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Contrasting effects of two mammalian soil engineers on microbial communities

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INTRODUCTION

Species invasions in Australia have resulted in huge range reductions for many native vertebrate species such as the greater bilby (*Macrotis lagotis*; Fig. 1a) and the burrowing bettong (*Bettongia penicillata*) that dig in the soil by burrowing, foraging for insect larvae or excavating seed caches. These 'ecosystem engineers' (Jones *et al.* 1997) have been shown to have marked, but variable effects on soil and ecosystem processes because the foraging pits they construct trap soil, litter, faeces, seed and nutrients (Boeken *et al.* 1995; Garkaklis *et al.* 2004; Eldridge & James 2009). This soil disturbance has been shown to alter water flows and sediment movement, and the capture and retention of organic matter, thereby influencing soil nutrient pools and habitat for plants (Eldridge & James 2009), invertebrates (Eldridge & Mensinga 2007), microbes (Eldridge *et al.* 2015) and fungi (Clarke *et al.* 2015). The loss of these soil engineers can be attributed to direct predation by feral carnivores such as the domestic cat (*Felis catus*) and red fox (*Vulpes vulpes*), the introduction of exotic pests such as the European rabbit (*Oryctolagus cuniculus*) with which they compete for food and habitat, and grazing by European livestock and vegetation removal (Johnson 2006).

Not all ecosystem engineers, however, have been affected equally. The short-beaked echidna (*Tachyglossus aculeatus*; Fig. 1b) is a soil-digging marsupial that has an extensive continental distribution because it is less susceptible to predation, and can use a wider range of habitats than the range restricted bilbies. Echidnas also have marked direct and indirect effects on microbial communities and ecosystem processes resulting from their soil foraging activities (pit construction; Eldridge & Mensinga 2007; Eldridge 2011). They construct generally large (up to 30 cm diameter), shallow (5–10 cm deep), circular-shaped depressions (Rismiller 1999) at densities up to 17 000 pits ha^{-1}, moving up to 32 t ha^{-1} of soil in the process (Eldridge 2011). Their pits are substantially shallower than those of bilbies, which are cylindrical-shaped and range from 10 to 25 cm deep.

Relatively little is known, however, about the effects of different Australian mammals on soil microbial communities in drylands, which occupy about three-quarters of the land mass of the continent. These drylands are highly susceptible to increases in aridity predicted under climate change scenarios, land use intensification and increasing livestock grazing under a drier climate. Ecologists have tended to overlook the extent to which surviving engineers such as the echidna might compensate for any negative ecosystem effects of the loss of native species such as bilbies. Over the past two decades, for example, land management agencies have tended to focus their attention, and limited resources, on strategies to re-establish viable populations of locally extinct animals such as bilbies (e.g. Manning *et al.* 2015) rather than focusing on the potentially more tractable and cost-effective task of enhancing habitat for surviving native animals such as echidnas.

Little is known about how foraging by bilbies and echidnas might influence soil microbial communities, and specifically, whether their effects are complementary. Herein, we compared microbial community composition of soils within echidna foraging pits with that in bilby pits and related this to the concentrations of enzymes associated with the cycling of carbon, nitrogen and phosphorus. Because these two animals dig different shaped pits, which differ in their capacity to trap organic matter, we expected to detect a greater relative abundance of bacterial phyla associated with the cycle of carbon and nitrogen in the shallower, organic-rich echidna pits than the deeper but narrower pits of the bilby, which trap less litter.

METHODS

Study area and field sampling

Our study was carried out at the Australian Wildlife Conservancy's Scotia Sanctuary in western New South Wales,

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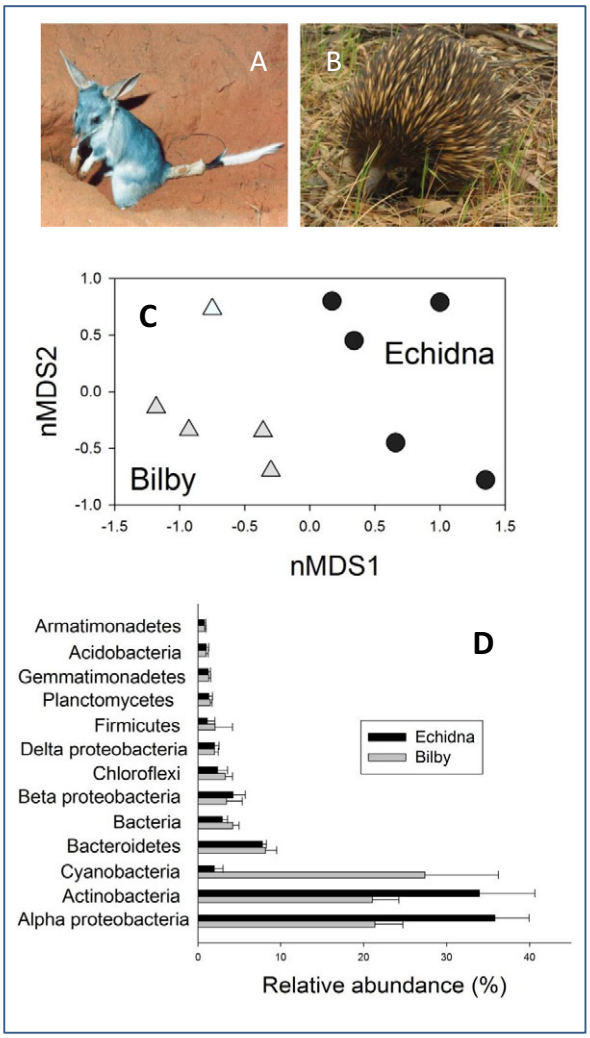

Fig. 1. (a) Short-beaked echidna (*Tachyglossus aculeatus*), (b) greater bilby (*Macrotis lagotis*), (c) 2-D nMDS biplot showing the separation of bilby and echidna foraging pits based on abundance of OTUs rarefied to 1600 reads. 2-D stress = 0.05, Permanova: $F_{1,8} = 3.09$, $P = 0.007$) and (d) mean (±SE) relative abundance of the main microbial phyla. [Colour figure can be viewed at wileyonlinelibrary.com]

Australia (33°24′S, 141°21′E). The sanctuary has large exclosures that exclude feral predators and livestock, and support low densities of native animals such as bilbies and echidnas. The soils are predominantly coarse-textured Quaternary alluvium deposited over low, west-east-trending sand dunes of calcareous and siliceous sands. Interdunal swales and plains are dominated by loamy, calcareous earths (Calcarosols). The vegetation is dominated by open woodlands with belah (*Casuarina pauper*), mallee (*Eucalyptus* spp.), variable shrub cover (e.g. *Senna*, *Dodonaea* and *Eremophila* spp.) and perennial grasses. The area is semi-arid, and rainfall is highly spatially and temporally variable and averages 243 mm year^{-1}, with an almost even distribution of rainfall.

Within the reserve where both bilbies and echidnas coexist, we collected about 5 g of soil from the uppermost 1 cm of the surface from echidna pits and bilby pits. We searched for an echidna pit, and then walked 10 m in a predetermined direction to locate the closest bilby pit and did this five times. The five bilby samples were pooled, as were the five echidna samples. This procedure was repeated another four times resulting in five pooled samples each of bilby and echidna pits. Soils were collected with a sterilized spatula, stored on ice and transported back to the laboratory. All analyses were performed on a subsample of the homogenized soil samples. We collected all soil samples from pits of a similar age, about 6 months old, based on information from detailed monitoring at the sites (Eldridge *et al.* 2012). Pit volumes and litter mass were also measured. Animals tend to forage close to the canopies of trees and shrubs where there are likely to be more resources (e.g. Eldridge 2011). 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 1995).

Molecular analyses

DNA was extracted from 50 mg of soil material using the FastDNA Spin Kit for Soil (MP Biomedicals, 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, 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 dataset (DeSantis *et al.* 2006). 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 16 000 sequences per sample. Uncorrected pairwise distances were calculated between sequence reads with the final clustering of OTUs (Operational Taxonomic Units) performed at an 0.03 distance threshold using the average neighbour algorithm (Schloss *et al.* 2011). Operational Taxonomic Units (OTUs) were defined as clusters of 97% sequence similarity.

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

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after 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.* 2013). 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.* (2013). Ammonium and nitrate were measured with auto-analysis following extraction with K_2SO_4. Labile carbon was measured following Weil *et al.* (2003). Potential relationships among the 13 most abundant phyla and the three enzymes were explored with Pearson's correlations and ANOVA. Non-metric multidimensional ordination (nMDS; PRIMER-E Ltd., Plymouth Marine Laboratory, UK; Bray-Curtis dissimilarity) was used to explore differences in OTU abundance between the pits of the two animals.

RESULTS AND DISCUSSION

We found significant differences in the bacterial communities in echidna and bilby pits at the OTU level ($P = 0.007$, Fig. 1c). In particular, three phyla, which accounted for 80% of the relative abundance of bacterial communities, were strongly associated with soil from either echidna (α-proteobacteria, actinobacteria) or bilby (cyanobacteria) foraging pits. Echidna pits were larger, containing five times more litter and had greater extracellular enzyme activities (e.g. β-glucosidase; Fig. 2). The relative abundance of actinobacteria in echidna pits was twice that in bilby pits (Fig. 2) and positively correlated with litter mass ($r = 0.61$) and phosphatase activity ($r = 0.74$; Table 1). Actinobacteria possess an impressive array of genes conducive to litter and soil organic matter breakdown, allowing them to resist stressful soil conditions and making them strong candidates to occupy niches created by echidna foraging (Trivedi *et al.* 2013). Similarly, α-proteobacteria were twice as abundant in echidna pits (Fig. 1d). α-Proteobacteria are predominantly copiotrophs that thrive in organic-rich soils rather than in mineral-rich soils (Fierer *et al.* 2007; Trivedi *et al.* 2013). Conversely, the increasing dominance of cyanobacteria corresponded to 15-fold greater ammonium concentrations, predominantly in bilby pits ($P < 0.001$; Table 1). This increased ammonium could have been due to bilby excreta deposited either directly while foraging, or blown into the pits. Unlike bilby excreta, echidna excreta comprise mainly compacted soil and invertebrate frass, and would therefore likely have low levels of nitrogen. Further, echidna scats are typically deposited in latrines (Sprent *et al.* 2006) rarely found in the pits. Cyanobacteria, either free-living or

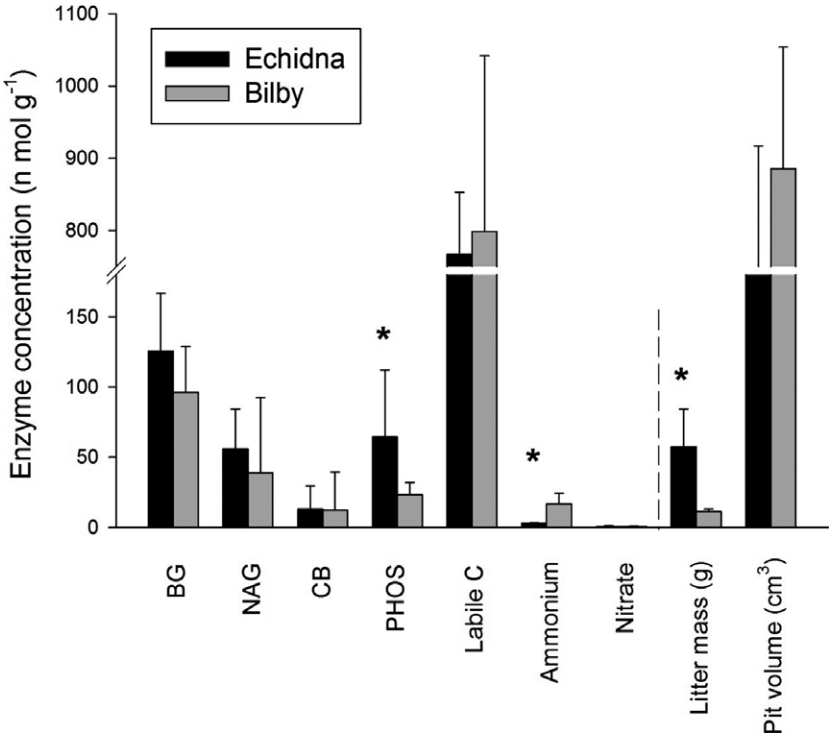

Fig. 2. Mean (±SEM) enzyme concentration (nmol g^{-1}), mass of trapped litter (g) and pit volume (cm^3) for echidna and bilby pits. BG, β-glucosidase; CB, cellobiosidase; NAG, N-acetyl-β-glucosaminidase; PHOS, phosphatase. *Indicates significant differences at $P < 0.05$.

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Table 1. Correlations (Pearson's *r*) among relative abundance of the main microbial phyla and litter mass, the four enzymes, and labile carbon (C), ammonium and nitrate; – not significant

Phylum	Litter mass	BG	NAG	PHOS	Labile C	NH_4^+	NO_3^-
Actinobacteria	0.61	–	–	0.74	–	−0.69	–
α-Proteobacteria	–	–	–	–	−0.82	−0.82	–
Cyanobacteria	–	–	–	–	0.84	0.84	–

BG, β-glucosidase; NAG, N-acetyl-β-glucosaminidase; PHOS, phosphatase.

associated with biological soil crusts are known to favour oligotrophic environments with sparse plant cover and may contribute to increase ammonia concentration in soil from N-fixation (Ochoa-Hueso *et al.* 2016).

Our results indicate that foraging pit morphology, and the capacity for pits to capture and retain organic matter, probably account for differences between the two mammalian engineers. Deep bilby pits had enzyme signatures resembling the subsoil (Eldridge *et al.* 2015) and a lower concentration of labile carbon ($r = -0.74$). The shallower but wider echidna pits (Eldridge 2011) trapped substantially more litter (Fig. 2; Travers & Eldridge 2016), and have been shown to retain more moisture than the soil surface, even after extended dry periods (Eldridge & Mensinga 2007). This would likely extend the period over which decomposition and mineralization occur. Echidnas often burrow into the nests of ants and termites, which are themselves ecosystem engineers that enhance soil physical and chemical properties through their central place foraging (Whitford & Eldridge 2013). It is probable, therefore, that some of the chemical differences that we measured in echidna foraging pits could result from the build-up of nitrogen and carbon due to invertebrate activity. The extent to which termites or ants might contribute to differences in fertility of echidna foraging pits is, however, largely unknown.

Overall, the two mammalian engineers supported different microbial life strategies; the bilby enhancing oligotrophic conditions and the echidna supporting copiotrophic conditions. The effect of their loss on ecosystem processes will depend, therefore, on the prevailing environment setting. Any loss of echidnas will reduce the potential for organic matter breakdown, thereby reducing the extent of fertile patches (Eldridge 2011) in drylands where soil nutrient availability is low (Delgado-Baquerizo *et al.* 2013) and where organic matter decomposition drives ecosystem processes (Whitford 2002). We argue that the loss of bilbies from drylands, however, is likely to be less significant because the matrix comprises extensive areas of bare soil where cyanobacteria predominate and oligotrophic phyla and enzymes are non-limiting. The loss of bilbies, however, from more mesic environments such as coastal regions or moist woodlands would likely have the opposite effect, given that the matrix is largely vegetated and soil disturbance is critical to create patches of oligotrophic soil with its associated microbial communities.

Together, our findings suggest that changes in the relative distribution and abundance of native mammals as a result of land use change and species invasion may alter microbial communities and ecosystem functions in drylands. Echidnas have not suffered the same levels of predation as other soil-disturbing animals, probably because they can exploit a wider range of habitats and the presence of a protective integument (Fleming *et al.* 2014). However, foxes and cats kill many young echidnas before they can breed (Rismiller 1999), and the use of intact road reserves by echidnas often brings them into contact with humans, resulting in substantial mortality. Programmes such as tree and shrub plantings that increase habitat complexity and connectivity for echidnas are likely to increase their survival, ultimately leading to substantial effects on litter capture, microbial community composition and processes such as decomposition and enzyme production.

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