

## ORIGINAL ARTICLE

# Soil-foraging animals alter the composition and co-occurrence of microbial communities in a desert shrubland

David J Eldridge<sup>1</sup>, Jason N Woodhouse<sup>2</sup>, Nathalie JA Curlevski<sup>2,3</sup>, Matthew Hayward<sup>4,5</sup>, Mark V Brown<sup>2</sup> and Brett A Neilan<sup>2</sup>

<sup>1</sup>Centre for Ecosystem Science, School of Biological, Earth and Environmental Sciences, University of NSW, Sydney, NSW, Australia; <sup>2</sup>School of Biotechnology and Biomolecular Sciences, University of NSW, Sydney, NSW, Australia; <sup>3</sup>Faculty of Science, Aquatic Processes Group, University of Technology, Ultimo, NSW, Australia; <sup>4</sup>Australian Wildlife Conservancy, Nichols Point, VIC, Australia and <sup>5</sup>School of Environment, Natural Resources and Geography; and School of Biological Science, Bangor University, Bangor, UK

**Animals that modify their physical environment by foraging in the soil can have dramatic effects on ecosystem functions and processes. We compared bacterial and fungal communities in the foraging pits created by bilbies and burrowing bettongs with undisturbed surface soils dominated by biocrusts. Bacterial communities were characterized by *Actinobacteria* and *Alphaproteobacteria*, and fungal communities by *Lecanoromycetes* and *Archaeosporomycetes*. The composition of bacterial or fungal communities was not observed to vary between loamy or sandy soils. There were no differences in richness of either bacterial or fungal operational taxonomic units (OTUs) in the soil of young or old foraging pits, or undisturbed soils. Although the bacterial assemblage did not vary among the three microsites, the composition of fungi in undisturbed soils was significantly different from that in old or young foraging pits. Network analysis indicated that a greater number of correlations between bacterial OTUs occurred in undisturbed soils and old pits, whereas a greater number of correlations between fungal OTUs occurred in undisturbed soils. Our study suggests that digging by soil-disturbing animals is likely to create successional shifts in soil microbial and fungal communities, leading to functional shifts associated with the decomposition of organic matter and the fixation of nitrogen. Given the primacy of organic matter decomposition in arid and semi-arid environments, the loss of native soil-foraging animals is likely to impair the ability of these systems to maintain key ecosystem processes such as the mineralization of nitrogen and the breakdown of organic matter, and to recover from disturbance.**

*The ISME Journal* (2015) 9, 2671–2681; doi:10.1038/ismej.2015.70; published online 1 May 2015

## Introduction

Australia has suffered one of the highest rates of global mammal extinctions over the past 200 years since European settlement (Woinarski *et al.*, 2012). Losses have been most pronounced in the critical weight range (35–5500 g) mammals, which were once common over large areas of continental Australia (Johnson, 2006). The loss of these animals, or the contraction of their ranges, has been attributed to multiple causes associated with European settlement and pastoral practices such as altered fire regimes, overgrazing by livestock, competition

with exotic pests including the European rabbit (*Oryctolagus cuniculus*) and predation by introduced species such as the domestic cat (*Felis catus*) and the red fox (*Vulpes vulpes*) (Johnson, 2006). Two species that have suffered substantial range restrictions are the greater bilby (*Macrotis lagotis*) and the burrowing bettong (*Bettongia lesueur*). Recent attempts have been made to reintroduce these animals into predator-proof exclosures within their former range in an effort to re-establish viable populations (James and Eldridge, 2007).

Many of Australia's locally extinct animals forage extensively in the soil for seeds, bulbs, invertebrates and fungi (Robley *et al.*, 2001; James *et al.*, 2011; Eldridge *et al.*, 2012). Foraging disturbs the soil surface and breaks up the surface crust (biocrust), altering rates of water infiltration, and creating small pits and depressions that trap water, soil, organic matter and seed (James *et al.*, 2009). These pits develop into patches of higher nutrients, with greater

Correspondence: DJ Eldridge, Centre for Ecosystem Science, School of Biological, Earth and Environmental Sciences, University of NSW, Sydney, NSW 2052, Australia.

E-mail: d.eldridge@unsw.edu.au

Received 5 October 2014; revised 5 February 2015; accepted 25 March 2015; published online 1 May 2015

concentrations of plant-available nitrogen and carbon than the surrounding soil matrix (James *et al.*, 2010) and often a different vegetation community (Lavelle *et al.*, 2006). Studies worldwide have shown that modification of the abiotic environment by these animals, a process referred to as ecosystem engineering (*sensu* Jones *et al.*, 1994), alters energy flows and resource availability, increases species richness, diversity and productivity, through niche construction, ultimately controlling the availability and distribution of resources to other organisms (for example, Whitford and Kay, 1999; Jones *et al.*, 2010).

An important process moderated by soil-disturbing animals in arid environments is the decomposition of organic matter. Litter and organic matter in these systems are spatially and temporally variable, and is often concentrated within the foraging pits of animals (James and Eldridge, 2007). Litter is a source of carbon, nitrogen and other trace elements, and provides habitat for a range of micro- and macro-invertebrates involved in the decomposition of organic matter (Haslem *et al.*, 2011). Litter falling into pits comes into close contact with soil, where it is held *in situ* more effectively than if it remained on the soil surface where it is subject to removal by wind and water (Whitford, 2002). Together with reduced evaporation resulting from lower temperatures in the pits than the undisturbed surface (Eldridge and Mensinga, 2007), this increases the time period over which soil moisture is optimum for microbial decomposition and nutrient mineralization (Steinberger and Whitford, 1983; Jacobsen and Jacobsen, 1998; Whitford, 2002). Litter remaining on the surface, however, is subject to photodegradation (Austin and Vivanco, 2006), potentially reducing the return of carbon to the soil organic pool.

Soil-disturbing animals therefore play an important role in bringing surface-resident organic matter into contact with soil microorganisms. The accumulation of litter in the pits is also likely to exert a strong selective pressure on microorganisms essential for the decomposition process. Given the marked differences in the biotic (litter cover and composition) and abiotic (for example, surface temperature, soil moisture) environments between pits and undisturbed soils, that is, those soils undisturbed by animal activity (for example, Vossbrinck *et al.*, 1979; Wallwork *et al.*, 1985; Eldridge and Mensinga, 2007), we expected that the pits would differ in the composition of soil microorganisms. For example, studies of foraging disturbances constructed by the short-beaked echidna (*Tachyglossus aculeatus*) indicate a greater diversity and abundance of microarthropods and higher rates of microbial respiration in the pits than undisturbed soil (Eldridge and Mensinga, 2007), suggesting that there are differences in the abundance or structure of microbial communities. Over time, pits collect organic matter, and research has shown that pits over about 12 months old have high levels of organic carbon.

This compares with young pits (<3 months old), which have relatively low levels of litter and organic matter (D J Eldridge, unpublished data). We would expect pit age to influence microbial community structure, as these old pits (~12 months) would have more time to establish seedlings and accumulate litter and microorganisms that are present on adjacent, undisturbed surfaces. Furthermore, older pits could provide a greater range of different environments, with differences in depth, shape and orientation, and therefore different soil chemistry and organic matter at varying stages of decomposition.

We compared the community structure of soil microbial communities in old and young pits with the undisturbed surface soil on two soil types in an area where bilbies and bettongs have been reintroduced into their former range. Both bilbies and bettongs construct pits while foraging for buried seed, invertebrates and plant roots. The pits of these two species are indistinguishable, and range from cylindrical-shaped excavations about 15-cm wide and up to 20-cm deep to shallow basin-like structures (Eldridge *et al.*, 2012). Pits are constructed only once, and unlike cache pits of heteromyid rodents (Geluso, 2005), are rarely reworked. Because pits vary in depth and shape, and are constructed in soils of different texture, they provide a range of different physical environments that influences the trapping and retention of litter and the breakdown of organic matter.

We hypothesized that the microbial community in pit soils would support more microorganisms commonly associated with decomposing litter. Conversely, we expected that the microbial community composition in undisturbed soils would support a community dominated by cyanobacteria, given the extensive cover of biocrusts on the soil surface. We used microbial network analysis to examine the structure of microbial communities, particularly in relation to resilience and reactivity (Ruiz-Moreno *et al.*, 2006; Bissett *et al.*, 2013). Examination of microbial networks improves our understanding of why undisturbed soils might be resistant to nutrient amendment, how microbial community structure is altered following pit construction, and how digging promotes nutrient enrichment within these microsites (James *et al.*, 2009).

## Materials and methods

### *The study area*

Our study was undertaken within the Australian Wildlife Conservancy's Scotia Sanctuary in southwestern, New South Wales, Australia (33°43'S, 143°02'E) where locally extinct bilbies and bettongs have been released into predator-proof enclosures. Soil samples were collected from two systems; (i) mallee (*Eucalyptus* spp.) west-east-trending dunes of Quaternary alluvium characterized by

calcareous and siliceous sands (Rudosols) and (ii) the inter-dunal swales and plains extending to these dunes, which are up to 500-m wide, comprised mainly of loamy, calcareous soils (Calcarosols). The vegetation on the dunes is moderately dense mallee (*Eucalyptus socialis*, *E. dumosa*) and the plains are dominated by open mallee woodland with scattered belah (*Casuarina pauper*) and sugarwood (*Myoporum platycarpum*), and a variable cover of shrubs such as punty bush (*Senna artemisioides*), hophbush (*Dodonaea viscosa*), turpentine (*Eremophila sturtii*), pinbush wattle (*Acacia burkittii*) and assorted bluebushes (*Maireana* spp.), depending on whether trees had been removed. Shrubs covered about 50% of the area of the plains. The climate in the area is semi-arid, with cool winters (mean  $\leq 17$  °C) and hot summers (mean 30 °C). Rainfall is highly spatially and temporally variable and averages 243 mm per year. Rainfall is evenly distributed between the six warmer months and the six cooler months.

#### Field sampling

The location, size, depth and age of all foraging pits constructed by bilbies and bettongs have been monitored at 36 large sites at the Scotia Sanctuary since 2007. Because sites were visited every 3 months, we were able to calculate the relative age of particular pits. In October 2009, we collected soil samples from six sites: three on sandy dunes and three on loamy plains. At each of the six sites, we sampled three microsites: (i) young foraging pits, that is, pits constructed since the previous measurements (<3 months old), (ii) old foraging pits, that is, pits older than 12 months and (iii) undisturbed non-pit surface soils at least 3 m from any pit. At each of the six sites, we sampled each microsite at 10 locations. For the young pits, soil was removed from the uppermost 10 mm layer of the soil surface or from the base of the pits after removing any existing organic material. Biocrust was not removed from the soil prior to sampling. Approximately 5 g of soil was collected with a sterilized spatula. The material from the 10 locations was then bulked and stored on ice before being transported back to the laboratory. The same procedure was used to collect samples from old pits and undisturbed surfaces. This resulted in a database of 18 bulked samples (3 replicate sites of 2 soil types  $\times$  3 microsites).

#### Molecular analysis

Environmental DNA was isolated from 500 mg of soil using the FASTDNA Spin Kit for Soil (MP Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions and stored at  $-80$  °C until use. DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the quality checked by PCR amplification of the 16 S rRNA gene using the primer pair 27 f/519r (Weisburg, 1991). Bacterial

and fungal specific tag-encoded FLX amplicon pyrosequencing of each sample was carried out using the primers 27 f/519r and funSSUF/funSSUR respectively (Lucero, 2011) on a Roche GS-FLX Titanium at the Research and Testing Laboratory (Lubbock, TX, USA). Sequence reads were analyzed using MOTHUR v1.22 ([www.mothur.org](http://www.mothur.org)) software package (Schloss *et al.*, 2009). Initial quality processing of 454 sequence reads was performed using the mothur implementation of PyroNoise (Quince *et al.*, 2011) using default settings. Sequences containing <200 bp, containing ambiguous bases and homopolymers longer than 8 bp in length were removed. The remaining sequences were aligned to either the bacterial or fungal alignments of the SILVA release 102 reference alignment. Chimeric sequences were identified and removed using the mothur implementation of uchime (Edgar *et al.*, 2011). The taxonomic identity of each unique sequence was determined by comparison against the SILVA release 102 reference database. Taxonomic assignment was made at each level, given a bootstrap value greater than 80, using the RDP classifier (Wang *et al.*, 2007). Sequences that failed to be classified at the phylum level or were classified as either Mitochondria, Archaea or Eukaryota/Prokaryota in the respective datasets, were removed. Sub-sampling was performed at a level of 400 sequences per sample for the bacterial dataset and 1300 sequences per sample for the fungal dataset. Implementation of this process resulted in the exclusion of a bacterial young loam soil sample and bacterial young sand soil sample, as these samples contained fewer than the 400 sequences required. To ensure a balanced design across the bacterial dataset, the corresponding samples were subsequently excluded from the bacterial old pit soil and bacterial surface pit soil sets (2 replicate sites of 2 soil types  $\times$  3 microsites). Uncorrected pairwise distances were calculated between sequence reads with the final clustering of operational taxonomic units (OTUs) performed at a 0.03 distance threshold using the average neighbor algorithm (Schloss and Westcott, 2011). The identity of each OTU defined at 0.03 distance threshold was obtained from the consensus of each sequence within that OTU at a confidence threshold of 80. From these data, two individual data matrices were generated, one for bacteria and one for fungi, each matrix containing every OTU and the number of reads assigned to it from each sample. In this instance, the relative proportion of each OTU was used as a proxy for abundance, as absolute abundance measures were not obtained.

#### Statistical analysis

We used permutation multivariate analysis of variance (PERMANOVA; Anderson *et al.*, 2008) to examine differences in the composition of a data matrix of 2500 bacterial OTUs, defined at 0.03



distance threshold, and a data matrix of 5895 fungal OTUs, defined at 0.03 distance threshold, in relation to microsite (undisturbed soils, young pits, old pits) and soil type (loam, sand). Relative abundance data were used after resampling, to ensure an equivalent number of sequences. The first stratum of this analysis considered soil type and the second stratum microsite and its interaction with soil type. Pairwise *a posteriori* comparisons were made, where necessary, using a multivariate analogue of the *t* statistic, the probability levels being obtained by permutation. We tested for differences in richness and diversity of taxa with a mixed-model ANOVA with the same structure as the PERMANOVA analysis. Richness and diversity data were checked for homogeneity of variance (Levene's test) and normality using diagnostic tests but no transformations were needed. For all analyses, significant differences between means were examined using Fisher's Protected Least Significant Difference test. The procedure was repeated for the fungal data.

The degree of association of OTUs with respect to microsite was measured with Indicator Species Analysis in R (De Cáceres *et al.*, 2012) using a data matrix consisting of 2500 bacterial OTUs and 5895 fungal OTUs. Indicator values combine information on relative abundance and frequency of species, and the indicator value is maximal (IV = 100%) when all individuals of a given species are restricted to a particular microsite (for example, old pit), and all samples from the particular microsite contain an occurrence of that species. Data (at the OTU level) were randomized among the treatments and a Monte Carlo randomization procedure performed with 1000 iterations to determine the statistical significance of the indicator values.

The degree of association of OTUs with respect to one another within each microsite was measured using the Pearson's correlation coefficient (*r*). Bacterial and fungal OTU tables, defined at 0.03 distance threshold, were separated on the basis of microsite, and then reduced by removing any OTUs that did not occur across at least 75% of available samples. A Pearson's *r* score and *P*-value were calculated pairwise for each bacterial OTU using the *rcor.test* algorithm, available from the *ltn* package (available from <https://cran.r-project.org/package=ltn>) as implemented in R version 3.0.2. For each correlation, *P*-values were generated and the false discovery rate was maintained below 5% using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Visualization of these interactions, incorporating taxonomic, abundance and microsite occurrence information, was made with the freely available Cytoscape package version 2.8.3 (available at: [www.cytoscape.org](http://www.cytoscape.org)). For each network, topological metrics of connectivity and density were calculated using the network analysis plug-in (Assenov *et al.*, 2008). Networks pre-embedded with sample and OTU-specific information are provided in the Supplementary Material.

## Results

### *Richness of bacterial and fungal taxa*

Most bacterial and fungal OTUs occurred at very low abundances, with a substantial number of abundances equal to one. Of the original 2500 bacterial OTUs after resampling, 320 (14%) contributed 50% of total OTU abundances. For fungal OTUs, 525 (9%) of the 5895 OTUs contributed 50% of total fungal abundances. There were no differences in bacterial OTU richness (that is, different number of OTUs) among the different soils ( $P=0.24$ ; range 238–332 OTUs) or among the three microsites ( $P=0.47$ ). Similarly, fungal richness did not vary with soil texture ( $P=0.81$ ; range 397–873) or among the three microsites ( $P=0.17$ ).

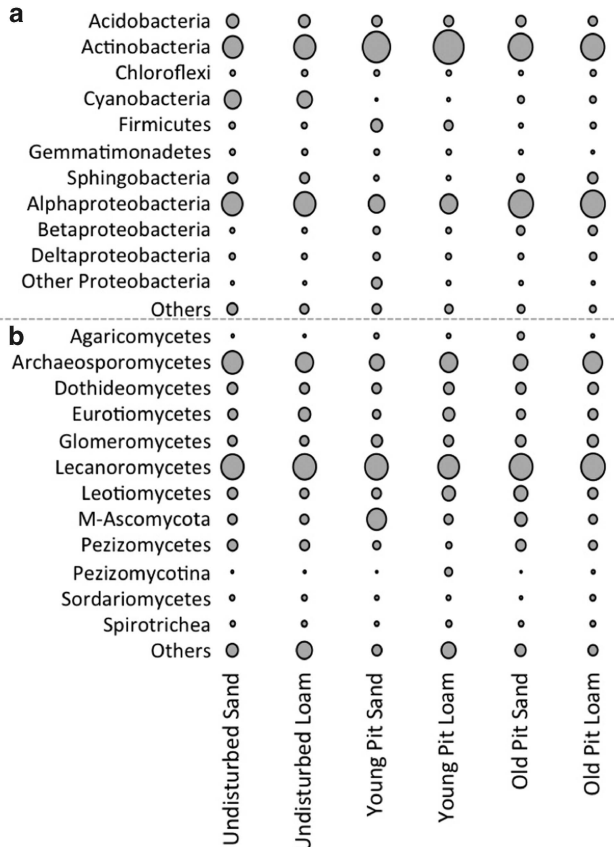
### *Community composition of bacterial and fungal taxa*

Bacterial communities were observed to contain a high proportion of Actinobacteria, and to a lesser extent, Alphaproteobacteria and Acidobacteria (Figure 1a). Cyanobacteria appeared to constitute a large proportion of the bacterial community, particularly in undisturbed soils. Fungal communities were observed to contain a high proportion of Lecanoromycetes, and to a lesser extent, Archaeosporomycetes (Figure 1b).

There was no significant difference in the composition of either bacterial or fungal OTUs assemblages between loamy and clay soils ( $P>0.30$ ). The composition of the bacterial assemblage did not vary among the three microsites ( $P=0.21$ ; Figure 2a), but there was a significant effect for fungi (Pseudo  $F_{2,8}=3.08$ ,  $P(\text{Perm})=0.003$ ). The composition of fungi in undisturbed soils was significantly different from that in old (pairwise  $t=2.14$ ,  $P=0.029$ ) or young ( $t=2.02$ ,  $P=0.02$ ) pits, but there was no significant differences between old and young pits ( $P=0.47$ ; Figure 2b).

### *Microsite indicators*

Six cyanobacterial OTUs (Gp I (3 OTUs), Gp X, Gp VII and an unclassified OTU) were indicators of undisturbed pits, as were the single *Asanoa* OTU (Actinobacteria), a *Segetibacter* OTU (Sphingobacteria) and an unclassified alphaproteobacterial OTU. A single *Hylangium* OTU (Myxobacteria, Deltaproteobacteria), *Microvirga* OTU (Rhizobiales, Alphaproteobacteria) and a Gp IV actinobacterial OTU were indicators of old pits. A single *Rubrobacter* OTU (Actinobacteria), *Ammoniphilus* OTU (Bacilli, Firmicutes) and *Actinaurispora* OTU (Actinobacteria) were indicators of young pits (Table 1). Overall, fungal taxa were better discriminators of the three microsites, with 20 orders containing 170 OTUs, with indicator values  $>0.70$ , and almost exclusively from sub-phylum Pezizomycotina. These included orders *Dothideales* (genera *Columnosphaeria*, *Delphinella*), *Chaetothyriales* (genus *Glyphium*), Lecanorales (genera *Sphaerophoraceae*, *Cladoniaceae*),

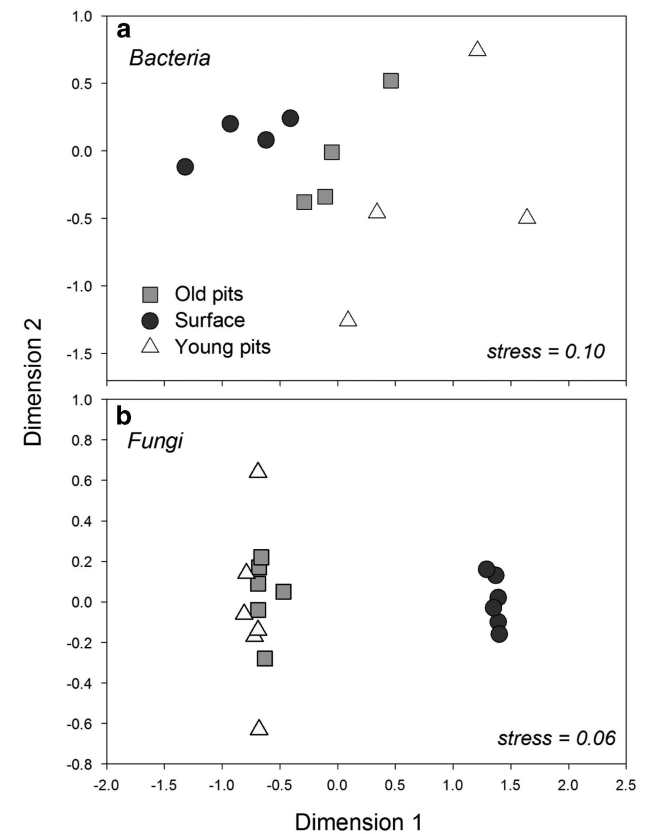


**Figure 1** Relative abundance of major (a) bacterial and (b) fungal taxa within each microsite. Larger circles indicate greater abundance.

*Myxotrichaceae* (genus *Geomyces*), *Mycocaliciales* (*Sphinctrina*) and *Pleosporales* (genera *Leptosphaeria*, *Trematosphaeria*, *Phaeosphaeria*). Ten fungal genera (particularly *Eupenicillium*, *Hamigera*, *Bionectriaceae* and an unclassified taxon from the family Bulgaria) were highly indicative ( $IV > 0.81$ ) of young pits. Old pits contained a wide range of different OTUs, with the orders *Chaetothyriales*, *Dothideales*, *Hypocreales*, *Lecanorales*, *Mycocaliciales* and *Pleosporales* having a large number of OTUs that were strongly indicative ( $IV > 69\%$ ) of older pits (Table 2).

#### Network analysis

Within the bacterial networks, the mean number of correlations between OTUs was greater in old pit soils (3.45) than either undisturbed (2.516) or young pit (1.294) soils, consistent with a larger number of OTUs co-occurring across the samples (Table 3). The majority of the associations present in young pit soils were between a small number of alphaproteobacterial and actinobacterial OTUs. Young pit soils returned the lowest values for network metrics of clustering (0), density (0.081) and centralization (0.050). Undisturbed soils and old pit soils were similar in relation to clustering (undisturbed = 0.566, old pits = 0.547), density (undisturbed = 0.084, old



**Figure 2** Multi-dimensional scaling biplot of the first two dimensions of an ordination of a reduced matrix of (a) 280 bacterial OTUs and (b) 135 fungal OTUs. Note the clustering of undisturbed samples for both bacteria and fungi.

pits = 0.088) and centralization (undisturbed = 0.089, old pits = 0.096) (Table 3).

Within the fungal networks, the highest mean number of correlations between OTUs (20.497) was observed in undisturbed soils, where many more OTUs (1814 OTUs) were present across multiple samples than in young (321) or old (485) pit soils. Similar to the bacterial networks, young pit soils returned the lowest values for density (0.009), but were also the most centralized (0.116). Old pit soils were the least clustered (0.472) and the least centralized (0.067). Undisturbed soils were similar to young pit soils in terms of clustering (undisturbed = 0.652, young pits = 0.647), whereas fungal young and old pit soils were only similar in relation to the mean number of correlations between OTUs.

## Discussion

Soil foraging by semi-fossorial animals in arid areas disrupts surface crusts, alters rates of water infiltration, and creates small pits and depressions that trap water, soil, organic matter and seed (James and Eldridge, 2007). We expected to detect substantial differences in the soil microbial community between

**Table 1** Bacterial taxa, to the level of genus, that are significantly associated with different microsites using Indicator Species Analysis

Order	Family	Genus	Microsite	IV	P-value	No of OTUs
Cyanobacteria	Family I	Group I	Undisturbed	0.866	0.047	3
Cyanobacteria	Family X	Group X	Undisturbed	0.866	0.046	1
Cyanobacteria	Unclassified	Unclassified	Undisturbed	0.866	0.049	1
Actinomycetales	Micromonosporaceae	Asanoa	Undisturbed	0.866	0.046	1
Cyanobacteria	Family VIII	Group VIII	Undisturbed	0.866	0.047	1
Sphingobacteriales	Chitinophagaceae	Segetibacter	Undisturbed	0.866	0.049	1
Alphaproteobacteria	Alphaproteobacteria	Alphaproteobacteria	Undisturbed	0.866	0.047	1
Myxococcales	Cystobacteraceae	Hyalangium	Old	1.000	0.008	1
Acidobacteria	Acidobacteria	Group IV	Old	0.913	0.030	1
Rhizobiales	Methylobacteriaceae	Microvirga	Old	0.812	0.028	1
Rubrobacterales	Rubrobacteraceae	Rubrobacter	Young	0.905	0.012	1
Bacillales	Paenibacillaceae	Ammoniphilus	Young	0.866	0.040	1
Actinomycetales	Micromonosporaceae	Actinaurispora	Young	0.866	0.040	1

Abbreviations: IV, indicator value; OTUs, operational taxonomic units.

intact undisturbed soils and recently excavated or older, more established pits in response to differences in plant and litter cover, organic matter decomposition and soil nutrient concentrations. Although we detected some significant differences in the fungal community composition between the soil surface and the pits (described below), there were no discernible differences in the bacterial community and in the fungal community between young and old pits, largely because of the high variability among microsites (Figure 1). Consequently, we undertook an analysis that would test whether the physical variability that is observed in pit soils, in regard to moisture and nutrient trapping, influenced the occurrence of individual species or the manner in which individual species exhibited correlations with one another. Indicator species analysis was implemented to identify specific OTUs that were more strongly associated with a particular microsite type. Critically, indicator species analysis has been previously shown to be suitable for identifying variable taxa where there was no prior assessment, or no significant variation, in the larger community composition (De Cáceres and Legendre, 2009; De Cáceres and Legendre, 2009; De Cáceres *et al.*, 2012). That it were possible to identify species that were statistically indicative of particular microsites when the multivariate (PERMANOVA) analysis was insignificant highlights the fact that there is substantial heterogeneity within microsites, and suggests a level of functional redundancy within microbial taxa that prevents large-scale perturbation of the community despite the loss of species. On the basis of the indicator species (De Cáceres and Legendre, 2009; De Cáceres *et al.*, 2012) and microbial network (Chaffron, 2010; Bissett *et al.*, 2013) analyses, there is sufficient evidence to suggest that pits may be associated with a reduction in autotrophic groups (Figure 1, Tables 1 and 2) that are compensated for by an emergence of taxa capable of decomposing organic material (Tables 1 and 2) and reduced resilience in the microbial communities (Table 3).

#### *Compositional differences between pit and undisturbed soils*

Consistent with information from arid soils worldwide, the bacterial community contained high proportions of Actinobacteria and Alphaproteobacteria (Figure 1a) (Yeager *et al.*, 2004; Kuske, 2012). At the community level, we detected no significant differences in bacterial community composition between pits and undisturbed soils (Figure 2). However, consistent with our first hypothesis, filamentous diazotrophic (Cyanobacteria GpI), bacocystous (Cyanobacteria GpVIII) and unicellular (Cyanobacteria GpX) cyanobacteria were found to be indicators of undisturbed soils (Table 1) with a reduction in the observed abundance of cyanobacterial sequence reads when soils were disturbed (Figure 1, Table 1). Cyanobacteria were present in undisturbed soils, however, the presence of these taxa as indicators was reflective of both a decrease in the abundance of cyanobacterial groups and a shift within the morphological and physiological nature of cyanobacteria between undisturbed and pit soils. Among the heterotrophic population, actinobacterial members of the *Rubrobacteridae* that are pioneers in biological crust formation (Yeager *et al.*, 2004) dominated both undisturbed and pit soils, with a single *Rubrobacter* OTU, an indicator of young pit soils. In addition to cyanobacterial groups, the Sphingobacterial genus *Segetibacter* has been previously affiliated with the decomposition of cyanobacteria- and plant-derived phytodetritus (Li *et al.*, 2011).

Fungal communities in undisturbed and pit soils comprised a wide range of saprotrophs, with *Lecanoromycetes*, the largest class of lichenized fungi, and to a lesser extent, *Archaeosporomycetes*, comprising about 80% of sequences across the three microsites (Figure 1b). Along with *Pezizomycotina*, these fungal taxa perform a diverse array of ecological functions including wood and litter decomposition, mycorrhizal associations and lichen symbioses, animal and plant pathogens (Spatafora *et al.*, 2006). Evidence for active recession, or at least competitive inhibition, of microbial groups from the old pits was

**Table 2** Fungal taxa, to the level of genus, that are significantly associated with different microsites using Indicator Species Analysis

Subclass	Order	Family	Genus	Microsite	IV	P	No of OTUs
Eurotiomycetidae	Eurotiales	Trichocomaceae	Unclassified	Undisturbed	0.94	0.002	1
Pleosporomycetidae	Pleosporales	Leptosphaeriaceae	Leptosphaeria	Undisturbed	0.91	0.006	8
Pleosporomycetidae	Pleosporales	Melanommataceae	Trematosphaeria	Undisturbed	0.91	0.006	4
Skeletonemataceae	Skeletonema	Unclassified	Unclassified	Undisturbed	0.91	0.002	1
Strombidiidae	Strombidium	Unclassified	Unclassified	Undisturbed	0.91	0.007	1
Dothideomycetidae	Dothideales	Dothioraceae	Delphinella	Undisturbed	0.90	0.003	3
Naviculaceae	Navicula	Unclassified	Unclassified	Undisturbed	0.90	0.003	4
Pleosporomycetidae	Pleosporales	Phaeosphaeriaceae	Phaeosphaeria	Undisturbed	0.88	0.013	6
Pleosporomycetidae	Pleosporales	Pleosporaceae	Pleospora	Undisturbed	0.88	0.011	4
Dothideomycetidae	Dothideales	Dothioraceae	Columnosphaeria	Undisturbed	0.86	0.012	17
Chaetothyriomycetidae	Chaetothyriales	Chaetothyriales	Glyphium	Undisturbed	0.86	0.012	19
Pleosporomycetidae	Pleosporales	Phaeotrichaceae	Phaeotrichum	Undisturbed	0.86	0.011	2
Chaetothyriomycetidae	Chaetothyriales	Chaetothyriales	Sarcinomyces	Undisturbed	0.86	0.011	2
Mycocaliciomycetidae	Mycocaliciales	Sphinctrinaceae	Sphinctrina	Undisturbed	0.86	0.012	11
Dothideomycetes	Tubeufiaceae	Letendraea	Unclassified	Undisturbed	0.86	0.012	10
Leotiomycetes	Myxotrichaceae	Myxotrichaceae	Geomyces	Undisturbed	0.85	0.012	6
Sporadotrichida	Halteriidae	Halteria	Unclassified	Undisturbed	0.85	0.014	7
Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Botryosphaeria	Undisturbed	0.82	0.016	1
Lecanoromycetidae	Lecanorales	Lecanorineae	Cladoniaeae	Undisturbed	0.82	0.016	1
Dothideomycetidae	Dothideales	Dothideales	Hortaea	Undisturbed	0.82	0.015	2
Xylariomycetidae	Xylariales	Xylariaceae	Hypoxylon	Undisturbed	0.82	0.023	1
Xylariomycetidae	Xylariales	Amphisphaeriaceae	Pestalospaeria	Undisturbed	0.82	0.012	1
Helotiales	Bulgariaceae	Bulgaria	Unclassified	Undisturbed	0.82	0.012	1
Dothideomycetes	Kirschsteiniotelia	Unclassified	Unclassified	Undisturbed	0.82	0.016	2
Pezizales	Pezizaceae	Peziza	Unclassified	Undisturbed	0.82	0.016	1
Sordariomycetidae	Magnaporthales	Magnaporthaceae	Pseudohalonestria	Undisturbed	0.81	0.021	3
Lecanoromycetidae	Lecanorales	Lecanorineae	Sphaerophoraceae	Undisturbed	0.81	0.018	5
Agaricomycetidae	Agaricales	Lycoperdaceae	Lycoperdon	Undisturbed	0.76	0.039	1
Dothideomycetidae	Dothideales	Dothioraceae	Delphinella	Old	0.91	0.004	1
Eurotiomycetidae	Eurotiales	Trichocomaceae	Chromocleista	Old	0.82	0.015	1
Chaetothyriomycetidae	Chaetothyriales	Chaetothyriales	Glyphium	Old	0.82	0.025	6
Dothideomycetes	Tubeufiaceae	Letendraea	Unclassified	Old	0.82	0.025	1
Chaetothyriomycetidae	Chaetothyriales	Chaetothyriales	Sarcinomyces	Old	0.80	0.025	1
Mycocaliciomycetidae	Mycocaliciales	Sphinctrinaceae	Sphinctrina	Old	0.80	0.027	3
Sporadotrichida	Halteriidae	Halteria	Unclassified	Old	0.80	0.024	2
Dothideomycetidae	Dothideales	Dothioraceae	Columnosphaeria	Old	0.79	0.025	5
Pleosporomycetidae	Pleosporales	Leptosphaeriaceae	Leptosphaeria	Old	0.79	0.030	4
Hypocreomycetidae	Hypocreales	Hypocreaceae	Hypocrea	Old	0.75	0.034	2
Xylariomycetidae	Xylariales	Amphisphaeriaceae	Pestalospaeria	Old	0.74	0.045	1
Eurotiomycetidae	Eurotiales	Trichocomaceae	Hamigera	Young	0.87	0.011	4
Hypocreomycetidae	Hypocreales	Bionectriaceae	Bionectriaceae	Young	0.86	0.013	2
Dothideomycetidae	Dothideales	Dothioraceae	Columnosphaeria	Young	0.86	0.013	1
Eurotiomycetidae	Eurotiales	Trichocomaceae	Eupenicillium	Young	0.84	0.016	5
Leotiomycetes	Myxotrichaceae	Myxotrichaceae	Geomyces	Young	0.82	0.021	1
Mycocaliciomycetidae	Mycocaliciales	Sphinctrinaceae	Sphinctrina	Young	0.82	0.012	1
Pleosporomycetidae	Pleosporales	Leptosphaeriaceae	Leptosphaeria	Young	0.79	0.041	2
Dothideomycetes	Tubeufiaceae	Letendraea	Unclassified	Young	0.79	0.026	2

Abbreviations: IV, indicator value; OTUs, operational taxonomic units. Only taxa with an IV > 0.75 are shown.

**Table 3** Metrics obtained from analysis of scale-free microbial networks of bacterial and fungal microbial communities

Taxon and microsite	OTUs	Edges	Mean number of neighbours	Clustering coefficient	Density	Centralization
<i>Bacteria</i>						
Old	40	70	3.450	0.547	0.088	0.096
Undisturbed	31	39	2.516	0.566	0.084	0.089
Young	17	11	1.294	0	0.081	0.050
<i>Fungi</i>						
Old	135	485	7.185	0.472	0.054	0.067
Undisturbed	177	1814	20.497	0.652	0.166	0.095
Young	81	321	7.926	0.647	0.009	0.116

Abbreviation: OTUs, operational taxonomic units. Edges represent the number of significant positive and negative Pearson correlation coefficients identified following implementation of the Benjamini-Hochberg procedure at a minimum false discovery rate of 5%.



found, with the insect and plant pathogenic fungi, *Delphinella*, *Leptosphaeria*, *Trematosphaeria* and *Columnosphaeria*, found almost exclusively in undisturbed and young pit soils. *Glomeromycetes*, which comprise arbuscular mycorrhizal species, represented about 3% of sequences in young pits and 2% of sequences in old pit and undisturbed soils.

#### *Community development with pit age*

*Rubrobacter*, *Ammoniphilus* and *Actinaurispora* were the only bacterial indicators of young pits and likely represent remnants of the sub-surface community. *Rubrobacter* is a cosmopolitan and abundant taxon in arid zone soils (Yeager *et al.*, 2004). The presence of *Ammoniphilus* and *Actinaurispora* in young pit soils is likely due to the deposition of plant material. *Ammoniphilus* has been reported as a strictly aerobic oxalotroph utilizing plant- and algae-derived oxalic acid as a sole carbon. *Actinaurispora* are known plant endophytes, inhabiting *Camptotheca acuminata* species (Zhu *et al.*, 2012). The family Micromonosporaceae, to which *Actinaurispora* belongs, however, has been tentatively correlated with increasing moisture content in arid and semi-arid soils (Bachar *et al.*, 2010), which may contribute to the presence of this species as an indicator of young soils. *Trichocomaceae* species were the primary fungal indicators of young pits. A single *Trichocomaceae* species was a key fungal indicator of undisturbed soils, suggesting that fungal communities of young pits contain residual surface taxa prior to the colonization and diversification of fungal communities observed in older pits. *Trichocomaceae* species are predominantly saprotrophic, have aggressive colonization strategies and a high tolerance to extreme environmental conditions such as soil drying, high temperature and metal toxicity (Houbraken and Samson, 2011). Their presence in young pits could indicate opportunistic colonization of recently disturbed soil.

On the basis of the criteria used to select the microsites, progression of the microbial community from young to old pits occurs over a period of 9–12 months. Over this time, although little change occurred within the microbial community composition between pit stages, a discernable difference was observed between the undisturbed and pit bacterial and fungal communities, irrespective of their age. Microbial richness among microsites, however, remained unchanged. Spore propagule density and arbuscular mycorrhizal fungi diversity are known to decline with increasing tillage associated with agriculture (Brito *et al.*, 2010; Schalamuk *et al.*, 2013). However, this was not reflected in our fungal species richness, which remained unchanged over time. The progressive accumulation of fungal species attached to organic matter and seed in the pits is consistent with the presence of several lichenized lecanoralean genera including *Parmeliaceae* and

*Myrangium* (Smith, 1948) and the epiphytic melanized taxon *Sarcinomyces* (Wollenzien *et al.*, 1997). The Lecanorales are predominantly lichen-forming fungi that are mycobionts of the genera *Xanthoparmelia*, *Parmotrema* and *Xanthoria*, which are common corticolous lichens of *Callitris glaucophylla* trees that occur in the study area (Filson and Rogers, 1979). These taxa are typically found on the soil surface or in the pits on detached plant material. At some sites, we also recorded the vagant lichen *Chondropsis semiviridis* from within the pits. This lichen, which has no attachment to the soil, moves freely along the surface by wind action (Eldridge and Leys, 1999). Similarly, *Cladonia* spp., another common soil lichen genus, was found on undisturbed surfaces. Along with the lichen genera *Endocarpon* and *Placidium*, it is one of the most common lichens forming biocrusts on stable soils in arid and semi-arid areas (Eldridge and Koen, 1998).

Despite our inability to discriminate between the bacterial community of old and young pits, we recorded three indicator species, *Hyalangium* and *Microvirga*, and a Gp IV Acidobacteria. The two proteobacterial species were indicative of the presence of established vascular plants. *Hyalangium*, belongs to the group of Myxobacteria that uses plant lignin and produces small bioactive molecules. *Microvirga* has been implicated in nodule formation, facilitating nitrogen-fixing processes within the rhizosphere (Ardley *et al.*, 2012). The occurrence of these groups in old pit soils is likely to enhance nitrogen fixation, presumably to levels greater than those in the undisturbed and young pit soils, and support the growth of vascular plants occurring in these microsites.

#### *Microbial co-occurrence in pit and undisturbed soils*

Our analyses thus far indicate that initial disturbance reduces the abundance of key photoautotrophic groups, and that over extended periods of time, capture of organic matter leads to changes in the abundance of some taxa, with increases in those taxa likely reflecting an increased capacity for the assimilation of organic carbon and nitrogen matter.

Resilience is the ability of a system to recover from large disturbances, typically over short time frames. Reactivity, however, is the capacity of a system to respond to small perturbations over extended periods. Under such circumstances, the apparent equilibrium may appear stable, despite moving to a new steady state over long time periods (Neubert *et al.*, 2009). Modularity, defined by the number and size of groups of highly interconnected nodes within a network, is positively correlated with reactivity, and negatively correlated with resilience (Ruiz-Moreno *et al.*, 2006). Analysis of both bacterial and fungal microbial networks revealed stark differences in modularity, reflected in the values of clustering, density and centralization, of microbial co-occurrence networks between undisturbed soils and pit soils at



different developmental stages (Table 3). Clustering coefficients and density (network connectivity) scores tending towards a value of 1 indicate a highly modular system whereas those tending towards zero represent the opposite (Bissett *et al.*, 2013). Low values of clustering and density associated with microbial communities from contaminated and reference estuarine sediments indicate historical community 'stress' contributing to functional redundancy and reduced correlations among species (Sun *et al.*, 2013). This was reinforced by marginally lower values, for each of these metrics, in contaminated sediments, with the suggestion that this anthropogenic perturbation has contributed an additional stress.

In the present study, bacterial species–species correlations within the young pit soils were almost nonexistent. A clustering coefficient of zero and a slightly lower density value were consistent with reduced modularity, and an increase in functional redundancy associated with a recent external stress (Sun *et al.*, 2013). In contrast, undisturbed and old pit soils were more consistent with increased modularity, suggesting a lack of functional redundancy, with greater species–species correlations, and increased clustering and density. This suggests to us that the bacterial community present in undisturbed soils and old pit soils are more reactive and less resilient than young pit soils. Within the fungal communities, the number of correlations among species, clustering coefficients and density, and hence modularity, were highest in surface soils and lowest in the old pit soils, suggesting that fungal communities within old pits are less reactive and more resilient. In contrast, the young pit soils exhibit reduced modularity, and increased resilience, suggesting that they are likely to respond to nutrient amendments over the short-term, thereby driving large and dramatic structural changes. This is largely because of the high degree of physical disturbance created when foraging pits are established. Within the old pit soils, the bacterial community has largely regained the modularity observed within the undisturbed soils. The fungal community, however, is apparently more resistant at this stage than in the undisturbed soils, suggesting it is able to continue to drive structural changes in response to events such as litter deposition.

A high level of centralization, as a consequence of the high frequency of centralized nodes, was observed among the fungal community in young pit soils (*Bulgariaceae*, *Myxotrichaceae*, *Trichocomaceae*, *Tubeufiaceae*) and among the bacterial community in undisturbed (*Rubrobacteraceae*, *Geodermatophilaceae*, *Bradyrhizobiaceae*) and old pit (*Rhodobacteraceae*, *Bradyrhizobiaceae*, *Geodermatophilaceae*, *Beijerinckiaceae*, *Comamonadaceae*, *Methylobacteriaceae*) soil (Supplementary Information). Centralized nodes have been proposed to represent keystone species, exhibiting a large influence of the 'information' transfer throughout the community (Bissett

*et al.*, 2013). It has been speculated that these nodes represent critical control points in the cycling of nutrients within the system (Ruiz-Moreno *et al.*, 2006; Bissett *et al.*, 2013). Thus, it is realistic to suggest that these centralized taxa act to stabilize the microbial community. It should be highlighted that these observations were made in the context of a small number of samples defining each microsite, as well as few sequence reads being available to identify species–species correlations. Our observations between the bacterial and fungal datasets suggest that these metrics are susceptible to sequence depth, and pre-treatment of the data by retaining only semi-ubiquitous (occurring across at least 75% of samples) OTUs. These values may also be influenced by the level of heterogeneity within microsites. Despite this, our analyses of network metrics from the bacterial communities suggested that the community structure of old pit soils reflect that of undisturbed soils. Over the long term, this would tend towards decreased responses to nutrient inputs into these soils. This, however, may be partially offset by frequent deposition of plant matter due to the establishment and growth of vascular plants within old pit soils, and subsequent assimilation of this matter by saprotrophic fungi.

## Conclusions

Our study suggests that digging by soil-disturbing animals is likely to create successional shifts in soil microbial and fungal communities, which could account for increases in organic matter of nitrogen in old foraging pits (James *et al.* 2009). The observed richness of fungal and bacterial OTUs, in undisturbed soils, and young and old pits did not differ, though fewer correlations, and hence an increased resilience, were observed between bacterial OTUs in young pits, and fungal OTUs in young and old pits. This suggests that these communities are more likely to respond over the short term to nutrient amendment, thus promoting nutrient enrichment and contributing to a form of patchiness that is critical for the functioning of arid systems. The action of soil-disturbing animals therefore leads to the development of a mosaic of different patches with a varying complement of microorganisms. Given the wide variety in pit size, depth, substrate and spatial configuration, this differential microbial activity will likely lead to the creation of a mosaic of patches of differing resource availability, analogous to larger surface-resident biotic patches such as hummocks and debris mounds. Our work suggests that microbial community composition and co-occurrence change with physical disturbance during the formation of foraging pits. Given the primacy of organic matter decomposition in arid and semi-arid environments, the loss of native soil-foraging animals from these systems may well impair the ability of these systems to maintain key ecosystem processes and to recover from disturbance.

## Conflict of Interest

The authors declare no conflict of interest.

## Acknowledgements

We thank Ivan Wong for field assistance and Samantha Travers for comments on an earlier draft of the manuscript. We acknowledge the considerable financial, logistic and technical support provided by the Australian Wildlife Conservancy through their Scotia Sanctuary. This research was supported by the Australian Government under ARC LP0882630.

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