

Mammalian engineers drive soil microbial communities and ecosystem functions across a disturbance gradient

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Summary

1. The effects of mammalian ecosystem engineers on soil microbial communities and ecosystem functions in terrestrial ecosystems are poorly known. Disturbance from livestock has been widely reported to reduce soil function, but disturbance by animals that forage in the soil may partially offset these negative effects of livestock, directly and/or indirectly by shifting the composition and diversity of soil microbial communities. Understanding the role of disturbance from livestock and ecosystem engineers in driving soil microbes and functions is essential for formulating sustainable ecosystem management and conservation policies.

2. We compared soil bacterial community composition and enzyme concentrations within four microsites: foraging pits of two vertebrates, the indigenous short-beaked echidna (*Tachyglossus aculeatus*) and the exotic European rabbit (*Oryctolagus cuniculus*), and surface and subsurface soils along a gradient in grazing-induced disturbance in an arid woodland.

3. Microbial community composition varied little across the disturbance gradient, but there were substantial differences among the four microsites. Echidna pits supported a lower relative abundance of Acidobacteria and Cyanobacteria, but a higher relative abundance of Proteobacteria than rabbit pits and surface microsites. Moreover, these microsite differences varied with disturbance. Rabbit pits had a similar profile to the subsoil or the surface soils under moderate and high, but not low disturbance.

4. Overall, echidna foraging pits had the greatest positive effect on function, assessed as mean enzyme concentrations, but rabbits had the least. The positive effects of echidna foraging on function were indirectly driven via microbial community composition. In particular, increasing activity was positively associated with increasing relative abundance of Proteobacteria, but decreasing Acidobacteria.

5. Our study suggests that soil disturbance by animals may offset, to some degree, the oft-reported negative effects of grazing-induced disturbance on soil function. Further, our results suggest that most of this effect will be derived from echidnas, with little positive effects due to rabbits. Activities that enhance the habitat for echidnas or reduce rabbit populations are likely to have a positive effect on soil function in these systems.

Key-words: animal foraging, arid, cyanobacteria, disturbance, ecosystem engineering, grazing, microbes, rabbits, soil disturbance

Introduction

Australia has suffered one of the highest rates of global mammal extinctions and range reductions over the past

200 years since European settlement (Johnson 2006). Most losses have been for small-to-medium (50–5500 g) herbivorous and insectivorous marsupials such as bandicoots, bettongs and bilbies (Johnson 2006) that foraged in the soil and had large impacts on plants and soils (Eldridge & James 2009). Despite these declines, and local extinctions, one native engineer, the short-beaked echidna

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(*Tachyglossus aculeatus* Shaw) has managed to survive over extensive areas of the continent (Strahan 1995).

Semifossorial ecosystem engineers such as echidnas have a major effect on resource availability through their non-trophic soil disturbing activities. Semifossorial animals directly alter plant germination, litter capture and infiltration through their digging activities (Eldridge & James 2009; James, Eldridge & Hill 2009) and have trophic, flow-on effects by preying on organisms such as invertebrates (e.g. Silvey, Hayward & Gibb 2015). At the ecosystem level, their digging activity can alter the flow of critical resources such as soil, organic matter, litter, water and seed, influencing ecosystem processes as broad as habitat availability, nutrient cycling, hydrology and productivity (Crooks 2002).

In addition to the loss of small-to-medium soil foraging mammals, invasion by the European rabbit (*Oryctolagus cuniculus* Linn.) and overgrazing by European livestock have resulted in substantial ecosystem disturbance. The European rabbit is a medium-sized feral, herbivorous lagomorph (Strahan 1995) that also forages in the soil, but constructs fewer, smaller foraging pits containing lower levels of plant available nutrients and a less diverse native plant assemblage than the pits of native animals (Eldridge & James 2009; James *et al.* 2011). European rabbits are predominantly herbivorous, unlike echidnas, whose diet is almost entirely termites and ants.

Australia has been grazed by European domestic livestock for only about 200 years. Consequently, the soils and vegetation have not co-evolved in the presence of domestic livestock. Livestock grazing changes plant species composition and therefore habitat for organisms, alters plant structure, increases soil erosion and modifies soil hydrological and biogeochemical processes (Fleischer 1994; Lunt *et al.* 2007; Eldridge *et al.* 2015) and microbial community structure (e.g. Bardgett & van der Putten 2014; Maestre *et al.* 2015). The direct and indirect effects of livestock grazing can have pronounced legacy effects on soils that reduce their capacity to maintain key ecological processes associated with organic matter decomposition and nutrient flows (Fleischer 1994). However, we know very little about the relative effects of human-induced disturbances, largely caused by grazing, on soil microbial communities and soil function.

Soil microbes are among the most abundant and diverse organisms on Earth and sustain critical processes associated with the cycling of carbon, nitrogen, phosphorus and other minerals. In highly functional systems, essential nutrients and minerals are synchronized in time (phase coherence) and matching in quantity (coupled), that is they occur in correct stoichiometric proportions. When systems become dysfunctional, such as through overgrazing, these relationships break down and resources become uncoupled. The ability of a system to adequately perform these functions simultaneously has been defined as multifunctionality (Lefcheck *et al.* 2015; Delgado-Baquerizo *et al.* 2016). We have little understanding of the extent to which any positive

effects of ecosystem engineers on soil function might partially offset any negative effects brought about by grazing-induced disturbance. Moreover, we know relatively little about the degree to which any engineering effects are species specific (i.e. native vs. exotic engineers, though see James *et al.* 2011) and thus whether a change from echidnas to rabbits might alter soil microbial communities and soil biochemical function.

Here, we compare how microbial community composition and enzyme activity vary among the foraging pits of echidnas and rabbits, and surface and subsurface soils, along a gradient in grazing-induced disturbance in an arid woodland. Although soil disturbance by ecosystem engineers in drylands has been shown to have relatively large effects on plant community structure (Whitford & Kay 1999; Eldridge & James 2009; James *et al.* 2011), its effects on soil microbial communities and therefore function are poorly known (though see Eldridge *et al.* 2015). Differences in effects between echidnas and rabbits could be due to the structural differences between their foraging pits. Echidna pit volumes are substantially greater, and they have been shown to trap and retain more organic matter than rabbit pits (Eldridge *et al.* 2012). Larger pits would be expected to have a greater range of microenvironments such as litter depths and soil moisture levels, resulting in greater niche partitioning, thereby supporting a richer microbial community. The greater volume of litter in echidna pits might also be expected to be correlated with a greater variety of plant forms with different chemistries (e.g. C : N ratios, phenols, cellulose and lignin concentrations; Bardgett, Freeman & Ostle, 2008) and more varied substrate quality, thereby supporting a richer bacterial community.

We compared microbial community composition and function (activity of four enzymes) in echidna and rabbit foraging pits with the surface and subsurface. The four enzymes are associated with the degradation of sugar (cellobiosidase) and cellulose (β -glucosidase), the cycling of nitrogen (*N*-acetyl- β -glucosaminidase), and the release of inorganic phosphorus from organic matter (phosphatase). Together these enzymes are a good proxy of the ability of soils to effectively carry out processes that make essential nutrients available to plants. We hypothesized that (i) the pits constructed by the native engineer promoted highly functional soils and microbial communities compared with those of the exotic rabbit; and (ii) the positive effects of the pits constructed by native mammal engineers can offset negative impacts of livestock grazing on soil functioning directly (*via* pit traits) and indirectly, via the soil microbial community.

Materials and methods

THE STUDY AREA

Our study was carried out at three large pastoral properties in western New South Wales, Australia (33°24'S, 141°21'E), with contrasting management histories and degrees of grazing-induced

disturbance (Table S1, Supporting Information). The properties ranged in grazing-induced disturbance from low (conservation reserve) to moderate (Research Station) to high (current pastoral property; Smith, Eldridge & Throop 2012). The low disturbance site was at the Australian Wildlife Conservancy's Scotia Sanctuary, where domestic livestock grazing has historically been very low. Rabbits and low densities of feral predators were present at Scotia outside large exclosures that supported low densities of native animals such as the burrowing bettong (*Bettongia lesueur*) and the greater bilby (*Macrotis lagotis*). The moderate disturbance site was a large paddock within the Nanya Research Institute (University of Ballarat) that was moderately grazed until 1995, after which all domestic herbivores were removed. Although it is managed as a research station, moderate densities of feral goats are still present on the property, but the area has recovered substantially since the mid-1990s (Westbrooke 2012). The site subjected to high disturbance was a large nearby pastoral property where domestic livestock grazing intensity is often very high, but is typical of many grazing properties in semi-arid eastern Australia (Smith, Eldridge & Throop 2012).

The soils are dominated by coarse-textured Quaternary alluvium distributed across two main geomorphic systems: (i) low, west-east-trending sand dunes with calcareous and siliceous sands (Rudosols) and (ii) interdunal swales and plains of loamy, calcareous earths (Calcarosols). Our study was confined to the swales and plains dominated by open woodlands with belah (*Casuarina pauper*), mallee (*Eucalyptus* spp.) and sugarwood (*Myoporum platycarpum*), and supporting a variable cover of shrubs (*Senna artemisioides*, *Dodonaea viscosa*, *Eremophila sturtii*) and perennial grasses (e.g. *Austrostipa* spp.). The climate in the area is semi-arid, with cool winters (mean ≤ 17 °C) and hot summers (mean 30 °C). Rainfall is highly spatially and temporally variable and averages 243 mm yr⁻¹, with an almost even distribution of rainfall throughout the year.

FIELD SAMPLING

At each of the low, moderate and high disturbance sites (hereafter 'sites'), we selected two locations, which were separated from each other by a distance of about 2 km (Fig. 1). At the first location, we searched for an echidna pit, then walked 10 m in a predetermined direction to locate the closest rabbit pit, another 10 m for a surface sample and a further 10 m for a subsoil sample. This procedure was repeated until we had sampled five each of echidna and rabbit pits, surface and subsoils from an area of about 2500 m² (50 by 50 m). The five samples were then bulked, to produce one sample for each of the four microsites. This process was repeated twice more at the first location (A) in two additional 2500-m² quadrats. These three large sampling areas were separated by distances of about 500 m. This resulted in three samples for each of the four microsites. We used a similar protocol at the second location (B), but sampled only two 2500-m² areas. Overall therefore, we collected a total of 20 samples: five echidna, five rabbit, five surface, five subsoil samples for each of the three sites (Fig. 1).

To sample soils and microbes, we collected a small amount of soil from (i) the uppermost 1 cm of an undisturbed soil surface (surface), (ii) the uppermost 1 cm of soil from within the foraging pit constructed by a rabbit and, (iii) an echidna, and (iv) subsoil collected at the depth of 8 cm beneath an area of undisturbed soil. This depth is to the average depth of rabbit or echidna foraging pits

(Eldridge *et al.* 2015) and represents a procedural control. Approximately 5 g of soil was collected from all microsites using a sterilized spatula. Soils were stored on ice before being transported back to the laboratory. All analyses were performed on a subsample of the homogenized soil samples. Animals tend to forage close to the canopies of trees and shrubs where there are likely to be more resources (e.g. Eldridge *et al.* 2012). All samples were taken from the edge of the canopy of woody plants, thus avoiding positions directly beneath the canopy. This removed any potential bias associated with differential effects on microbial communities in open areas compared with woody canopies, that is any 'fertile island' effect (Gallardo & Schlesinger 1992).

In order to reduce any confounding effect that different pit age (Eldridge *et al.* 2012, 2015) or pit size might have on microbial community composition or soil enzyme concentrations, we collected all soil samples from pits of a similar age, about six months old. We have been monitoring foraging pits at the Scotia Sanctuary quarterly for more than three years and can estimate pit age roughly by measuring attributes such as the mass of litter trapped organic matter, pit shape and the presence of loose surface material around the pit margins (Eldridge *et al.* 2012).

MOLECULAR ANALYSES

DNA was extracted from 50 mg of soil material using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's specifications. Amplicon sequencing of the 16S rRNA V1-3 region was performed using dual-indexed MiSeq compatible primers 27f and 519r. Amplicons were pooled using the SequalPrep Normalization Plate Kit (ThermoFisher Scientific, Waltham, MA, USA). Paired-end (2 × 300 bp) sequencing of pooled amplicons was performed on the MiSeq platform at the Ramaciotti Centre for Genomics, UNSW, Australia. Sequence reads were analysed using MOTHUR v1.22 (www.mothur.org) software package (Schloss *et al.* 2009). Initial quality processing of sequence reads was performed ensuring an average Q > 30 over an average window size of 50 bases. We removed sequences <200 bp containing ambiguous bases and homopolymers longer than 8 bp in length. The remaining sequences were aligned to the bacterial 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 Greengenes May 2013 release data set (DeSantis *et al.* 2006). Taxonomic assignment was made at each level, given a bootstrap value >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 data sets were removed. Subsampling was performed at a level of 16 000 sequences per sample. 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 neighbour algorithm (Schloss & Westcott 2011). The identity of each OTU defined at 0.03 a distance threshold was obtained from the consensus of each sequence within that OTU at a confidence threshold of 80.

LABORATORY ANALYSES

Phosphatase activity was measured by determination of the amount of p-nitrophenol (PNF) released from 0.5 g soil after

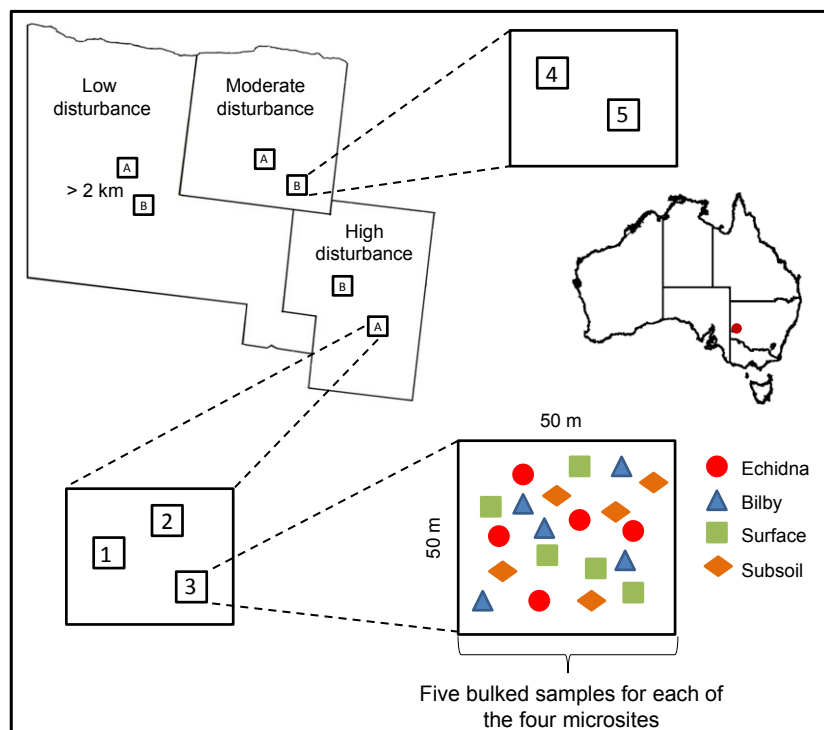


Fig. 1. Layout of the sampling design at the three locations along the gradient and the location of the study site in eastern Australia.

incubation at 37 °C for 1 h with the substrate p-nitrophenyl phosphate in MUB buffer (pH 6.5; Tabatabai & Bremner 1969; Bell *et al.* 2014). The activity of β -glucosidase was assayed following the procedure for phosphatase, but using p-nitrophenyl- β -D-glucopyranoside as a substrate and tris hydroxymethyl aminomethane instead of NaOH when preparing the buffer (Tabatabai 1982). *N*-acetyl-D-glucosamine and cellobiosidase activities were measured by fluorometry using 2.75 g of soil, as described in Bell *et al.* (2014).

STATISTICAL ANALYSES

We used two different approaches to assess how microbial communities changed along a disturbance gradient and among the four microsites: (i) a combination of univariate–multivariate approaches and (ii) systems-based (structural equation) modelling (SEM).

For the first analyses, we used permutational, multivariate analysis of variance (PERMANOVA; Anderson, Gorley & Clarke 2008) to examine differences in the composition of the 16S rRNA OTUs in relation to position within the disturbance gradient, microsite and their interaction. The analysis used a nested structure, with microsites (echidna and rabbit foraging pit, surface soils, subsoil) nested within the three levels of disturbance. Pairwise, *a posteriori* comparisons were made, where necessary, using a multivariate analogue of the *t* statistic, the probability levels being obtained by permutation. We then used non-metric multidimensional scaling ordination (nMDS) to derive the first two dimensions of the nMDS biplot and Shannon's diversity index as three measures of microbial community composition for use in a structural equation model (described below). The nMDS ordination was performed on log-transformed OTU abundance data using the PRIMER/PERMANOVA statistical package for Windows (PRIMER-E Ltd., Plymouth Marine Laboratory,

Plymouth, UK) using the Euclidean distance. The two-dimensional solution provided a suitable representation of the bacterial data (stress = 0.17).

We calculated an RII index (Armas, Ordiales & Pugnaire 2004) to explore overall effects of three of the four microsites (echidna, rabbit, surface) on microbial richness. The index provides the relativized difference between echidna pit, rabbit pit or surface soil and the corresponding subsoil sample. Our index was calculated as $(\text{Micr}_{\text{EC}} - \text{Micr}_{\text{SS}})/(\text{Micr}_{\text{EC}} + \text{Micr}_{\text{SS}})$, where Micr_{EC} and Micr_{SS} are values of microbial abundance in echidna pit soil and the subsoil, for a given site, respectively. Values of this index ranged from -1 to $+1$, with positive values indicating increases in microbial abundance within the pits of echidnas or rabbits, or on the surface, compared with the subsoil. Negative RII values indicate the opposite. An RII index was also calculated for rabbit pit soil and surface soil. We then compared differences in the average (across site) values of RII for echidna, rabbit and surface soils for those phyla that contributing >95% of total OTU abundance.

We explored potential relationships among the nine most abundant phyla, the two nMDS axes and the Shannon diversity index and concentrations of the four enzymes with Pearson's correlations. The degree of association of OTUs with microsite was assessed using Indicator Species Analysis with R ('Indicspec'; De Cáceres, Legendre & Moretti 2010). Indicator values combine information on relative abundance and frequency of OTUs, and the indicator value is maximal (IV = 100%) when all individuals of a given OTU are restricted to a particular microsite (e.g. echidna pit), and all samples from the particular microsite contain an occurrence of that OTU. Data (at the OTU level) were randomized among the microsites and a Monte Carlo randomization procedure performed with 1000 iterations in order to determine the statistical significance of the indicator values.

NETWORK ANALYSES

For network analysis, Pearson's correlation analyses were performed on samples subdivided on the basis of microsite ($n = 15$). For each of the four microsites across the gradient, we randomly selected a reduced set of OTUs that occurred across at least five samples and were collectively represented by at least 100 sequence reads. Pearson correlation coefficients and corresponding P -values were generated using the 'Hmisc' package (R Core Team 2012). Significant correlations were corrected for false discoveries with Bonferroni corrections. We reported the number of OTUs selected for these two analyses from each microsite, the number of OTUs involved in significant correlations and the number of significant correlations. Scale-free network visualization of these significant correlations was made using Cytoscape. The network analyser package contained within Cytoscape was used to generate metrics related to clustering, density and centralization, for each of the four microsites (Assenov *et al.* 2008).

STRUCTURAL EQUATION MODELLING

Structural equation modelling (Grace 2006) was used to explore the direct and indirect impacts of two exogenous variables, that is disturbance (high, medium, low) and microsite (echidna pits, rabbit pits, surface soils), and two endogenous variables (pit volume, microbial community) on soil function. The microbial community attribute comprised two components: (i) the composition of the microbial community (represented as the first two dimensions of the nMDS biplot), and (ii) Shannon's diversity index (calculated using operational taxonomic units; OTUs). The combined effect of these two components was represented as a composite variable, which allowed us to collapse the effects of these conceptually related variables into a single combined effect, aiding the interpretation of model results (Grace 2006). Our measure of function was the arithmetic mean of the standardized (z -transformed) concentration of the four soil enzymes. This approach, of averaging various functions to provide a multifunctionality index (*sensu* Maestre *et al.* 2012) is gaining widespread acceptance in ecology and soil science (Bradford *et al.* 2014; Wagg *et al.* 2014; Lundholm 2015) because it conveys the notion that complex processes can be distilled into a more easily translatable message that is not apparent when only a single function is used. It also provides a logical and straightforward measure of the average capacity of communities to sustain multiple functions simultaneously (Byrnes *et al.* 2014).

Structural equation modelling allowed us to test the plausibility of a causal model, based on *a priori* information (*a priori* model Fig. S1). In that model, we predicted that disturbance would have direct suppressive effects on function, as well as indirect effects, mediated by changes in the composite variable 'microbes'. We also predicted direct effects of microsites on function, and indirect effects, mediated by changes in pit volume and microbes. As previously indicated, we expected that echidnas would have a large positive effect on pit volume, given that their pits are substantially larger than those of rabbits and therefore trap more resources.

Before fitting empirical data to our *a priori* models, we examined the distributions of our endogenous variables, tested their normality and \log_{10} -transformed them where necessary. Overall goodness-of-fit probability tests were performed to determine the absolute fit of the best models. The goodness-of-fit test estimates

the long-term probability of the observed data given the *a priori* model structure. Thus high probability values indicate that these models are highly plausible causal structures underlying the observed correlations. Separate models with the strongest measures of fit [e.g. low χ^2 , high goodness-of-fit index (GFI), and high normal fit index (NFI)] were interpreted as showing the best fit to our data. We also calculated the standardized total effects of disturbance, echidna, rabbit and surface soils, pit volume, and nMDS1, nMDS2 and Shannon's index on function. This was calculated by summing all direct and indirect pathways between these variables and function. All SEM analyses were conducted using AMOS Software version 22 (IBM SPSS, Chicago, IL, USA).

Results

MICROSITE EFFECTS ON MICROBIAL COMMUNITY COMPOSITION IN RELATION TO LIVESTOCK DISTURBANCE

Compared with the subsoil, bacterial phyla varied across the different microsites, as assessed with the RII index (Fig. 2). Overall, the effects were similar across the disturbance gradient (Fig. S2). Cyanobacteria, Bacteroidetes and Proteobacteria were generally more abundant in the pits and surface than the subsoil, but Actinobacteria, Acidobacteria and Firmicutes showed the opposite response. Greater abundance of Cyanobacteria and Bacteroidetes in the surface and pits compared with the subsoil was most pronounced under low disturbance. Interestingly, echidna pits showed a much higher positive effect on RII Proteobacteria and negative effect on RII Cyanobacteria and Acidobacteria than rabbit pits. The RII for Cyanobacteria in echidna pits declined markedly with increases in disturbance (data not shown).

We found only slight differences in microbial community composition along the disturbance gradient (P

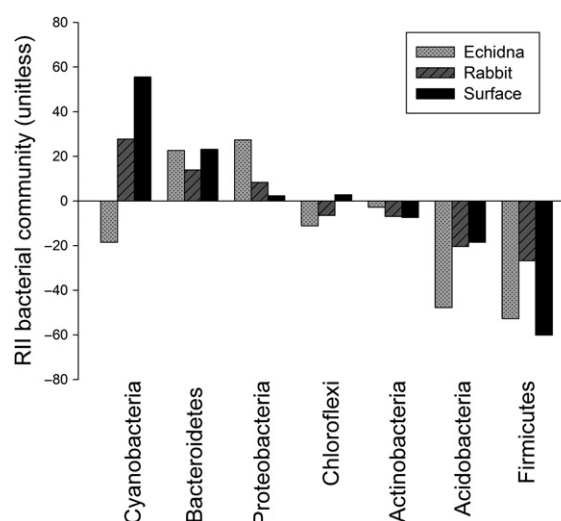


Fig. 2. Effects of microsite, as measured with the RII index, on bacterial composition (relative abundance of main phyla) averaged across the gradient. The seven phyla accounted for 98% of total OTU abundance.

(PERMANOVA) = 0.074). However, there were strong differences in the soil microbial communities among the different microsites ($F_{3,45} = 4.80$, $P = 0.001$) and a significant disturbance by microsite interaction (Pseudo- $F_{6,45} = 1.54$, $P = 0.009$). For example, all microsites differed in their bacterial composition under low disturbance ($t = 1.55$ to 1.99 , d.f. = 7, $P < 0.012$), but as disturbance increased, rabbit pits had a similar profile to the subsoil (moderate disturbance; $t = 0.89$, d.f. = 7, $P = 0.53$) or the surface soils (high disturbance; $t = 1.37$, d.f. = 7, $P = 0.073$).

Taxa from phylum Actinobacteria and class Alphaproteobacteria were significant indicators of echidna foraging pits, and to a lesser extent, the subsoil (Indicator Species Analysis; Table S2). Only two genera, *Modestobacter* (an Actinobacteria) and *Nitrosomonadaceae* (a Proteobacteria), were moderately indicative of rabbit foraging pits (Indicator Value: IV = 0.81–0.82). Cyanobacteria were moderately indicative of taxa present on the surface (e.g. Streptophyta, Xenococcaceae, Phormidiaceae), while Actinobacteria (e.g. *Acidimicrobiales*, *Actinobacteria*, *Virgisporangium*, *Mycobacterium*, *Streptomyces*, *Micrococcales*, *Rubrobacter* and *Gaiellaceae*; IV = 0.81–0.83), and to a lesser extent Firmicutes (*Bacillus*, *Ammoniphilus*, *Brevibacillus*; IV = 0.85–0.87), were indicative of subsoil environments (Table S2).

Across all microsites, a consistently large number of OTUs was involved in significant correlations (254.2 ± 19.6). Compared with surface soils, the mean number of neighbours was greatly reduced among the networks representing echidna foraging pits and subsoils. Echidna pit soils exhibited the lowest values of centralization, density and clustering (Fig. S3). Subsoil samples exhibited values of clustering, density and centralization lower than surface samples, but the values were not as low as those recorded from echidna pit soils. For rabbit pit soils, the mean numbers of neighbours, clustering and density were substantially greater than those of surface soils though centralization was lower (Table 1).

MICROSITE EFFECTS ON SOIL FUNCTIONS IN RELATION TO LIVESTOCK DISTURBANCE

Concentrations of β -glucosidase, *N*-acetyl-L-glutamine (NAG) and phosphatase were always greatest in echidna foraging pits ($F_{3,45} > 31.21$, $P < 0.001$), and there were some subtle differences in β -glucosidase and phosphatase among the other microsites along the gradient (disturbance \times microsite interaction: $F_{6,45} > 2.73$, $P < 0.024$;

data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.3936j>). Increasing soil enzyme activity was positively associated with increasing Bacteroidetes and to a lesser extent Proteobacteria, but decreasing Acidobacteria (Table 2).

STRUCTURAL EQUATION MODELLING

Before conducting SEM analyses, we simplified our microbial community metrics using multivariate analyses (nMDS). The first axis of the nMDS on bacterial OTUs was strongly positively correlated with Proteobacteria (Pearson's $r = 0.64$; $P < 0.001$) and Bacteroidetes (Pearson's $r = 0.56$; $P < 0.001$), but negatively correlated with five phyla, particularly Chloroflexi (Pearson's $r = 0.65$; $P < 0.001$) and Acidobacteria (Pearson's $r = 0.54$; $P < 0.001$; Table 2). The second nMDS axis was positively related to Proteobacteria (Pearson's $r = 0.59$; $P < 0.001$) and negatively related to Acidobacteria (Pearson's $r = 0.49$; $P < 0.001$), Gemmatimonadetes (Pearson's $r = 0.39$; $P = 0.030$) and Firmicutes (Pearson's $r = 0.31$; $P = 0.035$).

Our structural equation model explained 69% of the variance in soil function and showed a strong fit with the data ($\chi^2 = 1.55$, d.f. = 7, $P = 0.63$, NFI = 0.998 and GFI = 0.999). The model indicated (i) a slightly negative total effect of livestock disturbance on ecosystem function, (ii) a strong positive direct effect, and an indirect effect of echidna foraging on function, via changes in microbial community composition and richness, (iii) no direct effect of rabbit pits on function, and varied effects of rabbit pits on function mediated by changes in either microbial composition (increases) or richness (declines), (iv) a weak positive effect of echidnas on pit volume, but reductions by rabbits, and (v) an indirect positive effect of echidnas on function mediated by changes in both pit volume and microbial community composition [Fig. 3]. Echidna foraging had the strongest overall effect on function (standardized total effect (STE) = 0.58] followed by microbial community composition (nMDS2; STE = 0.39). The total effect of rabbit foraging pits and disturbance were extremely low (STE = 0.03 and 0.07, respectively).

Discussion

We examined microbial community composition along a gradient in grazing-induced disturbance (Eldridge *et al.* 2012; Smith, Eldridge & Throop 2012), predicting that

Table 1. Metrics obtained from analysis of scale-free microbial networks of bacterial communities among the four microsites along the gradient, independent of disturbance level. Values in parentheses represent the number of OTUs selected for the analysis

Microsite	OTUs	Edges	Mean number of neighbours	Clustering coefficient	Density	Centralization
Echidna	251 (279)	868	6.9	0.46	0.03	0.06
Rabbit	266 (269)	2897	21.8	0.73	0.08	0.12
Surface	272 (282)	2097	15.4	0.57	0.06	0.14
Subsoil	228 (250)	1034	9.1	0.51	0.04	0.08

Table 2. Pearson's *r* correlations among the nine most abundant phyla, the two nMDS axes and the Shannon diversity index (our measure of the microbial community), and the four enzymes

Phylum	nMDS1	nMDS2	Shannon diversity	BG	NAG	CB	Phos
Actinobacteria	ns	ns	0.43	ns	ns	ns	ns
Proteobacteria	0.64	0.59	-0.40	0.40	0.56	ns	ns
Cyanobacteria	ns	ns	-0.33	ns	ns	ns	ns
Bacteroidetes	0.56	0.27	ns	0.42	0.44	0.36	0.33
Acidobacteria	-0.54	-0.49	0.60	-0.40	-0.49	ns	-0.33
Firmicutes	-0.40	-0.31	ns	ns	ns	ns	ns
Chloroflexi	-0.65	ns	0.47	ns	-0.48	ns	ns
Planctomycetes	-0.46	ns	0.50	ns	0.35	ns	ns
Gemmatimonadetes	-0.36	-0.39	0.58	ns	ns	ns	ns

ns, not significant.

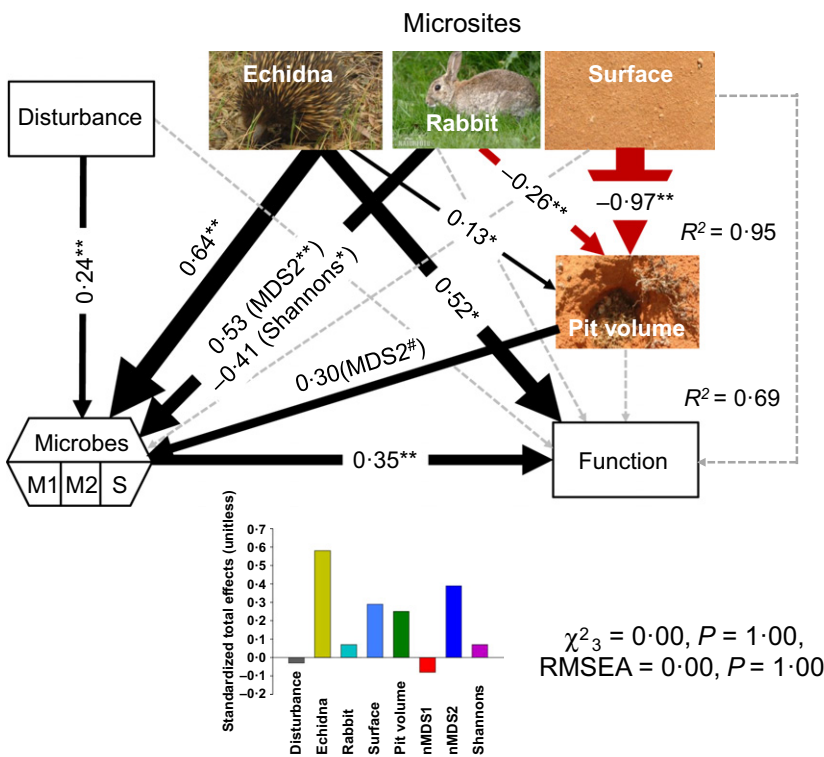


Fig. 3. Structural equation model for our study system. Boxes indicate measured variables and the hexagon a composite variable 'microbes' representing the combined effects of the first (M1) and second (M2) dimension of an nMDS biplot, and Shannons diversity, based on bacterial OTUs. Arrows indicate the effect of one variable upon another, with thicker arrows representing a stronger effect, either positive (unbroken black line), negative (broken red line) or non-significant (broken grey lines) effects. * $P < 0.05$; ** $P < 0.01$; # $P = 0.10$. Rabbit image courtesy of Nature Photo (<http://www.naturephoto-cz.com/wild-rabbit-photo-6264.html>).

positive effects from native engineering mammals would partially offset any changes in composition with increasing disturbance. Mammalian engineers were a major driver of microbial composition, but there were no clearly defined effects of the disturbance gradient. Our study showed that the foraging pits of echidnas were microbially and enzymatically more active than surface soil, subsoil or the pits of exotic rabbits, which now occupy large areas of Australia's drylands. Further, differences in the microbial signature between rabbit foraging pits and the subsoil dissipated with increasing disturbance. Most importantly, mammal engineers were able to offset any negative impact from livestock grazing on soil functioning in this study. Our results suggest that management practices that lead to changes in the relative proportion of these microsites, for example by enhancing habitat quality

and therefore densities of echidnas, or by reducing the rabbit populations, are likely to have substantial effects on microbial community composition and the processes that they mediate.

The activity of enzymes has been used widely as a proxy of organic matter degradation (Sinsabaugh *et al.* 2008). In our study, we measured the rate at which specific substrates were degraded by the activity of particular enzymes. Recent studies suggest that enzyme activity is largely controlled by specific microbial communities in the soil via shifts in the abundance of functional genes linked to particular microbial taxa (e.g. Trivedi *et al.* 2016). Thus, changes in microbial composition resulting from disturbance by mammals can markedly alter both microbial community composition and therefore ecosystem functioning. A critical question that remains to be

answered is whether indigenous engineers have a more positive effect on microbial communities and ecosystem function than exotic species. Our study supports current ecological theory suggesting that the invasion of exotics will produce fundamental shifts in ecosystem processes and functions compared with their native analogues (Mack *et al.* 2000; Ehrenfeld 2010).

Echidna pits had the greatest levels of four soil enzyme functions associated with the processing, stabilization and destabilization of soil organic matter and nutrient cycling in terrestrial ecosystems (Bell *et al.* 2014). Construction of foraging pits by echidnas therefore increases overall nutrient demand by soil micro-organisms. Consistent with these findings, our SEM models indicated strong direct and indirect effects of foraging by echidnas, our native engineer, on soil function but a relatively small and inconsistent effect on function of the rabbit, our exotic engineer. The positive effects of echidna pits on soil function were indirectly driven by a pit trait (pit volume) and microbial communities in our model. Part of the echidna effect was due to the mediating effect of pit volume, which relates to the capture, storage and decomposition of organic matter, which drives the productivity of semi-arid systems (Yonker *et al.* 1988). Echidna pits are moderately large, circular-shaped depressions that are substantially deeper than rabbit pits. Their large capacity means that they can capture and store large quantities of litter, organic matter, thus enhancing their capacity to influence soil nutrient contents (James, Eldridge & Hill 2009), water infiltration or soil moisture retention (Eldridge & Mensinga 2007; Eldridge & Whitford 2009). Supporting this notion, we detected a strong positive effect of pit volume on cellobiosidase (path coefficient = 0.97; Fig. S4c), the enzyme responsible for the degradation and processing of cellulose (Bell *et al.* 2014).

Moreover, our model showed a positive effect of echidna foraging on soil function via microbial community composition (i.e. nMDS2 – second axis of a nMDS), which was positively related to relative abundance of Proteobacteria and Bacteroidetes. Thus, echidna pits promoted the relative abundance of highly functional microbial groups such as Proteobacteria and Bacteroidetes (Trivedi, Anderson & Singh 2013). These groups are positively related to multiple soil enzyme activities in this study, with substantial roles in phosphorus mineralization, starch and cellulose degradation, and the breakdown of chitin. Chitin is a component of some soil microarthropods and the cell walls of fungi. Strong relationships among the relative abundance of the main taxa and enzyme function, particularly where these taxa are correlated with multiple functions, do not necessarily imply causation. Nevertheless, three lines of evidence support the notion that the positive effects from these microbes on soil function are critical components of the foraging pits of echidnas: (i) the presence of positive linear correlations between microbial taxa and enzyme activity, (ii) greater relative abundance of Proteobacteria and

Bacteroidetes groups associated with echidna pits, and (iii) previous literature highlighting these groups of microbes as highly functional microbial communities (Fierer, Bradford & Jackson 2007; Trivedi, Anderson & Singh 2013).

Our network analysis revealed that echidna pits had reduced modularity, consistent with an increased capacity to assimilate organic matter. Across the four microsites, there were marked differences in the number of correlations among different abundant OTUs. The greatest effect was seen in echidna pits where the average number of correlations per node was substantially reduced relative to surface soils. This reduced modularity was associated with decreases in the clustering coefficient, density and centralization of the resulting network (Eldridge *et al.* 2015). Modularity in network structures reflects both the capacity of the community to respond to short-term (reactive) and long-term (resilience) perturbations. Highly modular networks reflect more resilient systems with a dampening of any deviations from equilibrium (Ruiz-Moreno, Pascual & Riolo 2006). Subsequently, systems described by low modularity, such as our echidna pits, may be more susceptible to nutrient amendment with the microbial community moving towards another equilibrium. In support of this notion, echidna pits seem to promote fast-growing copiotrophic microbial communities such as Alphaproteobacteria and Bacteroidetes (Trivedi, Anderson & Singh 2013), which could break the coalescence of the microbial community by taking advantage of the beneficial conditions linked to echidna pits. The relatively moderate and positive path coefficient from pit volume to our measure of microbial composition (nMDS2) suggests that a link between the physical size of the pits (volume, and therefore width and depth) and environmental quality conducive to the dispersal of microbes associated with organic matter decomposition. Larger foraging pits from echidna (e.g. more litter and organic matter) may then represent an oasis for fast-growing and highly functional microbial communities from arid regions.

Unlike our results from echidna pits, the soil from rabbit pits had a microbial profile similar to the subsoil and was characterized by a greater relative abundance of Cyanobacteria, and to a lesser extent, Firmicutes and Chloroflexi. Increased cyanobacteria in rabbit pits might be expected for two reasons. First, rabbit-induced disturbance leads to the burial, rather than removal, of biocrust taxa, of which cyanobacteria are a major component (e.g. Rogers 1989). Secondly, rabbits tend to forage in open areas where biocrust cover is greater, compared with sites under tree and shrub canopies where echidnas often forage (Eldridge *et al.* 2012). Rabbit pit soils were similar to the subsoil in that there was an increase in the relative abundance of cellulolytic Bacillales (Firmicutes) (Medina *et al.* 2003) relative to surface soils. Rabbits tend to defecate in the pits, either during foraging or when attracted to previously disturbed soil. While rabbit faeces support a rich variety of bacteria (Abecia *et al.* 2005), many of

which are novel lineages, there is no evidence that these are dominated by Bacilli (e.g. Straw 1988; Abecia *et al.* 2005). Thus, unlike echidna foraging pits where exogenous recruitment is apparent, the impact of rabbit foraging is limited to exposure of the subsoil, with none of the ecosystem benefits that foraging by native soil disturbing animals provide (e.g. more litter, greater enzyme activity, higher soil C and N; James, Eldridge & Hill 2009; Eldridge & James 2009). Pit construction by rabbits is therefore analogous to soil disturbance by livestock, with few positive ecosystem benefits.

IMPLICATIONS FOR THE MANAGEMENT OF DRYLANDS

The positive total effect of echidna disturbance on soil functions (0.58) was eight times greater than the negative total effect reported from livestock disturbance (−0.07). Thus, our study provides evidence that the microsites constructed by native mammalian engineers can largely offset the negative impacts from livestock disturbance, by creating ecological refugia that promote both soil functions and highly functional microbial communities. Practices that alter the relative proportion of different microsites are likely therefore to have marked effects on ecosystem processes associated with the processing of nitrogen, carbon and phosphorus. We have previously shown that the foraging pits of echidnas provide a mechanism for coupling of sediment, seed and water in drylands (Eldridge 2011). The construction of foraging pits by echidnas may help to buffer the effects of increasing aridity on soil function by hosting microbial communities that promote processes associated with the sequestration and availability of soil carbon (Fierer, Bradford & Jackson 2007; Delgado-Baquerizo *et al.* 2016). Conversely, processes associated with rabbit-disturbed soil, which harbours microbial communities similar to subsoil, will have negative feedback processes on the soil. Combined with the removal of vascular plant cover, removal of woody plants and surface destabilization, this is likely to lead to conditions of increasing dryness consistent with increasing aridity (Delgado-Baquerizo *et al.* 2013).

The density of echidna foraging pits at the degraded end of the gradient has previously been shown to be substantially less than that at undisturbed sites (237 *cf.* 13 908 pits ha^{−1}; Huang 2007), and this is likely due to sparser vegetation cover and fewer resources (Eldridge *et al.* 2012). Thus, overgrazing is likely to reduce habitat quality for echidnas and lead to reductions in function when results are scaled up to the landscape scale. Management of these dryland ecosystems should aim to maintain habitat quality for soil disturbing animals that have a positive effect on ecosystem functions (echidnas) while controlling the European rabbit, whose activities are associated with a dysfunctional degraded environment. Our findings provide novel evidence that indigenous mammalian engineers are key drivers of distinctive soil microbial

communities and function, and emphasize the need to establish effective policies to protect native mammalian engineers as a key community that provide further protection to microbial communities and ecosystem functioning in arid regions.

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Data accessibility

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.3936j> (Eldridge *et al.* 2016).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Current and historic data on the abundance and types of herbivores and omnivores across the gradient.

Table S2. Bacterial taxa, to the level of genus, that are significantly associated with different microsites across the entire gradient using Indicator Species Analysis.

Fig. S1. Conceptual *a priori* meta-model (*sensu* Grace 2006) for the response variable function.

Fig. S2. Effects of disturbance, as measured with the RII index, on bacterial composition (relative abundance of main phyla) averaged across the three non-subsurface microsites.

Fig. S3. Scale-free network depicting significant positive Pearson's correlations between soil enzyme activity levels and abundant 16S rRNA OTUs.

Fig. S4. Separate structural equation models for the four functions.