterial-enriched top soil would facilitate biocrust recovery of this mine site."

R1: **Methods** need revision for clarity, specific points to address are addressed in responses below

Responses: The methods have been completely revised into a clear sequence under revised headings as follows:

2.0 Methods

2.1 Background and site description

2.2 Field Methods

2.3 Ecophysiological properties of biocrust cyanobacteria

2.4 Cyanobacterial community structure

2.5 16S rDNA profiling of native undisturbed biocrust microbiomes

2.6 Cyanobacterial tolerance to stockpiling

Specific points addressed below:

R1: 'Fig 1 (11 sites) vs. 10 sites sampled'

Response: Figure description now states that Site 11 was not used.

R1: "natural samples vs. stockpile samples"

Response: In the last paragraph of the introduction we had already clearly stated they were natural undisturbed samples versus stockpile samples that had been crushed and buried.

"The overall goals of the biocrust research program were to: (a) evaluate specific roles of natural, undisturbed biocrusts in ecosystem function at the mine site; (b) determine cyanobacterial community structure in terms of key species that drive early

colonisation, biogeochemical cycling and soil stabilisation; and (c), to investigate the effects of stockpiling topsoil on cyanobacterial survival after burial and subsequent recovery."

R1: 'Explain crust types'

Response: We have added a description into the first section 'Site description and background'

5 "The biocrusts at J-A had been previously classified into three primary successional stages representative of the five biocrust types found growing across the landscape (Doudle et al., 2011); Types 1–2: light coloured, thin cyanobacterial crust in early stages of development; Type 3: cyanobacterial crust, well established, intermediate stages of development; Types 4–5: biocrust, well established with cyanolichens and/or green algal lichens and mosses, late successional stage of development (additional descriptions available in supplementary Table S1). Study locations were selected from the main vegetation 10 associations across the three soil types and Lake Ifould (a dry salt lake)."

R1: 'p5, line 2 what type of tool?'

Response: Inserted "Within each area eight 10 cm diameter samples were selected at random and removed to a depth of 1 cm using metal scraper (n=80), air dried (>40°C), and stored in Petri dishes."

R1: 'p7, line 3 what type of chlorophyll and improve description'

15 Response: Chlorophyll a defined throughout. Description of methods improved: "Chlorophyll *a* concentration of the biocrusts were determined following resurrection (by moistening) using a 1:5 ratio of (dry weight) biocrust to Dimethyl sulfoxide (DMSO) (Barnes et al., 1992) with samples placed in a warm bath (65°C) for a two-hour dark extraction, followed by centrifuging for five minutes (5000 g RCF). Chlorophyll *a* concentration was calculated using Wellburn's (1994) equations."

R1: 'p6, lines 10-11 clearly explain use of penetrometer'

20 Response: Description revised to read "A pocket penetrometer (8 mm foot) was used to determine the compressive strength (kg cm-2) of the dry intact biocrusts. Four measurements were taken from each sample location, providing 12 replicates per site. The measurement was taken at the point when the crust was broken, and the foot penetrated the crust surface."

R1: 'Explain measurements of photosynthetic activity from what you did'

Response: This has been revised to read "Photosynthetic performance (recorded as yield, YII) was measured using pulse-25 amplitude modulated (PAM) fluorometer (Pocket PAM; Gademann Instruments, Germany). The aim was to demonstrate photosynthetic yield (YII) indicative of active growth of the biocrusts, using the detection of chlorophyll fluorescence from photosystem II (PSII). The sensor was placed onto the biocrust and once started, a series of short pulses of excitation light at high intensity that is amplified resulting in a brief closure of PSII and the measurement of fluorescence yield based on the Genty parameter which is the quantum yield (YII) of the charge separation of PSII (Genty et al., 1989) and recorded on a scale 30 of 0–1 for all photosynthesis. Allowing a short space of time between readings, this process was completed several times for each sample."

R1: 'More detail in sequencing methods and was sequencing done for stockpile samples?'

Response: Please note that sequencing was not used in stockpiles as unfortunately there was insufficient budget to cover this. Now the methods are rewritten this is clearer.

Page 5, Line 11: Biocrusts were collected using a paint scraper and spatula which were wiped between each sample using 70% ethanol. Figure 3 shows how biocrusts were selected.

More information on DNA library generation: Sentence updated to - Molecular libraries of the 16S rDNA V123 hypervariable region generated via PCR as per Chilton et al., (2017) and submitted to the Ramaciotti Centre for Genomics (UNSW, Australia) for a 2x300 bp sequencing run on an Illumina MiSeq instrument.

Clearer statistical analyses: Methods section has been re-structured. Each approach now has the specific statistical analyses used under that section.

This section in the methods has been rewritten as follows:

"2.3 16S rDNA profiling of native undisturbed biocrust microbiomes

For genomic profiling of naturally occurring successional biocrust communities, a location adjacent to Site 9 was visually determined to contain Bare, Early (Types 1-2) or Late (Types 3-5) stages of development (Table S2). Biocrust successional features were determined by morphological attributes of pigmentation, thickness and surface roughness as well as the presence/absence of lichens and mosses (Fig. 3), (Chilton et al., 2017). Bare stage was defined by loose soil particles with no biocrust structure. Samples were collected in July 2014. For each successional stage, three replicates were collected that were representative of SMUs 1–3 where a 10 cm^2 plot with 95% coverage of the desired biocrust stage was excised to the depth of the crust and non-aggregated soil discarded (Fig. 4). Samples were processed at UNSW, Sydney.

Each biocrust replicate for Bare, Early and Late stages of development were homogenised and genomic DNA extraction performed using the FASTDNA Spin Kit for Soil (MP Bio Laboratories, USA) according to the manufacturer's instructions. Molecular libraries of the 16S rDNA V123 hypervariable region generated via PCR as per Chilton et al., (2017) and submitted to the Ramaciotti Centre for Genomics (UNSW, Australia) for a 2x300 bp sequencing run on an Illumina MiSeq instrument. Sequencing data was processed using Mothur version 1.34.0 (Schloss et al 2009) and described in detail in Chilton et al., (2017). Singleton and doubleton OTUs were removed and samples rarefied to 8598 sequences each across 3785 OTUS. The curated Greengenes database (McDonald et al 2012) was used to assign taxonomy to OTUs. Diversity values were derived using the DIVERSE function within the Primer package (Anderson et al 2008) upon standardized OTU values. ANOVA with post hoc Tukey's tests was used to test for significant differences between stages. Multivariate analyses were performed in Primer upon a Bray-Curtis dissimilarity matrix generated from square-root transformed abundance data. Samples were represented in two and three-dimensional space within a non-metric multidimensional scaling plot (nMDS). Pair-wise, a posteriori comparisons of factor Stage were performed using the PERMANOVA function with 9999 Monte Carlo permutations. Homogeneity of dispersion for each stage was tested using PERMDISP."

R1: 'Please provide the type of statistical analysis for each specific section'

Response: see above and further descriptions added throughout revised methods (refer to main ms).

Results

R1: Please present your results step by step

Response: The results section has been revised to reflect the methods section, main headings as follows:

3.0 Results

3.1 Ecophysiological properties of biocrust cyanobacteria

5 **3.2 Cyanobacterial community structure**

3.3 16S rDNA profiling of native undisturbed biocrust microbiomes

3.4 Cyanobacterial tolerance to stockpiling

R1: p8, line 24 recast sentence "in Table 1…"

10 Response: this sentence has been removed as it was unnecessary.

R1: p8, line 25 'what do you mean by ecologically significant…'

Response: the word "ecologically" has been removed

R1: p9 define chlorophyll type, add mean concentration (missing), surface area reporting preferred

Response: chlorophyll *a* inserted, mean concentration added. In this case we reported in $\mu g\ g^{-1}$ soil as we needed to compare

15 with disturbed topsoil and topsoil stock piles. It was later used (ms in preparation) to define the concentrations per g soil to add in restoration trials. I understand that globally comparisons by surface area are easier however we were constrained by the requirements of the mine project. We do however have some earlier biomass area data done as part of an honours project that was carried out as a preliminary study that I will add to final ms revisions.

R1: p9, line 6 – had T2 been introduced previously?

20 Response: T2 had been defined in two figure descriptions "T2 = 2YO Topsoil stockpile originating from SMU 3" however in the script two-year has also been described as 2YO stockpile (see below) and further down has had (T2) added for clarity of reference between results and figures.

R1: clarify sites in this section

Response: The first sentence now reads "Soil pH across the three soil management units (SMUs) ranged from 8.4–8.6 while

25 the two-year old (2YO) topsoil stockpile was higher at 8.9 (Table 1)."

R1: p9, line 7 incorrect use of Levene's test, explain analysis

Response: this was removed and correctly identified as not relevant, analysis described in revised methods

R1: please state clearly what methods were used for each section i.e. community structure, stockpiles, were polyphasic approach used across all of these?

30 Response: This has been clarified in revised methods and explained in previous section (sequencing was not used in stockpiles as unfortunately there was insufficient budget).

R1: p9, line 19 'unicellular…' what genera does this refer to?

Response: inserted "(e.g. *Chroococcidiopsis, Acaryochloris, Xenococcaceae*)." These are also identified in Figure 7.

R1 p9, line 20 – explain differences in richness, evenness and diversity according to….

The methods section has been re-structured to better reflect which statistical analyses were used for which set of data. Please find the relevant sentence: "Diversity values were derived using the DIVERSE function within the Primer package (Anderson et al 2008) upon standardized OTU values. ANOVA with post hoc Tukey's tests was used to test for significant differences between stages."

R1: p9, line 24-25 also 26-30 remove sentences, not relevant to results or stating what we know.

Response: sentences removed

R1: p10, lines 7-8 soil texture can influence community structure…not in results?

Response: The results relate to soil types rather than textures and written as soil types (SMU 1-3, soil management units) which were previously determined by a soil consultant and described in detail in methods section 2.1 (see below)

R1: p10, lines 8-9 ideas are opposite?

Response: this sentence clarified

R1: p10, line 16 'introduce soil textures, please do it before…'

Response: Soil management units are now fully described in Methods Section 2.1

"The landscape has been characterised into three distinct soil types associated with vegetation communities (Table 1) called soil management units: SMU1 – deep calcareous yellow sands associated with dune ridges; SMU2 – shallow calcareous sandy loams and, SMU3 – deep calcareous sandy loam (Goode, 2009; Doudle et al., 2011)."

"The landscape has been characterised into three distinct soil types associated with vegetation communities (Table 1) called soil management units: SMU1 – deep calcareous yellow sands associated with dune ridges; SMU2 – shallow calcareous sandy loams and, SMU3 – deep calcareous sandy loam (Goode, 2009; Doudle et al., 2011)."

R1: Section 3.5 'too many numbers in community structure data'

Response: This section has been refined as follows:

Of the 21 species more than half (12 species) of cyanobacteria were identified in SMU 1 where four primary genera made up 75% of the community: *Symploca*, *Schizothrix*, *Scytonema* and *Symplocastrum* (for more detail see Fig. S4). Cyanobacterial crusts from the dune regions on SMU 1 (deep calcareous yellow sands) were representative of crust types 1–3; patchy, brittle (when dry) early-successional crusts as well as formed dark crusts that were mid to late-successional and included cyanolichens (also see Doudle et al., 2011).

Cyanobacterial crusts from the chenopod shrublands and open woodlands in SMU 2 (shallow calcareous sandy loam) represented a broad range of crust types (2–5) but overall could be described as late-successional. Lichens and mosses were highly visible (also see Doudle et al., 2011). There were 21 cyanobacteria recorded: four were primary genera that made up 63% of the community including: *Schizothrix*; *Porphyrosiphon*; *Scytonema* and *Symploca* (for more detail see Fig. S5).

Cyanobacterial crusts from the open woodlands in SMU 3 (deep calcareous sandy loam, Fig. 2c) represented a broad range of crust types (2–5) but like SMU 2 could also be described as late-successional. Lichens and mosses were highly visible (see Doudle et al., 2011). There were nine cyanobacteria recorded of which four were primary genera that made up 85% of the community: *Symploca*, *Porphyrosiphon*, *Scytonema* and *Schizothrix* (for more detail see Fig S6). Cyanobacteria with the capacity to fix nitrogen contributed to 77% of the community structure.

Cyanobacterial crusts from Site 6 were from the 2YO topsoil stockpile that had originated from SMU 3 (deep calcareous sandy loam) would be described as early successional crusts with some seasonal mosses. There were eight cyanobacteria recorded of which four were primary genera that made up 84% of the community: *Symploca*; *Symplocastrum*; *Porphyrosiphon*; and *Scytonema* (also see Fig. S7). It was interesting to note that *Symplocastrum* was co-dominant with *Symploca* whereas in the other communities it ranged between 8-13%. Sub-surface species *Schizothrix* (found in top 5 mm) only contributed to 4% of the richness compared to 10-20% elsewhere. Cyanobacteria with the capacity to fix nitrogen (*Symploca*, *Porphyrosiphon*, *Scytonema* and *Brasilonema*) contributed to 61% of the community.

R1: p11, line 1 2YO topsoil acronym query

Response: this has been addressed early in results (see above)

R1: p11, line 13 'you did not sample Stigonema…'

Response: This paragraph has been recast to read: "Examination of stockpile soil samples via microscopy revealed five cyanobacterial morphotypes corresponding with the genera: *Nostoc, Scytonema, Microcoleus, Porphyrosiphon* and *Leptolyngbya* (Fig. 17). Average morphotype richness was highest in stockpiled samples at and above 10 cm depth for all stockpile ages (Figs. 13, 14)."

R1: p12, line 1 'area mentioned….where has it been introduced previously..'

Response: In methods area has been described as 'area of coverage' – "To determine cyanobacterial growth rates and richness, wet-mounts for each sample were examined under 16 x magnification for cyanobacterial thalli and colony size was estimated via area of coverage of the field of view."

Subsequently, the references to the word area in results were changed to cover or coverage to better reflect the description in the methods.

R1: p12, lines 19-20 remove sentence

Response: Sentence removed

R1: p12, lines 21-22 use of the term growth rates

Response: above mentioned sentence highlighted refers to growth that was measured over time (via area of coverage).

Discussion

R1: Please rewrite

Response: The discussion has been rewritten with the useful comments by R1 incorporated. The full discussion is in ms below:

Tables and Figures: All corrections have been made (see ms below)

Responses to R2

We would like to thank Reviewer 2 for their appreciation of this manuscript and helpful comments to improve its quality.

R2: It would be worth adding information on the distribution of temperature and rainfall

Response: We have now included the Bureau of Meteorology graphs for temperature, rainfall and evaporation (as this is extreme) for Tarcoola, the nearest recording station for the last 20 years. Due to the large number of figures we have provided brief in text descriptions and placed the figure in supplementary material.

R2: No reference concerning the major soil types and surface as they could influence rehabilitation of biocrusts

Response: We have now included a key reference to the history and nature of the soils from J-A (Hou and Warland, 2005) and incorporated a section in the revised discussion that focuses on the role of the soil elements investigated in rehabilitation especially in terms of biocrusts. It should be noted at the stage of our research that the first rehabilitation site had not commenced thus we were investigating the natural soil environment of the biocrusts (undisturbed) compared to stockpiles of topsoil which were due to be used in the near future (and subsequently a few hectares have undergone rehabilitation).

Microbial Biobanking
Cyanobacteria-rich topsoil facilitates mine rehabilitation

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10 **Abstract**

Restoration of soils post-mining requires key solutions to complex issues where the removal or disturbance of topsoil incorporating soil microbial communities can result in a shift in ecosystem function. This research was in collaboration with Iluka Resources at Jacinth-Ambrosia (J-A) mineral sand mine located in a semi-arid chenopod shrubland in southern Australia. At J-A assemblages of microorganisms and microflora inhabit at least half of the soil surfaces and are collectively known as
15 biocrusts. This research encompassed a polyphasic approach to soil microbial community profiling focused on 'biobanking' viable cyanobacteria in topsoil stockpiles to facilitate rehabilitation. We found that cyanobacterial communities were compositionally diverse topsoil microbiomes. There was no significant difference in cyanobacterial community structure across soil types. As hypothesised, cyanobacteria were central to soil micro-processes, strongly supported by species richness and diversity. Cyanobacteria were a significant component of all three successional stages with 21 species identified from ten
20 sites. Known nitrogen-fixing cyanobacteria *Symploca, Scytonema*, *Porphyrosiphon, Brasilonema, Nostoc* and *Gloeocapsa* comprised more than 50% of the species richness at each site and 61% of the total community richness. In the first study of its kind we have described the response of cyanobacteria to topsoil stockpiling at various depths and ages. Cyanobacteria are moderately resilient to stockpiling at depth and over time, with average species richness greatest in the top 10 cm of the stockpiles of all ages, and more viable within the first six weeks, indicating potential for biocrust re-establishment. In general,
25 the resilience of cyanobacteria to burial in topsoil stockpiles in both the short and long term was significant, however in an arid environment recolonization and community diversity could be impeded by drought. Biocrust re-establishment during mine rehabilitation relies on the role of cyanobacteria as a means of early soil stabilisation. At J-A mine operations do not threaten the survival of any of the organisms we studied. Increased cyanobacterial biomass is likely to be a good indicator and reliable metric for the reestablishment of soil micro-processes.

30

1.0 Introduction

Soil disturbance results in a loss of resources from arid ecosystems and often has long lasting effects on soil stability, nutrient cycling and surface hydrology (Bowker, 2007; Tongway and Hindley, 2004). Restoration of ecosystem function post disturbance requires appreciation of the dynamic functional status of the landscape prior to disturbance (Tongway and Ludwig, 1996), as well as an understanding of the net accumulative effects of disturbance on the components of that system. To evaluate the net effects of disturbance, the severity and periodicity need to be understood whereby small but frequent disturbances may have an accumulative effect, whilst rare but severe disturbance may permit natural recovery between events. Subsequently, it is necessary to appreciate the micro-processes that will assist in the restoration of soil function and to monitor recovery along the way. Microbial communities drive micro-processes that impact on soil ecosystem function on several levels with feedbacks that can considerably assist arid landscape rehabilitation.

Highly structured, diverse biocrust communities are a significant asset to arid soil ecosystems providing a protective, nutrient rich layer closely integrated into the soil surface (Delgado-Baquerizo et al., 2013; Maestre et al., 2012). As biocrusts develop in structural complexity, the diversity of organisms is regulated by water infiltration from rainfall, temperature, light and disturbance (Belnap and Eldridge, 2001; Büdel et al., 2009; Elbert et al., 2012). Early successional biocrusts are mainly composed of bacteria, algae and cyanobacteria whereas late successional biocrusts can incorporate lichens, mosses, liverworts, algae, fungi and bacteria (Büdel et al., 2009). Cyanobacterial re-establishment is a key indicator of early soil surface re-stabilisation, regulation of soil moisture and the balancing of soil carbon and nitrogen (Elbert et al., 2012).

In arid environments, excretions of extracellular polymeric substances (EPS) by microorganisms such as cyanobacteria, form stratified layers of organic and inorganic material that link and bind soil particles together (Issa et al., 2007; Rossi et al., 2017). Cyanobacteria in arid landscapes are exceptionally well-adapted to desiccation. Their polysaccharide sheaths and EPS production perform a vital role in maintaining cyanobacterial cell integrity, exchange of information and absorption of water during rehydration (Rossi et al., 2017). EPS has adhesive properties that binds non-aggregated soil particles into a protective encrusted surface that reduces the destructive impacts of wind and water (Eldridge and Leys, 2003; Rossi et al., 2017). Cyanobacterial biofilms provide stabilisation of initially disturbed surfaces that pave the way for diverse microbial communities, and form bioactive crust-like layers assimilated into the soil (e.g. Büdel et al., 2009; Rossi et al., 2017; Bowker et al., 2014). EPS forms the bulk of the biocrust structure. EPS more than doubles the biocrusts compressive strength and increases cohesiveness by up to six times (Hu et al., 2002).

Physical disturbance can profoundly disrupt biocrust integrity, composition and physiological function where the impact is governed by site characteristics, severity, frequency and timing (Belnap and Eldridge, 2001). Mining disturbance alters the biophysical state of the biocrust community through excavation, crushing, mixing and burial. The negative impacts of biocrust

Moved (insertion) [2]

Moved (insertion) [3]

Moved (insertion) [4]

Moved up [3]: As biocrusts develop in structural complexity, the diversity of organisms is regulated by water infiltration from rainfall, temperature, light and disturbance (Belnap and Eldridge, 2001; Büdel et al., 2009; Elbert et al., 2012). Early successional biocrusts are

Moved up [4]: Early successional biocrusts are mainly composed of bacteria, algae and cyanobacteria whereas late successional biocrusts can incorporate lichens, mosses, liverworts, algae, fungi and bacteria (Büdel et al., 2009).

Moved up [2]: Highly structured, diverse biocrust communities are a significant asset to arid soil ecosystems providing a protective, nutrient rich layer closely integrated into the soil surface (Delgado-Baquerizo et al., 2013; Maestre et al., 2012).

burial within the natural environment as a result of environmental stress and disturbance have been previously studied. For example, significant declines were recorded in cyanobacterial richness and abundance that was linked to a reduction in soil nutrient concentrations (Rao et al., 2012; Williams and Eldridge, 2011). Following disturbance, restoration and regrowth of biocrusts can take place unassisted, seasonally driven and may take many years (Belnap and Eldridge, 2001; Belnap and Gillette, 1998). Alternatively, assisted biocrust restoration places emphasis on the recovery of ecosystem function and necessarily addresses environmental constraints. This incorporates the knowledge of 'potential condition' based on experience with sites of ecological similarity that have undergone disturbance and recovery (Bowker, 2007). Biocrust recovery can be altered by dust deposition, fire and climatic conditions (Weber et al., 2016). When biocrusts recover naturally soil properties change. For example, in Southern African and Spanish rangelands an incremental accumulation of soil nutrients, organic matter and a build-up of silt and clay lead to the development of a resilient and multi-functional biocrust (Büdel et al., 2009; Maestre et al., 2012; Weber et al., 2016).

Mine rehabilitation is a complex process that involves many levels of understanding of difficult issues relating to ecosystem function where the removal or burial of the bioactive soils can have knock-on effects for rehabilitation efforts such as native seedling establishment (Jasper, 2007; Tongway and Ludwig, 1996). Successful ecological restoration of arid mining sites relies on a holistic approach where biocrust recovery to pre-disturbance levels is integral and can serve as an indicator of the integrity of the ecosystem (Tongway, 1990). Research into biocrust disturbance with a focus on recovery post-mining is rare. In the Namaqualand arid lands (Namibia, South Africa) low rainfall and high winds impact the rehabilitation of degraded lands following diamond mining and grazing (Carrick and Krüger, 2007). These researchers found that cyanobacteria and non-vascular plants that form a living and protective surface crust were crucial to surface stabilisation. Jasper, (2007) also recognised the importance of soil microbial communities including cyanobacteria in post-mine rehabilitation in the Jarrah forests of south-western Australia. In the Czech Republic and Germany chrono-sequential studies of old brown coal mine sites found in younger sites that green algal biofilms and a diverse range of cyanobacteria initiated the rehabilitation of the soils (Lukešová, 2001). In serpentinite mine tailings (New South Wales, Australia), McCutcheon et al., (2016) showed filamentous cyanobacteria accelerated carbonate mineral precipitation and stabilised the tailings. They demonstrated cyanobacteria had the capacity to adsorb magnesium while acting as a nucleation site and sequestered carbon. In our current study preliminary research identified that in undisturbed chenopod shrublands at the edge of the Nullarbor Plain (South Australia) biocrusts cover more than 45% of the soil surfaces between the grass plants and post-mining rehabilitation needs to investigate their role (Doudle et al., 2011). It follows that there is a real need for a focus on practical approaches that contribute to the restoration of soil function and measure relevant aspects of success through soil microbial communities and biocrust reestablishment, especially cyanobacteria (for example: Setyawan et al., 2016; Mazor et al., 1996; Fischer et al., 2014; Chiquoine et al., 2016; Doherty et al., 2015; Harris, 2003; Tongway and Hindley, 2004; Zhao et al., 2014).

As a component of the broader landscape biocrust communities maintain spatial structure on the macro-scale through maintenance of landscape organisation (Bowker, 2007; Gilad et al., 2004; Tongway, 1990). On the micro-scale cyanobacterial species richness contributes to soil ecosystem function through micro-processes including carbon fixation through photosynthesis, atmospheric nitrogen fixation in a biological-available form, micro-nutrient breakdown and release, soil particle cohesion, regulation of moisture and soil surface structure (Delgado-Baquerizo et al., 2013; Elbert et al., 2012; Hu et al., 2002; Maestre et al., 2012 and others).

In the mining process the crushing and burial of the biocrust leads to the inability of crust organisms to photosynthesise due to the lack of light. It does not appear that the effects of topsoil stockpiling on biocrust organisms such as cyanobacteria, and their recovery time, has previously been investigated. Should biocrust organisms remain inactive while they are wet, cell death and decomposition may occur (Kidron et al., 2012; Rao et al., 2012). Nevertheless, in dry conditions, cyanobacteria and algae are known to remain desiccated and viable for up to millions of years (Vishnivetskaya et al., 2003).

This project is based on developing mine rehabilitation plans that will achieve improved long-term outcomes. The restoration of landscape function and the accompanying need for the restoration of the soil ecosystem that included biocrusts directed the establishment of this biocrust research project. As cyanobacterial communities develop their species richness, abundance and structure in response to their environment (e.g. Aboal et al., 2016; Büdel et al., 2009; Williams et al., 2014; Williams and Büdel, 2012), it was important to examine the biocrust community structure and survival. Within this design a polyphasic approach considers the essential ecosystem services provided through the reestablishment of biocrusts. The conceptual design is based on the net ecosystem benefits that must be achieved through biocrust regeneration. It follows that cyanobacterial inoculum in the topsoil stockpiles would be central to early stabilisation of mobile surfaces subjected to the potential impacts of wind and rain-splash erosion. We sought to determine whether shallow 'biobanks' of cyanobacterial-enriched top soil would facilitate biocrust recovery of this mine site.

The overall goals of the biocrust research program were to: (a) evaluate specific roles of natural, undisturbed biocrusts in ecosystem function at the mine site; (b) determine cyanobacterial community structure in terms of key species that drive early colonisation, biogeochemical cycling and soil stabilisation; and, (c) to investigate the effects of stockpiling topsoil on cyanobacterial survival after burial and subsequent recovery. We hypothesised that cyanobacteria would be essential as ecosystem engineers in soil habitat formation with a clear distinction in community structure between soil types and biocrust successional stages. It was also anticipated that in the topsoil stockpiles there would be a low survival rate especially at depth and over time.

2.0 Methods

2.1 Background and site description

Jacinth-Ambrosia (J-A) heavy mineral sand mine is located on the eastern edge of the Nullarbor Plain, South Australia. The mine is located across the boundary of two Regional Reserves within the Eucla Basin region. The climate is semi-arid with a mean rainfall of 185 mm, mean maximum temperature of 27.6°C and minimum of 12.1°C (further detail provided in supplementary Figure S1, bom.gov.au). Tertiary sediments deposited in marine and terrestrial settings and the soil distribution of the area reflects the geological history, with at least five marine transgression and regression events depositing 40-50 m of sediments (Hou and Warland, 2005). The landscape is broadly undulating with low open woodlands that have a shrub understorey; there are chenopod shrub lands as well as dune fields that consist of parallel dunes and inter-dune swales (Doudle et al., 2011; Gillieson et al., 1996). Prior to mining disturbance, the landscape is superficially homogenous chenopod-dominated vegetation but functionally patchy on the fine scale where the soil surfaces are extensively colonised by biocrusts. At J-A biocrusts primarily consist of lichens, mosses and cyanobacteria cover around 45% of the landscape surfaces or 2,000 hectares of the mining lease, providing a significant contribution to ecosystem function and stability (Doudle et al., 2011).

The landscape has been characterised into three distinct soil types associated with vegetation communities (Table 1) called soil management units: SMU1 – deep calcareous yellow sands associated with dune ridges; SMU2 – shallow calcareous sandy loams and, SMU3 – deep calcareous sandy loam (Doudle et al., 2011; Hou and Warland, 2005). The biocrusts at J-A had been previously classified into three primary successional stages representative of the five biocrust types found growing across the landscape (Doudle et al., 2011). Types 1–2: light coloured and patchy (Type 1), thin and fragile cyanobacterial crusts (Type 2) in early stages of development; Type 3: cyanobacterial crust, well established, intermediate stages of development; Types 4–5: biocrust, well established with cyanolichens and/or green algal lichens and mosses, at a late successional stage of development (additional descriptions available in supplementary Table S1). Study locations were selected for: (a) vegetation communities, (b) soil types and underlying geology, (c) biocrust successional types, (d) areas identified for future mine operations and a dry salt lake (Lake Ifould). The dry salt lake would potentially identify cyanobacterial species adapted to saline conditions similar in nature to ground water.

In the process of mining and preparation of the J-A site for future rehabilitation, topsoil (0–100 mm) is stored in low stockpiles, generally less than two metres in depth where stockpiles are comprised of topsoil sourced from only one vegetation type. Later stockpiles will be returned to the surface of mined areas in the rehabilitation process, and the cyanobacterial activity therein must be quantified to enable educated planning and decision making regarding biocrust re-establishment. If low levels of biocrust organisms are detected below the top few centimetres, the addition of propagated biocrust organisms (e.g. cyanobacteria) to returned topsoil may be warranted. Activity and species richness within the stockpiled top soils may also vary with age and this may also influence the establishment of vegetation in the rehabilitation process.

2.2 Field Methods

The mine research program was conducted over a 16-month period from late May 2011 to September 2012 and was comprised of four approaches described in the following sections 2.3 to 2.4:

2.3 Ecophysiological properties of biocrust cyanobacteria

5 *2.3.1 Field Sampling*

To characterise the cyanobacterial communities, nine sites were selected sampling the three SMUs (Table S1) and five crust types (Table S2). A single sample set was taken from a two-year-old topsoil stockpile regarded as an early (Type 1) biocrust representing recovery two years post disturbance. Photos were taken at the time of sampling (Fig. 2). Preliminary identification of biocrust types had been determined by Doudle et al., (2011) and provided the baseline data for biocrust sampling from Sites 10 1–10 (Fig. 1, Table S1). Within each site eight 10 cm diameter samples were selected at random and removed to a depth of 1 cm using metal scraper (n=80), air dried (>40°C), and stored in Petri dishes. Each Petri dish contained approximately 80 g of crust. The samples were packed to avoid crust disruption and transported to The University of Queensland's Central Analytical Laboratory at Gatton.

15 *2.3.2 Biocrust biophysiochemical properties*

For each site, duplicate sub-samples were taken from each Petri dish for each biophysiochemical analysis. Soil was fine sieved (1.70 mm) and analysed for total C and N and C:N ratio using a high temperature digestion in a vario MACRO Elemental Analyser (Elementar). Soil pH and electrical conductivity (EC) samples were prepared using a 1:5 (soil to water) ratio and shaken for one hour. Following shaking, samples were left to stand for 30 min and EC measured using a Crison Conductivity 20 Meter 525. The sample was mixed again, and pH was measured with a TPS pH meter MC-80 using an ionode IJ44C electrode. Chlorophyll *a* concentration of the biocrusts were determined following resurrection (by moistening) using a 1:5 ratio of (dry weight) biocrust to Dimethyl sulfoxide (DMSO) (Barnes et al., 1992) with samples placed in a warm bath (65°C) for a two-hour dark extraction, followed by centrifuging for five minutes (5000 g RCF). Chlorophyll *a* concentration was determined using Wellburn's, (1994) equations.

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A pocket penetrometer (8 mm foot) was used to determine the compressive strength (kg cm^2) of the dry intact biocrusts. Four measurements were taken from each sample location, providing 12 replicates per site. The measurement was taken at the point when the crust was broken, and the foot penetrated the crust surface.

30 Photosynthetic performance (recorded as yield, YII) was measured using pulse-amplitude modulated (PAM) fluorometer (Pocket PAM; Gademann Instruments, Germany). The aim was to demonstrate photosynthetic yield (YII) indicative of active growth of the biocrusts, using the detection of chlorophyll fluorescence from photosystem II (PSII). The sensor was placed

onto the biocrust and once started, a series of short pulses of excitation light at high intensity that is amplified resulting in a brief closure of PSII and the measurement of fluorescence yield based on the Genty parameter which is the quantum yield (YII) of the charge separation of PSII (Genty et al., 1989) and recorded on a scale of 0–1 for all photosynthesis. Allowing a short space of time between readings, this process was completed several times for each sample.

5 **2.2 Cyanobacterial community structure**

The dried crust samples were resurrected (wet up until they were active) in the glasshouse for three to five days. Following resurrection each of the samples were analysed microscopically. Species richness was assessed with light microscopy using multiple wet mounts. In this study community species richness and structure were assessed at two scales. Each of the ten sites had field site replicates (n=30) were all divided into four microsites (n=120) which provided 12 replicates per site. A minimum 10 of two wet-mount slides incorporated six representative portions of the cyanobacterial colonies. Therefore, cyanobacterial colonies examined for each of the ten field sites totalled 144 (12 reps. x 12 colonies, total n=1,440). For the dominant land type, Chenopod shrubland (Site 8), there were an additional 10 x 6-cell multi-well plates. These were treated similarly where two slides were examined from each of the 60 multi-wells (n=120). In total > 2,184 cyanobacterial colonies were examined.
Initial inspection of the biocrust and the separation of individual species were made using an Olympus SZH10 microscope at 15 70 x magnification. Cyanobacterial filaments or colonies were carefully extracted with forceps to recover sufficient material that included important morphological features such as their colour, encasing sheaths as well as cellular structure. Live material was examined by Nomarski differential interference contrast (DIC) microscopy with a Jenaval (Jena Zeiss) and an Olympus BX51 compound microscope (magnifications 400–1000 x). Photomicrographs were taken using an Olympus SC100 digital microscope camera, and morphological measurements of vegetative cells were made from digital images of live material taken 20 at 400 x magnification using Olympus cellSens® digital imaging software.

Identification was performed to a species level (wherever possible) in the laboratory using the following taxonomic references: Anagnostidis and Komarek, (2005, 2005); Sant'Anna et al., (2011); Skinner and Entwisle, (2002). It was often necessary to record the closest named species as attributes varied somewhat to temperate climate and aquatic specimens described in 25 literature. Nitrogen fixing cyanobacteria were identified based on the three recognised types: (1) heterocystous species (those with specialised N-fixing cells); (2) non-heterocystous species that fix N aerobically and; (3) non-heterocystous species that fix N anaerobically (Bergman et al., 1997; Stal, 1995).

Using a graticule, abundance was ranked on a scale of 1–8 where the main taxa are ranked in decreasing order of the relative 30 percentage area occupied in a single view (Biggs and Kilroy, 2000). More than one species could be dominant, and all other taxa were ranked in relation to the dominant taxa as abundant, common, occasional and rare.

To determine similarities between cyanobacterial communities a cluster analysis and nMDS were conducted using Primer v6 (Clarke & Gorley 2001) and that the SIMPROF routine was used to determine significance between clusters.

2.3 16S rDNA profiling of native undisturbed biocrust microbiomes

For genomic profiling of naturally occurring successional biocrust communities, a location adjacent to Site 9 was visually
5 determined to contain Bare, Early (Types 1-2) or Late (Types 3-5) stages of development (Table S2). Biocrust successional features were determined by morphological attributes of pigmentation, thickness and surface roughness as well as the presence/absence of lichens and mosses (Fig. 3), (Chilton et al., 2017). Bare stage was characterised by loose soil particles with no visible biocrust structure. Samples were collected in July 2014. For each successional stage, three replicates were collected that were representative of SMUs 1–3 where a 10 cm^2 plot with 95% coverage of the desired biocrust stage was
10 excised to the depth of the crust and non-aggregated soil discarded (Fig. 4). Samples were processed at UNSW, Sydney.
Each biocrust replicate for Bare, Early and Late stages of development were homogenised and genomic DNA extraction performed using the FASTDNA Spin Kit for Soil (MP Bio Laboratories, USA) according to the manufacturer's instructions. Molecular libraries of the 16S rDNA V123 hypervariable region generated via PCR as per Chilton et al., (2017) and submitted to the Ramaciotti Centre for Genomics (UNSW, Australia) for a 2x300 bp sequencing run on an Illumina MiSeq instrument.
15 Sequencing data was processed using Mothur version 1.34.0 (Schloss et al 2009) and described in detail in Chilton et al., (2017). Singleton and doubleton OTUs were removed and samples rarefied to 8598 sequences each across 3785 OTUS. The curated Greengenes database (McDonald et al 2012) was used to assign taxonomy to OTUs. Diversity values were derived using the DIVERSE function within the Primer package (Anderson et al 2008) upon standardized OTU values. ANOVA with post hoc Tukey's tests was used to test for significant differences between stages. Multivariate analyses were performed in
20 Primer upon a Bray-Curtis dissimilarity matrix generated from square-root transformed abundance data. Samples were represented in two and three-dimensional space within a non-metric multidimensional scaling plot (nMDS). Pair-wise, a posteriori comparisons of factor Stage were performed using the PERMANOVA function with 9999 Monte Carlo permutations. Homogeneity of dispersion for each stage was tested using PERMDISP.

2.4 Cyanobacterial tolerance to stockpiling

25 Sampling was carried out in March 2012 with samples sourced from topsoil stockpiled from areas with *Acacia papyrocarpa* (Western Myall) over-storey (Table S3). Stockpiles established at three different time points (9 months, 20 months and 29 months) as well as corresponding undisturbed sites were sampled in triplicate at six depths (0–2, 2–4, 4–6, 10, 25 and 50 cm). This resulted in 18 replicates for each soil depth for each stockpile time point. Holes were dug to >50 cm depth with a shovel and the exposed profile removed with an ethanol-wiped spatula before taking a soil sample. A second sample at 50 cm was
30 autoclaved to serve as a culturing control. Samples were stored in paper bags and processed at The University of Queensland. For each sample, 20 g of soil was set up in petri dishes with 9 ml of water and sealed with Parafilm®. Petri dishes were incubated for six weeks at 26°C under a 12-hour photoperiod regime and rotated weekly to prevent site specific effects.

Samples were maintained as wet by the addition of sterile water within a laminar flow cabinet. To determine cyanobacterial growth and richness, wet-mounts for each sample were examined under 16 x magnification for cyanobacterial thalli and colony size was estimated via area of coverage of the field of view. Where multiple morphotypes were present in a colony, the microscopic relative abundance of each was used to divide the cover between the morphotypes accordingly. Where no growth was observed, five soil samples were taken randomly from the sample, mounted and examined under 400 x magnification for the presence of cyanobacterial cells. ANOVAs were performed with post hoc Tukey's tests performed to determine any differences between treatments.

3.0 Results

3.1 Ecophysiological properties of biocrust cyanobacteria

Soil pH across the three soil management units (SMUs) ranged from pH 8.4–8.6 while the two-year old (2YO) topsoil stockpile was higher at pH 8.9 (Table 1). Electrical conductivity ranged from 92–140 µS cm^{-1}. There were no significant differences in pH and EC between field samples and intact samples (data not shown). Total nitrogen was typically <0.1% across all sites and total carbon ranged between 1–2% with higher percentages generally found across SMU 2 and 3 (Table 1). The ratio between carbon and nitrogen was the greatest across SMU 3 and the 2YO topsoil stockpile, also originating from SMU 3.

For the intact undisturbed biocrusts mean chlorophyll *a* concentrations (10.33–13.64 µg g^{-1} soil) differed across all sites and SMU 1 biocrusts were significantly lower ($p = 0.05$) compared to SMU 2 and 3 (Fig. 4). The mean chlorophyll concentrations of biocrusts sourced from T2, the 2YO topsoil stockpile (7.49 ± 1.01 µg g^{-1} soil) were almost half the concentration of SMU 3 (13.53 ± 1.74 µg g^{-1} soil), which was the origin of the topsoil.

The penetrometer index of crust compressive strengths across the ten sites showed that Sites 4, 9 and 10 in SMU 1 were significantly different from each other as well as significantly lower than SMU 2, SMU 3 and T2, 2YO topsoil stockpile sites (Table 2). The means for SMU 1–3 were also significantly different ($p < 0.0001$), and it was confirmed by a student's t-test that the difference was between SMU 1 and SMU 2 and 3.

The photosynthetic yield measurements showed there was variability between sites (Fig. 5). The yields recorded all fell within the expected range and when data was separated into their respective SMU it provided a clearer picture of the variability. SMU 3 had the lowest mean overall while there were no differences between the others.

3.2 Cyanobacterial community structure

A total of 21 cyanobacterial species were identified across the ten sites using microscopy (Table 5; Figs. S2–S9). The majority of species richness and abundance was comprised of four filamentous genera: *Symploca* (18%), *Schizothrix* (16%), *Porphyrosiphon* (16%) and *Scytonema* (16%). Secondary cyanobacteria present variously occupied <1 to 10%, and overall

made up 34% of the community. The known nitrogen-fixing cyanobacteria *Symploca, Scytonema*, *Porphyrosiphon, Brasilonema, Nostoc* and *Gloeocapsa* comprised more than 50% of the species richness at each site and formed 61% of the total community richness (Fig. 10). In this study cyanobacterial community structure was tested against the three different soil types to determine whether soil type was influential in determining community structure. There was no significant difference
5 in cyanobacterial community structure across soil types, however the results do suggest some spatial structuring exists across the three SMU (Figs. 11, 12). The structural relationship between all samples shows greater similarities rather than dissimilarities, of which there are only a small number of samples that are significantly different (Fig. 12). Soil type did not explain these differences. This suggests that most of the species could potentially be found anywhere across the three zones and their richness and abundance is controlled by other factors. *Symploca* occurred more frequently and was more abundant
10 in the majority of the samples examined therefore was the most significant contributor to the community (data not shown). Individual sites generally displayed similar trends although there was some variability occurring between sites.

Of the 21 species more than half (12 species) of cyanobacteria were identified in SMU 1 where four primary genera made up 75% of the community: *Symploca*, *Schizothrix*, *Scytonema* and *Symplocastrum* (for more detail see Fig. S10). Cyanobacterial
15 crusts from the dune regions on SMU 1 (deep calcareous yellow sands) were representative of crust types 1–3; patchy, brittle (when dry) early-successional crusts as well as formed dark crusts that were mid to late-successional and included cyanolichens (also see Doudle et al., 2011).

Cyanobacterial crusts from the chenopod shrublands and open woodlands in SMU 2 (shallow calcareous sandy loam)
20 represented a broad range of crust types (2–5) but overall could be described as late-successional. Lichens and mosses were highly visible (also see Doudle et al., 2011). There were 21 cyanobacteria recorded: four were primary genera that made up 63% of the community including: *Schizothrix*; *Porphyrosiphon*; *Scytonema* and *Symploca* (for more detail see Fig. S11).

Cyanobacterial crusts from the open woodlands in SMU 3 (deep calcareous sandy loam, Fig. 2c) represented a broad range of
25 crust types (2–5) but like SMU 2 could also be described as late-successional. Lichens and mosses were highly visible (see Doudle et al., 2011). There were nine cyanobacteria recorded of which four were primary genera that made up 85% of the community: *Symploca*, *Porphyrosiphon*, *Scytonema* and *Schizothrix* (for more detail see Fig S12). Cyanobacteria with the capacity to fix nitrogen contributed to 77% of the community structure.

30 Cyanobacterial crusts from Site 6 were from the 2YO (T2) topsoil stockpile that had originated from SMU 3 (deep calcareous sandy loam) would be described as early successional crusts with some seasonal mosses. There were eight cyanobacteria recorded of which four were primary genera that made up 84% of the community: *Symploca*; *Symplocastrum*; *Porphyrosiphon*; and *Scytonema* (also see Fig. S13). It was interesting to note that *Symplocastrum* was co-dominant with *Symploca* whereas in the other communities it ranged between 8-13%. Sub-surface species *Schizothrix* (found in top 5 mm) only contributed to 4%

of the richness compared to 10-20% elsewhere. Cyanobacteria with the capacity to fix nitrogen (*Symploca*, *Porphyrosiphon*, *Scytonema* and *Brasilonema*) contributed to 61% of the community.

3.3 16S rDNA profiling of native undisturbed biocrust microbiomes

5 Microbial community profiling using high through-put sequencing revealed cyanobacteria comprised a significant component of all three stages forming the majority of sequences in Early and Late stages (Fig. 6). There was a diversity of morphotypes observed including simple filamentous, heterocystous and unicellular types (Fig. 7). The most abundant genera identified were *Leptolyngbya, Phormidium, Tolypothrix, Nostoc, Brasilonema, Chroococcidiopsis* and *Acaryochloris*. Unclassified Nostocaceae were dominant within Bare soils while Early stages observed a relative even increase in *Phormidium*,

10 *Brasilonema* and the unicellular genera (e.g. *Chroococcidiopsis, Acaryochloris, Xenococcaceae*). Late stage biocrusts showed a slight resurgence of Nostocaceae. There was no significant difference in the richness, evenness or diversity between the three stages (Table 3). However, there were significant differences in the composition and structure of the communities of each stage. The Bare stage was significantly different to the crusted stages (Fig. 8, Table 4) while resolution between the Early and Late stages was less clear. Ordination of the samples within three dimensions showed Early and Late stages grouped separately

15 however, PERMANOVA failed to identify a significant difference (Table 3).

3.4 Cyanobacterial tolerance to stockpiling

Examination of stockpile soil samples via microscopy revealed five cyanobacterial genera being: *Nostoc, Scytonema, Microcoleus, Porphyrosiphon* and *Leptolyngbya* (Fig. 17). Average genera richness was highest in stockpiled samples at and above 10 cm depth for all stockpile ages (Figs. 13, 14). These differences were significant at 50 cm in all stockpiles (29-month

20 $F_{(1,5)}$ = 7.00, p= 0.024; 20-month $F_{(1,5)}$ + 8.37, p = 0.016; 9-month $F_{(1,5)}$ = 18.00, p = 0.002) and 25 cm in the twenty- and nine-month old stockpiles ($F_{(1,5)}$ = 32.73, p = 0.000; F = 16.20, p =0.000) respectively. Conversely, after nine and twenty months of stockpiling, average genera richness was higher in stockpiled samples when compared with undisturbed samples except for 20-month old 4–6 cm samples (Figs. 13 a, b) but this difference was only significant in the nine-month old stockpile at 10 cm depths ($F_{(1,5)}$ = 8.27, p = 0.017). High variability between replicates accounted for the lack of significance in the rest.

25

When comparing average genera richness at different depths between stockpiles, richness was greater in material stockpiled for the least amount of time above 10 cm depth but not at or below this level (Fig. 13a). Average genera richness was variable within adjacent undisturbed areas (Fig. 13b). The variability in genera richness within replicates was high as shown in the error bars of Figures 14a–c. This variability was evident in the lack of significant difference in species richness at any depth between

30 stockpiles of different ages. The cover of genera was relatively constant between sites in the undisturbed samples, but in stockpiled samples *Nostoc* cf *commune* was more prevalent ($F_{(1,5)}$ = 5.97, p = 0.012).

Nostoc cf. *commune, Nostoc* yellow, *Microcoleus* and *Leptolyngbya* were present in more stockpiled samples than undisturbed samples (Fig. 15). Conversely, *Scytonema* and the black form of *Leptolyngbya* were more prevalent in undisturbed samples (Fig. 16). When all samples were combined, *Nostoc* cf *commune* had the greatest cover in both stockpiled and undisturbed samples followed by yellow *Nostoc* in stockpiles only. The coverage by the remaining genera was similar for most other genera although *Microcoleus* and *Leptolyngbya* had greater cover in stockpiled areas and *Porphyrosiphon* had slightly higher coverage in undisturbed samples. The cover of *Scytonema* was almost identical in stockpiled and undisturbed areas and this lack of difference was supported statistically ($F_{(1,17)} = 0.00$, $p = 0.969$).

Nostoc cf. *commune* exhibited the best survival response in stockpiling as it had significant coverage in samples from all depths in all stockpile ages. *Nostoc* had the greatest coverage in the oldest stockpiles, followed up by the most recently created stockpiles. These differences were significant from depths of 6 cm to the surface (4–6 cm $F_{(1,5)} = 28.83$, $p = 0.000$; 2-4 cm $F_{(1,5)} = 4.89$, $p = 0.023$; 0-2 cm $F_{(1,5)} = 3.72$, $p = 0.049$). These patterns were not reflected in the total coverage by *N.* cf. *commune* in corresponding undisturbed areas. The *Leptolyngbya* genus black morphotype only occurred between 20 and 40 cm depth and were found in only one stockpile samples but were present in six samples from three adjacent areas. *Stigonema* genus was found in six stockpile samples spanning all ages and in only one adjacent sample in all cases in the upper 10 cm of the soil profile.

Nostoc cf. *commune, Porphyrosiphon, Microcoleus* and *Scytonema* were the first genera to develop to an identifiable stage (Fig. 17). Filaments of the *Stigonema* genus were found in low numbers and appeared to be recently formed. It was only present in samples examined in the latter stages of the identification process. The yellow form of *Nostoc* exhibited a much slower rate of development than *Nostoc* cf *commune* and could only be definitively determined as a form of *Nostoc* when examined after 13 weeks of incubation.

Samples from undisturbed areas sourced at depths from between 10 cm and 50 cm initially showed no visible signs of growth when examined after six weeks of incubation. Six weeks later cyanobacterial growth was evident yet in many cases had not advanced to the point where morphotypes could be distinguished.

4.0 Discussion

In terms of rehabilitation the natural capital in topsoil that has been removed in the mining process is often not recognised or poorly understood. Soils properties are crucial in the re-establishment of a raft of ecosystem services that include fertility, structure, climate regulation and biodiversity (Dominati et al., 2010). Cyanobacteria are ecosystem engineers in that they have the capacity to provide many of these crucial ecosystem services (Jones et al., 1994). This research demonstrated that the cyanobacterial communities in the J-A biocrusts were compositionally diverse topsoil microbiomes that substantially

contributed to the Myall-chenopod biomes. We had hypothesised that cyanobacteria would be central to soil micro-processes and this was strongly supported by extensive species richness and diversity. In the first study of its kind we have shown the response of cyanobacteria to topsoil stockpiling at various depths and ages. In this study we have shown cyanobacteria are moderately resilient to stockpiling at depth and over time, providing they are manipulated dry (W. Williams, unpublished data). Cyanobacteria from the top 10 cm were found to be more viable within the first six weeks and showed potential for biocrust re-establishment. We found greater cyanobacterial richness in the nine and 20-month stockpiles compared to undisturbed samples adjacent to the stockpiles. In general the resilience of cyanobacteria to burial in topsoil stockpiles in the longer term appeared good, however in an arid environment recolonization and community diversity could be impeded by drought (Williams and Büdel, 2012)

In this study cyanobacterial community diversity and abundance was not related to soil and landform type. At J-A any of the cyanobacteria could conceivably occur anywhere across the landscape, with their relative abundance likely determined by microenvironments and microhabitats such as light (sun and shade) and chemical gradients (Stal, 2003), as well as moisture availability (Büdel et al., 2018) and soil particle size (Büdel et al., 2009). Cyanobacterial community richness and abundance were not affected by soil type as shown by the similarity between early stage (SMU 1) and late stage (SMU 3), further supported though the sequenced samples where there were no significant differences. Contrary to our hypothesis there was not always a clear distinction in community structure between soil types and biocrust successional stages, notably in the early and late stages.

4.1 Cyanobacterial community structure

Key cyanobacteria indicating biocrust formation and development were *Leptolyngbya, Phormidium, Tolypothrix, Nostoc, Brasilonema, Chroococcidiopsis* and *Acaryochloris*. These genera have consistent morphological traits with those observed via microscopy. Notably, the identification of *Brasilonema* was supported with sequencing data and had not been previously recorded in Australian soils. Simple filamentous types are often attributed with the primary crust building role, able to span inter-particle gaps within the soil via supra-cellular structures (Garcia-Pichel and Wojciechowski 2009). Sequencing data showed *Phormidium* was the dominant cyanobacterium for this role and it is likely that *Symploca* identified though microscopy was the principal *Phormidium* present. *Microcoleus* sp. and *Porphyrosiphon* were also identified as early colonisers however these genera are currently poorly resolved phylogenetically (Garcia-Pichel et al 2013) but share critical morphological features enabling biocrust formation and maintenance.

The cyanobacterial richness at J-A was determined according to their morphological features. In many cases these features (e.g. outer protective sheaths, UV protection, EPS production) provided the basis of attributes that pertained to fundamental survival strategies. Environmentally induced strategies of arid land cyanobacteria reflect their habitat, these survival traits have

developed over a long evolutionary history. Many primary (common to abundant) and secondary (uncommon) cyanobacteria recorded at J-A exhibited thick gelatinous sheaths (*Porphyrosiphon, Schizothrix, Microcoleus, Nostoc*) or were associated with the production of EPS (*Symploca, Nostoc, Schizothrix, Leptolyngbya*). Filamentous cyanobacteria formed the major part of the J-A crust structure with tufts, webs or creeping masses closely intertwined (e.g. *Porphyrosiphon*, *Symploca*, *Scytonema*, *Schizothrix*, *Microcoleus*). These are often assimilated with unicellular forms (e.g. *Gloeocapsa*, *Chroococcus*, *Chroococcidiopsis*) or gelatinous colonies of *Nostoc.*

Twenty-one cyanobacteria were recorded from 13 genera. Four species were unicellular and the remaining seventeen were filamentous. Some cyanobacteria found at J-A (*Microcoleus paludosus, Nostoc* sp.*, Gloeocapsa*) had also been recorded at Lake Gilles (SA) about 400 km southeast of J-A (Ullmann and Büdel, 2001). Although *Microcoleus* species were recorded at J-A they did not dominate the biocrust compared with many reports from the United States, Asia and elsewhere (e.g. see Belnap and Eldridge, 2001). This infers that the early colonisers such as *Microcoleus* would not play a dominant role in early stabilisation and colonisation of the soil. At J-A *Symploca* and *Scytonema* appeared to be an important colonising cyanobacterium in the biocrusts and have been recorded as playing a key role in carbon sequestration in northern Australian cyanobacterial crusts (Büdel et al., 2018).

The taxonomic status of *Brasilonema* remained uncertain and may be a variety of *Scytonema*, however, genomic data supported morphological identification and the type has also been recorded in other terrestrial habitats globally. Due to its similar morphological attributes and genomic data, in this study we called this cyanobacterium *Brasilonema* (Fiore et al., 2007; Vaccarino and Johansen, 2012). *Nostoc commune* var. *flagelliforme* had been recorded at J-A along with *Nostoc commune* across the shallow and deep sandy loams. Although *N. flagelliforme* appeared rarely, it had been previously documented from sites in south-western South Australia, Western Australia, Northern Territory (Skinner and Entwisle, 2002) and Victoria (W. Williams, unpublished data). A joint Spanish-Australian study has now shown that both *Nostoc commune* and *N. flagelliforme* contain the same genomic markers and cannot be separated, rather the spaghetti-like tubes that are unique ecotype likely associated with aridity (Aboal et al., 2016). This is supported by the semi-arid environment at J-A and it may be more widespread in Australia than previously recorded as it is often only clearly visible following rains.

4.2 Cyanobacterial tolerance to stockpiling

Physical disturbance of biocrusts occurs on a large scale at the J-A mine site with the removal and temporary stockpile storage of topsoil. This type of mechanical disturbance results in the burial and translocation of the biocrust. The impacts of burial within the natural environment are rarely studied. In China artificial sand burial at shallow depths showed there were significant reductions in chlorophyll concentration, UV synthesis, total carbohydrates (EPS) and damage to photosynthetic activity (Rao et al., 2012). In a semi-arid grassland in Australia, wind-borne sand burial of cyanobacterial crusts during a severe drought

resulted in a significant reduction in surface dwelling cyanobacteria and significant reductions in biological-available nitrogen (Williams and Eldridge, 2011).

Here we have shown that without further disturbance, a proportion of cyanobacteria can survive stockpiling for over two years. This is not surprising due to the recognised ability of cyanobacteria to survive in extreme environments. In previous studies, cyanobacteria have been grown from samples sourced at 18 cm depths in Japanese rice paddy soils (Fujita and Nakahara, 2006), 50 cm in the UK (Esmarch, 1914), and 70 cm depths in the USA (Moore and Karrer, 1919). Yet, the cyanobacteria from the surface depths of the stockpiles (i.e. <10 cm) appeared more resilient in the short-term.

The species sampled at J-A have a proven track record of survival under extreme conditions. *Microcoleus* and *Leptolyngbya* have survived and remained viable after up to three million years frozen in lake sediments in permafrost (Vishnivetskaya et al., 2003). Vegetative *Nostoc commune* material retains viability following several decades of storage in desiccated form (Bristol, 1919; Lipman, 1941). Reactivation of vegetative material after decades of storage was successful but several months (Lipman, 1941) to a year (Bristol, 1919) of incubation can be necessary for growth to take place. These results were reflected in the current study where growth was not observed in the undisturbed areas below 10 cm depth for several months. It may be that the longer the period of inactivity, the longer time taken for reactivation to occur (Billi and Potts, 2002; Williams et al., 2014), or less material is viable therefore it takes longer to rebuild colonies (Agrawal and Singh, 2002).

Akinetes are desiccation resistant cells produced by certain filamentous cyanobacteria that can survive for long periods. *Nostoc* and *Scytonema* produce akinetes (Kaplan-Levy et al., 2010; Tomaselli and Giovannetti, 1993) but many of the other species sampled in this study cannot, therefore alternative survival methods are in action. Heterotrophic growth is also possible for some cyanobacteria (Flores and Herrero, 2010). Cyanobacteria can survive in darkness through utilisation of alternate carbon sources in drinking water systems (Codony et al., 2003) and this may also be true for soil cyanobacteria (Reisser, 2007). *Nostoc* have the potential to grow at low light in caves and under ice (Dodds et al., 1995) and even in darkness (Huang et al., 1988). Belnap and Gardner, (1993) reported *Microcoleus vaginatus* sheaths at depths to 10 cm and considered the sheaths to be remnant from a time when the surface was lower than the current day due to a lack of chlorophyll. It is possible that heterotrophic growth was still occurring at these depths for which chlorophyll is unnecessary.

The species richness in taxa at depths in undisturbed areas was like that of surface samples yet with much slower growth. The fact that these organisms took much longer to grow than those sampled from upper layers would suggest that they have grown from vegetative material that has been photosynthetically inactive for long periods. Long term inactivity of vegetative material can result in long lag times for growth following re-activation (Bristol, 1919; Lipman, 1941; Shaw et al., 2003) and this was observed in species sourced from depths that are incapable of akinete production.

In the context of rehabilitation, it is not practical to store stockpiles at very shallow depths, however in the longer term under optimum conditions a diverse range of cyanobacteria did recover, indicating the depth of burial is an important feature of recovery over time. Gradual removal of the topsoil from the stockpiles at shallow depths may facilitate and provide a greater opportunity for microbial recovery. The timing of topsoil amendments would be crucial in terms of moisture availability for the initial cyanobacterial resurrection and to facilitate enough growth to stabilise the newly laid soil surfaces.

5.0 Conclusions

Biocrusts and cyanobacteria are a major component of the J-A landscape that protect and enhance soil function. These studies focused on the cyanobacterial community structure at J-A and its recovery following topsoil stockpiling. It was apparent that the top few centimetres of the stockpiles were the most responsive to cyanobacterial regeneration. Detecting increases in key species and shifts of community structure will likely provide more informative and robust verification of desired rehabilitation outcomes. Cyanobacterial species richness is an important measure of biocrusts that incorporate micro-processes central to a healthy and functional soil ecosystem. Increased cyanobacterial biomass is likely to also be a good indicator and reliable metric for biocrust reestablishment. Yet, diversity indices derived from sequencing data of the whole bacterial community are poor measures of biocrust formation and development.

Cyanobacteria are well adapted to long periods without water, optimisation of short growing seasons, wet-dry cycles, low water potentials, tolerance of high UV and low light intensities, fluctuating temperatures and in marine type habitats, high salinity. Cyanobacterial strategies central to survival include EPS production, spectral adaptation, nitrogen fixation and motility. Biocrust re-establishment during mining rehabilitation relies on the role of cyanobacteria as a means of early soil stabilisation. Provided there is adequate cyanobacterial inoculum in the topsoil stockpiles their growth and the subsequent crust formation should take place largely unassisted. Nevertheless, even from low-profile stockpiles re-establishment of biocrusts would be dependent on rainfall. It follows that the timing of rehabilitation would be important so as to take advantage of favourable climatic conditions. At J-A microbial biobanks have been created through shallow scraping of topsoil, low-profile topsoil stockpiles and direct-return that facilitate the survival of cyanobacteria we studied. Ongoing monitoring of biocrust recovery is important as it provides an effective means of measuring important soil restoration processes.

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Table 1: Intact biocrust soil physicochemical descriptions for all sites EC = electrical conductivity in µS cm^{-1}; total percentage of nitrogen present (N%), total percentage of carbon present (C%) and carbon to nitrogen ratios (C:N) for all sites.

Vegetation	**Soil**	**SMU**	**Site**	**pH**	**EC**	**N%**	**C%**	**C:N**
Mallee	Deep calcareous yellow sand		4	8.6	92	0.07	1.02	15.1
Mallee		1	9	8.5-8.6	107	0.07	0.93	13.7
Mallee			10	8.5-8.6	118	0.09	1.37	15.8
Myall	Shallow calcareous sandy loam		1	8.4-8.5	122	0.10	1.20	11.7
Mallee		2	5	8.4	133	0.13	1.84	13.8
Chenopod			8	8.6	124	0.10	1.32	12.8
Myall	Deep calcareous sandy loam		2	8.5	113	0.09	1.09	12.1
Myall		3	3	8.6	114	0.09	1.99	21.4
Myall			7	8.5-8.6	140	0.10	1.76	18.3
-	Topsoil stockpile	Origin SMU 3	-	-	-	-	-	-
Myall			6	8.9	119	0.08	1.57	19.6

Table 2: Biocrust compressive strengths measured with a penetrometer, means and standard deviations (SD) for SMU 1–3 (kg cm^{-2}) tests with p-values, values in bold that are different from 0 with a significance level alpha=0.05, NS = not significantly different.

SMU	Means ± SD	SMU 1	SMU 2	SMU 3
SMU 1	2.79 ± 1.41	**0**	**0.001**	**0.005**
SMU 2	3.75 ± 0.79	**0.001**	**0**	NS
SMU 3	3.97 ± 0.70	**0.005**	NS	**0**

Table 3: Cyanobacterial mean (± Standard Error) of richness (Margalef's index), evenness (Pielou's index) and diversity (Shannon index) across successional stages.

	Bare		Early		Late	
	Mean	SE	Mean	SE	Mean	SE
Richness (d)	167.4	5.344	146.8	5.818	142.8	15.69
Evenness (J')	0.816	0.011	0.791	0.005	0.788	0.027
Diversity (H')	5.977	0.103	5.692	0.064	5.642	0.281

Table 4: Permutational analysis of variance (PERMANOVA) of pair-wise comparisons of Bray-Curtis dissimilarity between biocrust stages and bare soil. P(MC) = probaility values obtained using 9999 Monte Carlo permutations. Permdisp showed no significant differences in variation of spread (pseudo *F*=3.8068, *P*(perm) = 0.068).

Groups	**t**	**P(perm)**	**Unique permutations**	**P(MC)**
Bare, Early	2.6216	0.0979	10	0.0107
Bare, Late	2.5742	0.0959	10	0.0120
Early, Late	1.2793	0.0953	10	0.1993

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Table 5: Diversity across sites on a presence absence basis for all seasons and Lake Ifould (salt lake). Different species attributed to a genus (i.e. sp. 1,2,3) have all been separated based on their morphological features and size but could not be positively identified.

Cyanobacterium	***SMU 1***	***SMU 2***	***SMU 3***	***T2 Stockpile***	***Lake Ifould***
Aphanothece	-	-	-	-	x
Brasilonema	x	x	x	x	x
Chroococcidiopsis	-	x	-	x	-
Chroococcus sp. 1	x	x	x	x	-
Chroococcus sp. 2	-	-	-	-	x
Gloeocapsa	x	x	-	-	x
Leptolyngbya	x	x	-	-	x
Microcoleus cthonoplastes	-	-	-	-	x
Microcoleus paludosus	x	x	x	x	x
Microcoleus sociatus	-	-	-	-	x
Microcoleus vaginatus	x	x	-	-	x
Nostoc commune	-	x	x	x	x
Nostoc flagelliforme	-	x	-	-	-
Nostoc pruniforme	-	x	-	-	-
Nostoc sp.	x	x	-	-	-
Porphyrosiphon sp. 1	x	x	x	x	-
Porphyrosiphon sp. 2	-	x	-	-	x
Schizothrix sp. 1	-	x	-	-	x
Schizothrix sp. 2	x	x	x	x	-
Schizothrix sp. 3	-	-	-	-	x
Scytonema sp. 1	x	x	x	x	-
Scytonema sp. 2	-	x	-	-	x
Scytonema sp. 3	-	x	-	-	-
Scytonema sp. 4	-	-	-	-	x
Symploca sp. 1	x	x	x	x	x
Symploca sp. 2	-	x	-	-	-
Symplocastrum sp. 1	x	x	x	x	x
Symplocastrum sp. 2	-	-	-	-	x
Species richness	**12**	**21**	**9**	**10**	**18**

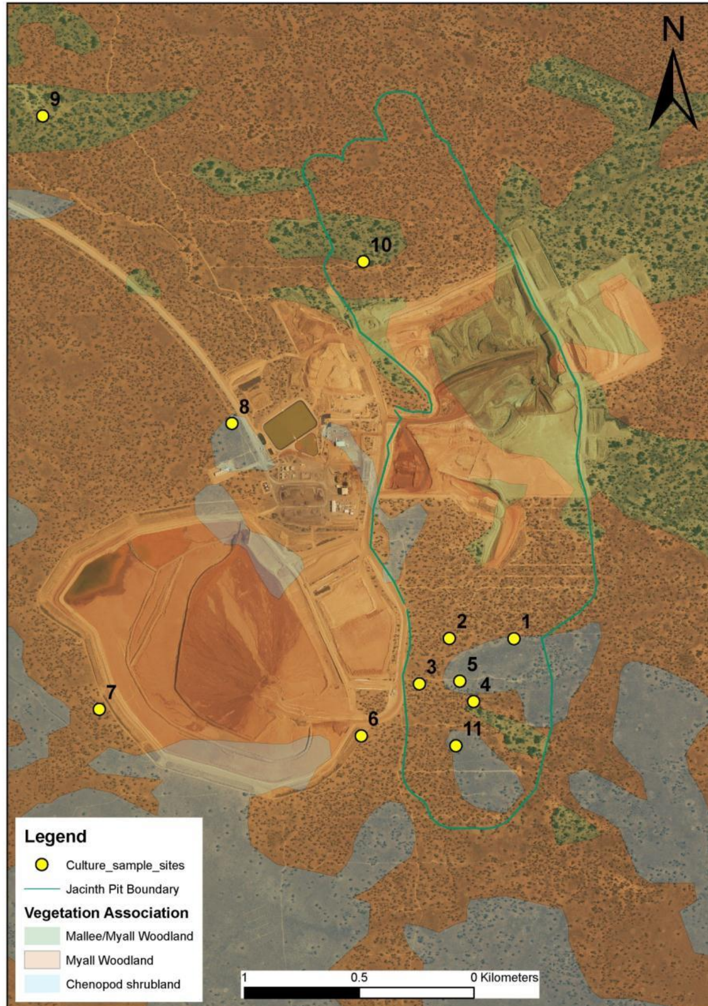
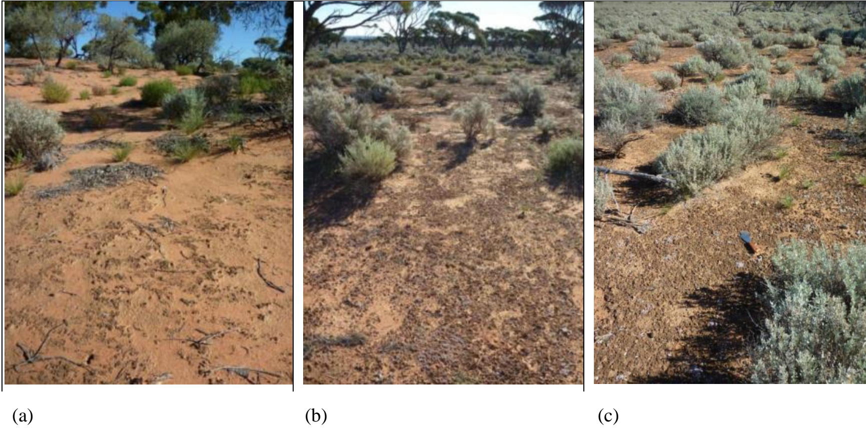


Figure 1: Image of biocrust sample sites located within the vegetation associations described in Table S1 (supplied, S. Doudle). Site 11 was initially investigated but later discarded as it was a fourth replicate of the Chenopod Shrubland.



(a) (b) (c)

Figure 2: (a) SMU 1 (Sites 4, 9 and 10): Type 1–3 Biocrusts on deep calcareous yellow sands (dunes); (b) SMU 2 (Sites 1, 5 and 8): Primarily types 4 and 5 biocrusts on shallow calcareous sandy loam; (c) SMU 3 (Sites 2, 3 and 7): Types 1-5 biocrusts on deep calcareous sandy loam (Photographs by S. Doudle, 2011)

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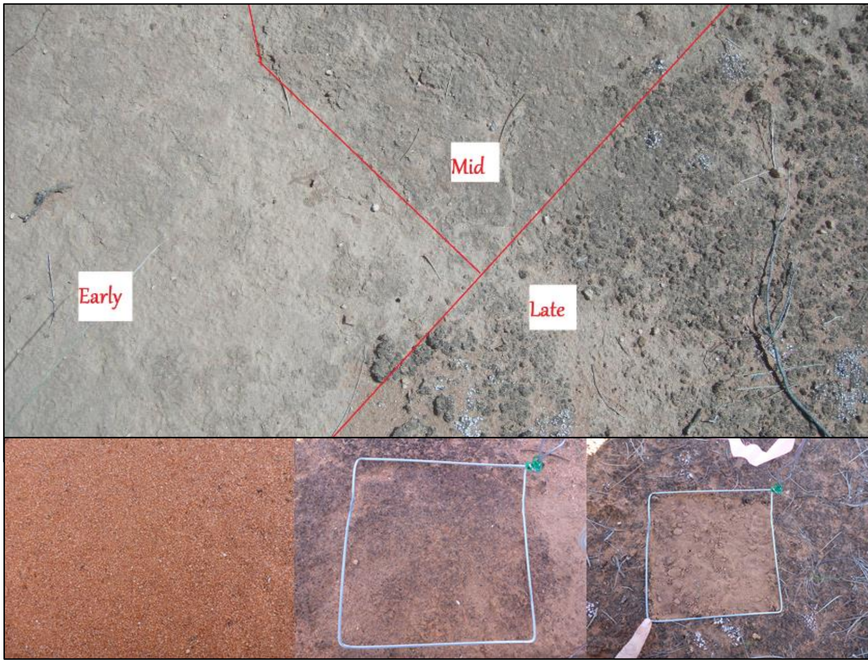

25 Figure 3: Different biocrust stages (top) north of Stockpile 19, adjacent to Site 9; (b) Bare (lower left), Early (centre) and Late (right) stages showing biocrust sample already removed from Late stage.

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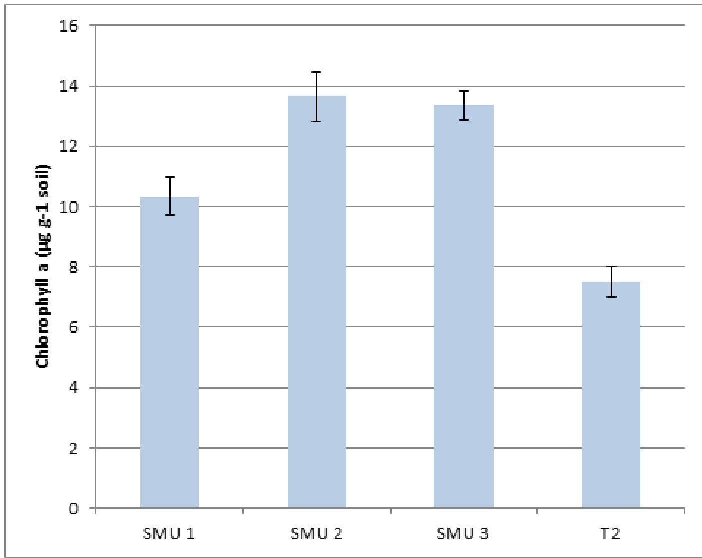

5 Figure 4: Chlorophyll concentration (µg g^{-1} soil) following resurrection (2 weeks) after a desiccation (dry) period of 6 months. Mean values and standard error of the mean (SEM), SMU = Soil Management Units where SMU 1 = Sites 4, 9, 10; SMU 2 = Sites 1, 5, 8; SMU 3 = Sites 2, 3, 7; T2 = 2YO Topsoil stockpile (originating from SMU 3).

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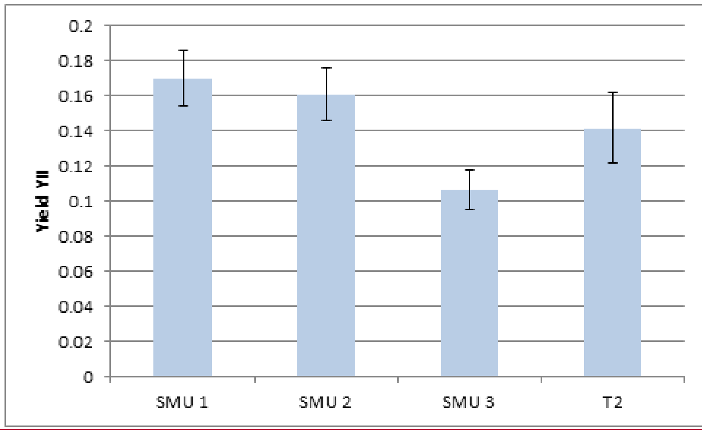

Figure 5: Photosynthetic yield (YII) of photosystem II (PSII) for SMU 1–3 and T2 (Site 6 2YO topsoil stockpile), mean values and standard error of the mean (SEM).

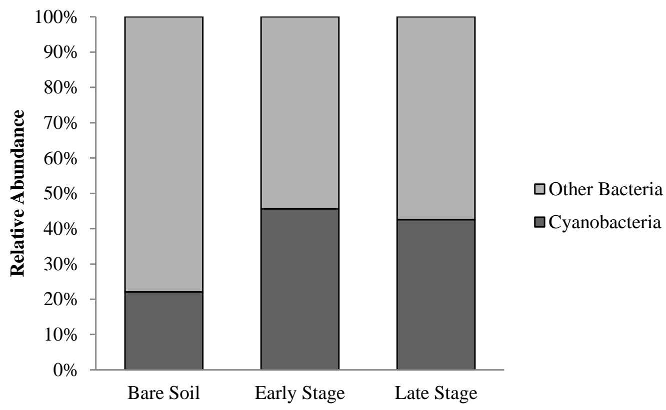

Figure 6: Relative abundance of cyanobacteria to other bacteria within Bare soil and Early and Late stage biocrusts

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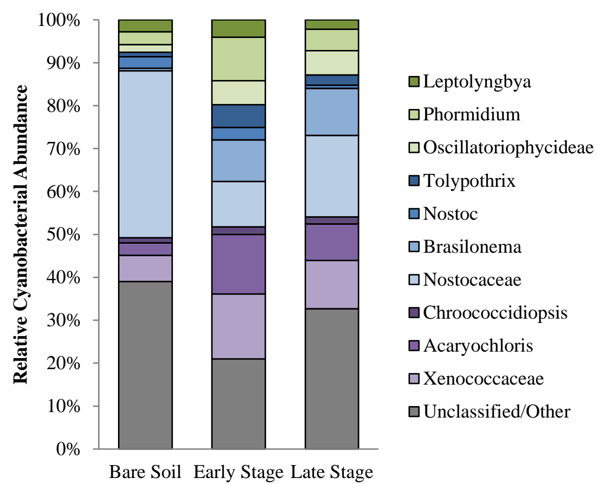

Figure 7: Abundance of cyanobacterial genera and groups. Green = Simple filamentous types, Blue = Heterocystic types, Purple = Unicellular. Grey = Unclassified/Other includes chloroplasts.

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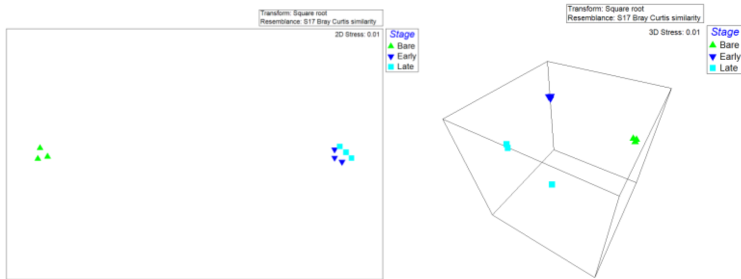

Figure 8: Non-metric Multidimensional Scaling of Bare soil and Early and Late Stage biocrusts within two dimensions (left) and three dimensions (right).

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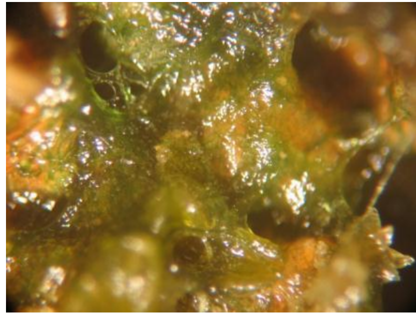

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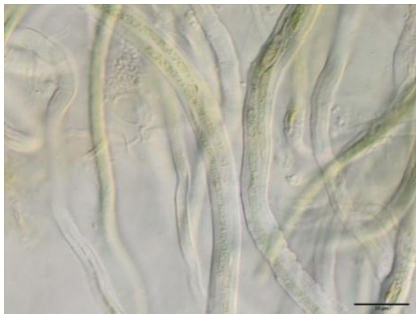

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Figure 9: (a) Predominantly subsurface-dwelling cyanobacteria *Microcoleus paludosus* forming a network of filaments across the soil surface during times of adequate moisture and light; (b) Masses of fine filaments of surface dwelling *Symploca* embedded into EPS that provides a gelatinous and sticky biofilm to create the basis of the biocrust; (c) *Schizothrix* contained in thick sheaths; (d) biocrusts were typically 1-10 mm thick and could be easily removed in pieces from the soil, white arrow points to tiny black cyanobacterial colony with UV protection in amongst green colonies; (e) gelatinous EPS covering cyanobacterial colonies; (f) a probe is used to lift cyanobacterial crust that is held together by surface and subsurface network of filaments.

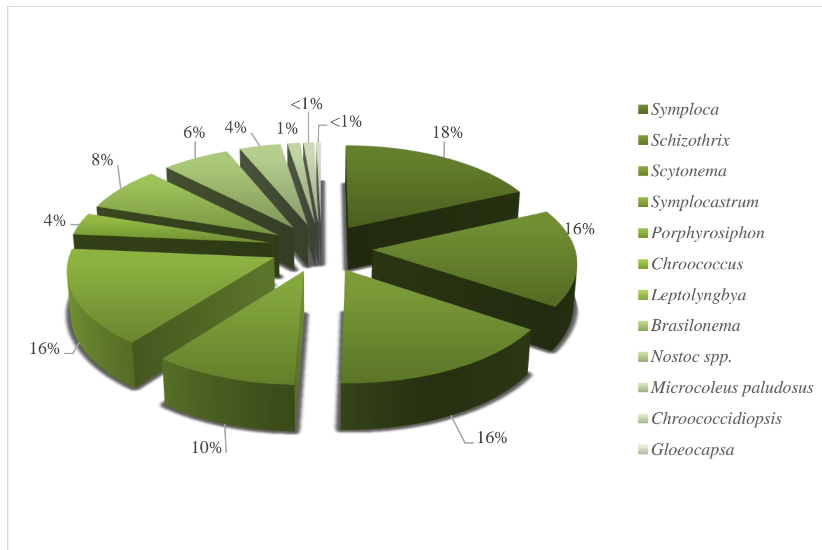

Figure 10: Cyanobacterial community structure across all sites expressed as a percentage of the total community based on mean richness and abundance scores. N-fixing cyanobacteria contributed to 61% of the community structure.

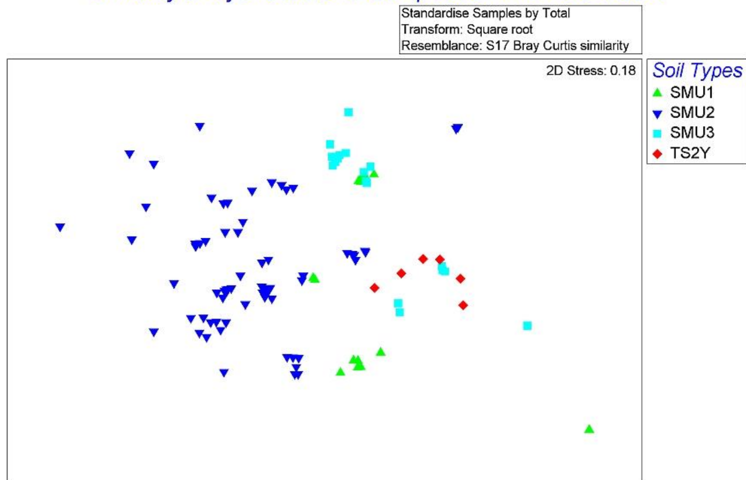

Figure 11: Cyanobacterial community structure based on indexed abundance and diversity across all sites displayed in an nMDS plot (Bray Curtis similarity). SMUs 1-3 refer to key soil management units; SMU 2 appeared to have more range in the clusters compared to the closer groupings of SMU 1 and 3. TS2Y is the 2YO topsoil stockpile (T2); note these are clustered more closely.

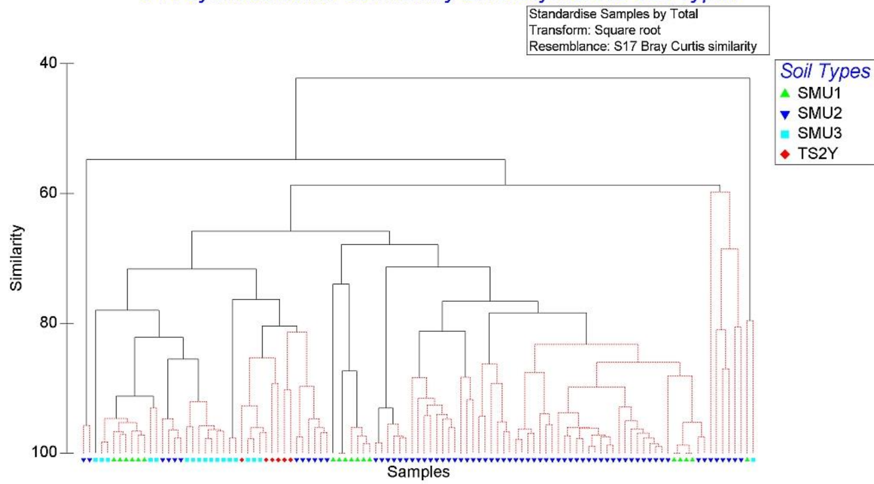

Figure 12: Similarities between samples within their SMU's are displayed in a Bray Curtis dendrogram. Black continuous lines show significant differences between samples (p = 0.05) and lighter lines indicate that most samples were nor significantly different to each other.

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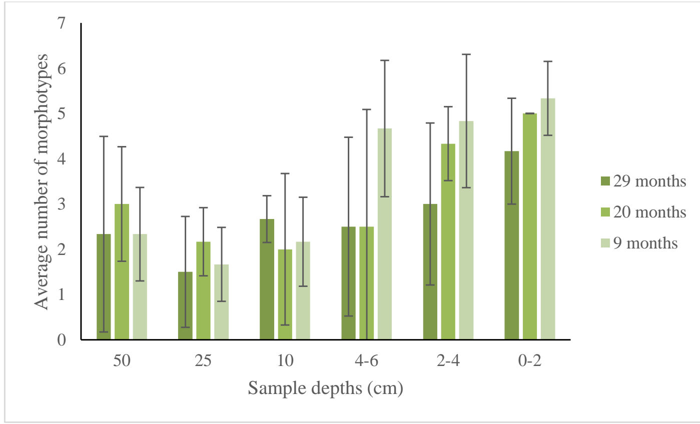

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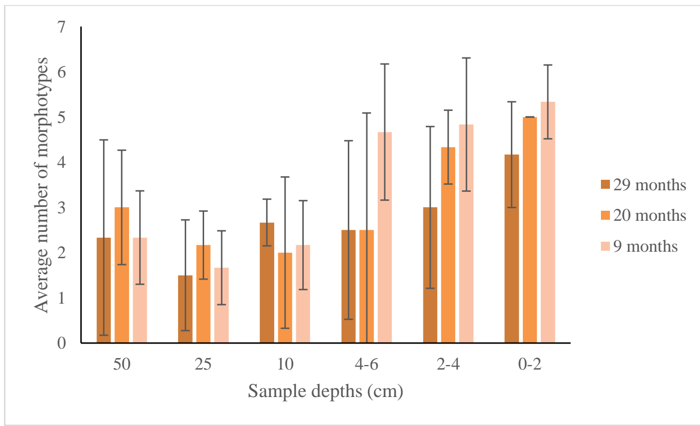

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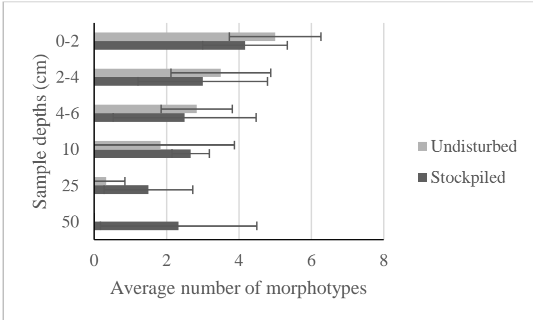
Figure 13: Cyanobacterial diversity in: (a) undisturbed and, (b) stockpiles of different ages.



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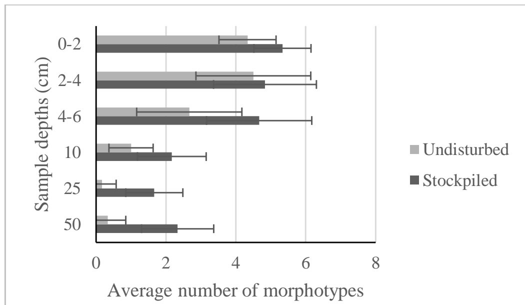

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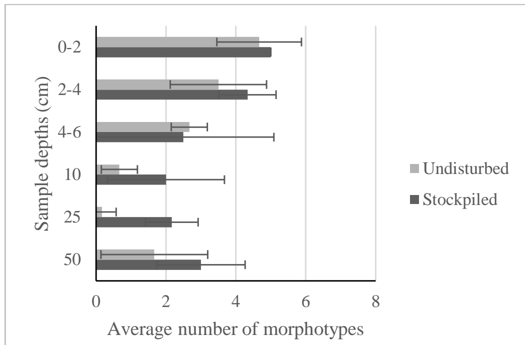
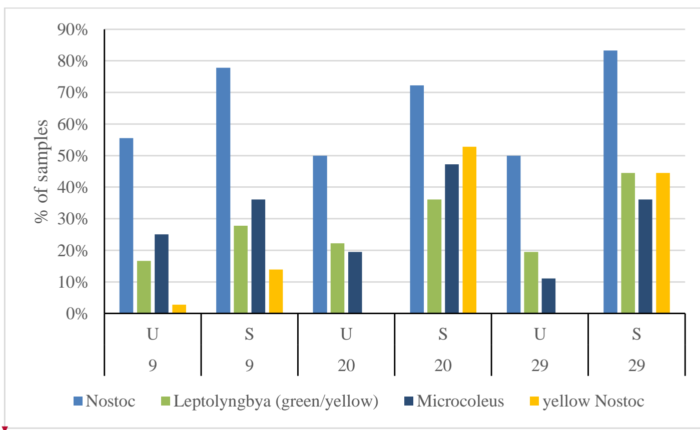


(c)

Figure 14: (a) cyanobacterial species richness following 29 months of stockpiling; (b) cyanobacterial species richness following 20 months of stockpiling; (c) cyanobacterial species richness following 5 months of stockpiling.



5 Figure 15: Morphotypes identified in a higher percentage of stockpiled samples (S) when compared with undisturbed samples (U) of varying ages (9, 20 and 29 months)

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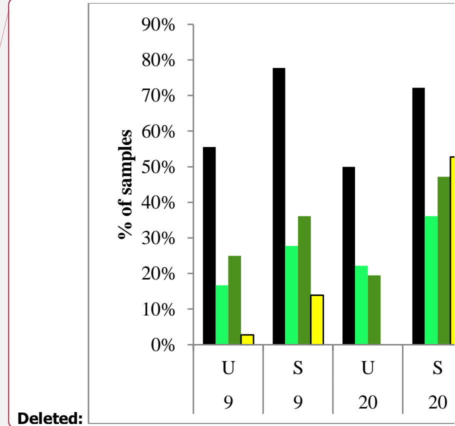

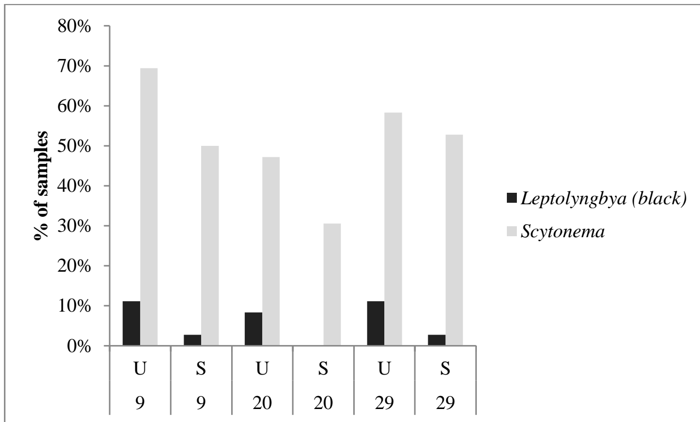

Figure 16: Morphotypes identified in a higher percentage of undisturbed samples (U) when compared to stockpiled samples (S) of varying ages (9, 20 and 29 months)

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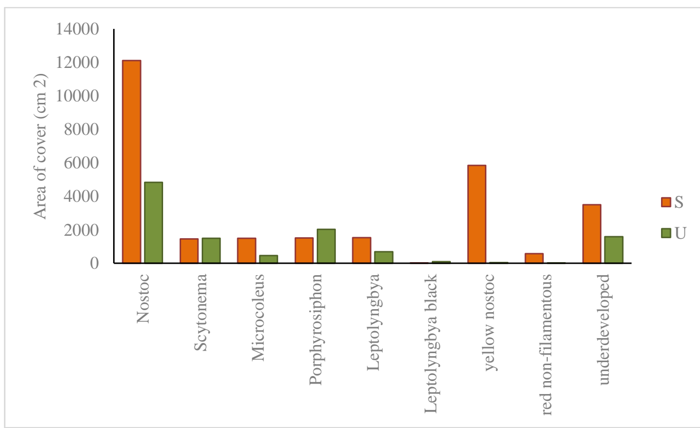

Figure 17: Total area of cover for each cyanobacterial morphotype in all stockpiled (S) and all undisturbed (U) samples

Acknowledgements

This project was funded and supported by Iluka Resources Ltd. and the Jacinth-Ambrosia Rehabilitation team. Thanks to Sam Doudle, Iluka Rehabilitation Specialist for her recognition of the importance of biocrusts at Jacinth-Ambrosia and for initiating this research program. This was a UniQuest project that was carried out at The University of Queensland's School of Agriculture and Food Sciences, Gatton Campus and the University of New South Wales. Especially appreciated was the strong support, project advice and laboratory resources provided by Assoc. Prof Vic Galea and Katherine Raymont. Special thanks to Dr Glenn McGregor for technical advice, assistance with cyanobacterial identification and the use of specialist microscopes and facilities at the Ecosciences Precinct (Qld), as well as report editing. Special thanks to David Tongway who assisted in Landscape Function Analysis field work, together with Colin Driscoll and two anonymous reviewers who provided valuable insights and editing to greatly improve this manuscript.

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