Experimentally testing the species-habitat size relationship on soil bacteria: a proof of concept.

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Abstract

The species-area relationship is one of the most widely reported ecological theories accounting for biodiversity of plants and animals. However, we lack solid experimental data demonstrating whether this key ecological theorem also applies in the microbial world. Here, we conducted a microcosm study to evaluate the role of habitat area in driving the diversity, abundance, composition and functioning (i.e., four enzyme activities linked to organic matter decomposition) of soil bacterial communities. Thus, we aim to evaluate whether the principle of species-area relationship is potentially applicable to soil microbes. We established a fully factorial experimental design of three island sizes (~9, 50 and 150 cm^2) by two sterile soils (low, high resources). After six months of glasshouse incubation, habitat-area was positively related to bacterial richness, relative abundance of Chloroflexi, Verrucomicrobia and δ-proteobacteria, and soil functions in both soils. Soil with higher resources always had the greatest bacterial richness and functions. Our findings provide a proof of concept by demonstrating the potential importance of both habitat-area and resource availability in driving soil bacterial biodiversity and functioning.

Key words: Bacterial diversity; Decomposition; Ecological theory; Miseq Illumina; Extracellular enzyme activities; quantitative PCR.

1. Introduction

The relationship between habitat-area and number of plant and animal species is one of the most consistent ecological patterns in terrestrial ecosystems (MacArthur & Wilson 1967; Hoyer & Canfield 1994; Brunet & Medellin 2001). Larger islands support a greater absolute number (i.e. not standardized to a common area) of plant and animal species than smaller islands (MacArthur & Wilson 1967). As this popular theorum was developed without explicitly considering the microbial world, much less is known about the extent to which microbe diversity (i.e. number of species) conforms to predictions of Island Biogeography Theory (Green & Bohannan 2006; Barberan et al. 2014). Bell et al. (2005) provided the first evidence that habitat size could drive diversity of bacteria using water-filled tree holes as its island model. However, the observational nature of this study and the multiple confounding factors surrounding the selected type of island led to serious criticism of this study (Fenchel and Finlay 2005). In addition, Zinger et al. (2014) and Barreto et al. (2014) provided evidence that in aquatic environments, bacterial communities display a taxa-area relationship; however their results are also based on observational correlations.

Observational relationships have been questioned because of the inability to establish a cause-and-effect relationship between explanatory and responses variables. In other words, an experimental proof of concept for the microbial species-area relationship is needed to support future studies aiming to detect these types of relationships in real world ecosystems. Importantly, island vary in their availability of resources (e.g., soil fertility). Given the importance of resource availability in shaping the diversity and functioning of terrestrial ecosystems (Tilman et al. 1982; Waldrop et al. 2006; Maestre et al. 2015), any attend to evaluate the link between island size, and microbial diversity and function, needs to account for resource availability as a potentially important regulator of these relationships. Considering that soil microbes are major drivers of the rates and stability of key soil processes such as organic matter decomposition and nutrient cycling (Bodelier et al. 2011; Singh et al. 2009; Bardgett & van der Putten 2014; Delgado-Baquerizo et al. 2017), improving our understanding of the ecological patterns driving soil microbial diversity is essential to predict changes in ecosystem functioning under changing environments.

Herein we posit that habitat-area drives the diversity (i.e. number of species – richness) and functioning of soil microbes. Specifically, we hypothesized that i) larger islands provide more space for microbial colonization resulting in greater microbial diversity and functioning; and ii) resource availability plays an essential role during island colonization (i.e. islands with higher amount of resources result in a higher soil microbial diversity and functioning).

2. Methods

2.1 Study design

To test our hypotheses, we conducted a microcosm study in which we evaluated the role of habitat-area in driving the diversity, abundance, composition and functioning (enzyme activities) of bacterial communities. We established a fully factorial experimental design with two factors: island size (three levels: ~9, 50 and 150 cm^2) and soil type, including relatively low (Soil A) vs. high (Soil B) nutrient availability (Table 1; Fig. 1a). Soils for this study were collected during March 2014 from two semiarid woodlands (*Eucalyptus* spp.) in eastern Australia. At each site, a composite soil sample (twenty soil cores) was collected (top 20 cm) under tree canopies. The full description of the site characteristics and soil properties are available in Table 1. We found significant differences ($P < 0.05$) in all soil variables between the two locations in this study (Table 1). Soil properties were measured using standardized protocols as described in Maestre et al. (2012).

Following field sampling, the soil was highly homogenized, sieved (<2 mm mesh) and sterilised using gamma radiation (50kGy; see Delgado-Baquerizo et al. 2016a for a similar approach). Soils were re-sterilised seven days later (Gamma radiation, 50 kGy) to remove all microbial spores. We used gamma radiation because it causes minimal changes to the physical properties of soils compared to other methods such as autoclaving (Wolf et al. 1989; Lotrario et al. 1995). Sterilised soil diluted in nutrient medium (peptic digest of animal tissue 1.5 g L^{-1}, yeast extract 1.5 g L^{-1}, sodium chloride 5 g L^{-1}, beef extract 1.5 g L^{-1} each from DIFCO laboratories, USA) exhibited no growth 5 days after incubation at 28 °C.

2.2. Microcosm construction

Microcosms were constructed by carefully placing sterile soil in petri dishes of three sizes (1cm depth). Five replicates were established, resulting in 30 microcosms (two soil types x three island sizes x five replicates). Microcosms were placed close to each other (~5 cm) in a random spatial grid (6 x 5). The position of each microcosm in the grid was changed about every 5 days to avoid positional effects. Microcosms were placed indoors in a glasshouse, watered regularly with autoclaved sterile water, and incubated for six months to allow "natural" microbial colonization (i.e. by airborne microbial spores). Given that current empirical evidence suggests that microbial succession occurs from days to a few months (e.g., Edwards et al. 2014, Vorıskova & Baldrian 2013, Jurburg et al. 2017), we assume here that six months should be a reasonable incubation period over which to obtain a late successional microbial community in our soils. We collected all our soils after a six months incubation period, which ultimately allowed us to directly compare the microbial

communities in our microcosms at this point of time. Moisture content was adjusted and maintained at 50% water holding capacity during the duration of the experiment. By moistening the soils, we aimed to maintain microbial activity while avoiding water saturation and anoxic conditions.

2.3. Soil bacterial community and functioning

After incubation, we collected and homogenized the entire surface soil to 1-cm depth from each microcosm. We then extracted the DNA from 0.25g of soil/sample (Powersoil® DNA Isolation Kit, Mo Bio Laboratories, Carlsbad, CA, USA) to characterize bacterial diversity, composition and abundance. The abundance of bacteria was measured using quantitative PCR (qPCR) on a Carber Rotor-Gene 6000 cycler Real-Time PCR (Qiagen, Doncaster, Vic. Australia) and the Eub 338 – Eub 518 primer set as described in Fierer et al. (2005).

The diversity and composition of bacteria (16s rRNA) were determined using Miseq Illumina profiling of ribosomal genes (Illumina Inc.) and the 341F/805R (Herlemann et al. 2011) primer set. After visual assessment of the quality of all Illumina R1 and R2 reads using FastQC (Andrews, 2010), low quality regions (Q<20) were trimmed from the 5' end of the sequences (1 bp from R1 and 22 bp from R2) using SEQTK (https://github.com/lh3/seqtk). The paired ends were subsequently joined using FLASH (Magoc & Salzberg 2011). Primers were removed from the resulting sequences using SEQTK and a further round of quality control was conducted in MOTHUR (Schloss et al. 2009) to discard short sequences (<380 bp), as well as sequences with ambiguous characters or more than 8 homopolymers. Operational Taxonomic Units (OTUs) were built at 97% sequence similarity using UPARSE (Edgar, 2013). Singletons were discarded, as well as chimeric sequences identified by the UCHIME algorithm using the recommended SILVA gold 16S rRNA gene (Edgar et al. 2011). OTU abundance tables were constructed by running the usearch_global command (http://www.drive5.com/). Taxonomy was assigned to OTUs in MOTHUR using the naïve Bayesian classifier with a minimum bootstrap support of 60% and the Greengenes database version 13_8 (DeSantis et al. 2006). The OTU abundance tables were rarefied to an even number of sequences per sample (16853). Alpha diversity metrics were then calculated using MOTHUR (Schloss et al. 2009). The number of bacterial sequences obtained from two of the samples (replicates #2 and #5 for the medium island size in soil B) was too low to estimate microbial diversity accurately, so they were not used in further analyses.

Finally, four soil functions (i.e., extracellular enzyme activities) linked to soil organic matter decomposition: β-glucosidase (Starch degradation; BG), β-D-cellobiosidase (Cellulose degradation; CB), Phosphatase (P mineralization; PHOS) and N-acetyl-β-D-glucosaminidase

(Chitin degradation; NAG) were measured from 1g of soil using fluorometry as described in Bell et al. (2013).

2.4. Statistical analyses

We first tested for differences between soil type and island sizes in bacterial richness (number of OTUs as defined by 97% sequence similarity), abundance (qPCR), community composition (at the OTU level), relative abundance of main bacterial taxa and function using independent two-way permutational multivariate ANOVA (PERMANOVA) with soil type and island size as fixed factors. We then used non-metric multidimensional ordination (nMDS) and a two-way PERMANOVA (Anderson 2001) with soil type and island size as fixed factors and Bray-Curtis dissimilarity metric to explore overall differences in microbial composition (at the OTU level) across island sizes and soil types. PERMANOVA and nMDS analyses were done using PRIMER-E Ltd. & PERMANOVA version 6 (Plymouth Marine Laboratory, UK). We used Pearson correlations to test relationships among island area and diversity, abundance, composition and functioning (enzyme activities) of bacterial communities to further explore the role of the species-area relationship in driving soil bacterial features and function. Abundance of bacteria (qPCR), CB and the relative abundance of β- and δ-Proteobacteria were log-transformed prior to analyses to achieve normality (Shapiro-Wilk test). Finally, we evaluated the relative importance of island area per se and soil type in driving bacterial composition at the OTU level using variation partitioning analyses (Legendre et al. 2012) using island area and soil type (a categorical variable with 0 and 1) as predictors of bacteria features.

3. Results

We found a strong relationship between habitat area and diversity of bacteria in soil (Fig. 1). Larger islands had more bacterial diversity than smaller islands for both Soils A and B ($P < 0.001$; Fig. 1b; Table 2). Similar results were found when we explored the correlation between island area and the richness of main bacterial taxa (at the OTU level) independently (Table S1). Conversely, island size did not significantly influence the total abundance of bacteria (i.e. number of gene copies g^{-1} soil measured using qPCR; Fig. 2). In addition, we found that soil B –which had greater resource availability (e.g. organic matter, inorganic P and available N), but similar soil pH, texture and bulk density than Soil A– always exhibited the greatest bacterial richness across island sizes (Fig. 1b).

In all cases, our microcosms were dominated by similar taxa of bacteria belonging to phyla Actinobacteria, Bacteroidetes, and α- and β-Proteobacteria. On average, *Arthrobacter oxydans* (Actinobacteria) and *Massilia sp.* (β-Proteobacteria) were the two dominant

205 microbial species in all microcosms, accounting together for 25.2 and 15.8% of the relative
206 abundance of bacteria in Soils A and B, respectively. Island size still affected the composition
207 of bacteria at the OTU level (Fig. 1c). Increases in island size were associated with an
208 increase in the relative abundance of less abundant bacterial taxa such as Chloroflexi,
209 Verrucomicrobia and δ-Proteobacteria and reduced the relative abundance of dominant
210 groups such as β-Proteobacteria in both soil types (Table 2; Fig. 3). Similarly, island size was
211 negatively correlated with the relative abundance of dominant phylum Bacteroidetes and
212 positively related to the minority phylum Cyanobacteria in Soil A (Table 2; Fig. 3). Even so,
213 our variation partitioning model indicated that island size and soil type (i.e. resource
214 availability) have a relative low control on the final identity of bacterial species in the
215 microcosms (Fig. S1).

216 When enzyme activity was detected, island size was positively related to soil function
217 (enzyme activity; Table 2). Note that we were only able to detect the activity of CB in Soil A
218 (Fig. 4). Larger islands had greater levels of enzyme activity related to starch (BG), cellulose
219 (CB) and chitin (NAG) degradation and P mineralization (PHOS) than smaller islands (Fig.
220 4). Thus, soil types largely influenced soil functions, having soil B the highest enzyme
221 activity (Fig. 4).

222 **4. Discussion**

223 Our results provide solid evidence, from an experimental approach, that, as predicted by
224 Island Biogeography Theory, larger islands supported a greater diversity of bacteria, but also
225 greater diversity of bacterial taxa from different phyla than smaller islands in two different
226 soil types. Our findings offer a proof of concept for the microbial species-area relationship
227 under experimental conditions. As such, our work provides some of the first experimental
228 evidence that island size could be a driver of microbial diversity. However, we acknowledge
229 that this is only the first step in understanding these types of relationships in terrestrial
230 ecosystems under real world conditions, a research question to be addressed by future studies.

231 Several mechanisms can potentially explain the strong microbial species-area
232 relationship reported in our study, including larger microcosms receiving more colonizers and
233 stochastic processes. Because the likelihood of a “propagule” arriving is area-dependent,
234 larger islands would be expected to support a more diverse bacterial community by
235 enhancing the likelihood that different bacteria would settle on these islands. Moreover,
236 larger islands may also support a larger number of independent colonization events across an
237 island, thus increasing the chances of greater bacterial co-existence. Larger islands would be
238 expected to support greater range of microhabitats, thus supporting more species, as reported

for plants and animals (Ricklefs & Lovette 1999). However, the fact that our soil was strongly mixed, sieved and homogenized prior to microcosm preparation could reduce the importance of this aspect of our results. Moreover, soil pH would be expected to influence the diversity of bacteria in our two soils (Lauber et al. 2009), however, the fact that both soils have similar neutral pH values (pH 6-7), likely limit the influence of this factor on our results. Similarly, Soils A and B showed similar values for bulk density and soil texture (Table 1). Both factors may have influenced the diversity of bacteria in our soils (Bach et al. 2010; Delgado-Baquerizo et al. 2016b).

Interestingly, we found that soil B, which had the most resources (e.g. organic matter, inorganic P and available N; Table 1), exhibited the greatest bacterial richness across island sizes after 6 months of colonisation, suggesting that resource availability can influence bacterial diversity. This result is consistent with the notion that resource availability can strongly influence soil microbial diversity, and accords with empirical results for plants and animals (Tilman et al. 1982; Waldrop et al. 2006; Maestre et al. 2015). However, this result does not necessarily mean that the soil with more resources will continue to support a diversity of microbes in the longer term, or that diversity was always greatest during the early stages of the incubation. Therefore, our results are limited by the fact that we only conducted measurements at a single time point.

Island size did not significantly influence the total abundance of bacteria per gram of soil (via qPCR), suggesting that the effects of habitat size on bacterial communities are not associated with bacterial abundance *per se*, but only with diversity. Of course, larger islands had a larger amount of soil and therefore, a larger total abundance of bacteria. This result suggests that the abundance of bacteria per gram of soil may be related more to the quantity of resources held in the soil substrate rather than microcosm size. However, the total number of phylotypes, in a comparable amount of soil (0.25g), is likely influenced by microcosm size, because a larger substrate would be more likely to be colonized by airborne microbial communities, and therefore have greater subsequent horizontal colonization within the plate.

Our microcosms were dominated by two bacterial species *Arthrobacter oxydans* (Actinobacteria) and *Massilia* sp. (β-Proteobacteria). Both species have been found to have a high dispersal capacity via airborne deposition (Favet et al. 2013; Stone et al. 2016). Even so, island size strongly influenced the composition of soil bacteria. For example, island size increased the relative abundance of minority phylum such as Chloroflexi, Verrucomicrobia, δ-Proteobacteria (both soils) and Cyanobacteria (Soil A), but reduced the dominance of major groups such as β-Proteobacteria (both soils) and Bacteroidetes (Soil A). These results further

support the notion that larger islands may increase the likelihood of simultaneous colonization of different bacterial communities, including less abundant groups, which seem to be limited in the smallest islands for both soils. Strikingly, our variation partitioning model suggested that island size and soil type have a relative low control on the final composition of bacteria in the microcosms. The relatively low capacity of island size and soil type to predict the resulting microbial community in our microcosms may be related to the high similarity in bacterial taxa found across different microcosms, i.e. the dominant greenhouse bacteria landing on all microcosms. Thus, almost half of the bacterial OTUs were found to be ubiquitous across all island sizes, i.e., these "species" were detected at least once in each island size class, 44.4% for Soil A and 45.6% for Soil B. This strongly limits the statistical power of island size and soil type in our model to predict changes in the bacterial community composition in our soils. An alternative to this is that the resultant colonization process may be highly stochastic and likely modulated by the airborne microbial pool present in the glasshouse.

While we were able to detect the activity of all enzymes measured in soil B, only the activity of a single enzyme was detected in soil A. When enzyme activities were detected, island size was also significantly and positively related to soil function linked to organic matter decomposition in both soil types, with soil B exhibiting the highest soil functioning. The decomposition of organic matter is the consequence of a strong interaction between microbial diversity (Delgado-Baquerizo et al. 2016b) and resource content (Schimel et al. 2005; Delgado-Baquerizo et al. 2016c). In support of this notion, when enzyme activity was detected, we found an overall positive relationship between soil bacterial diversity and function in both soils (Table S2). The reported lack of detection in activity of some enzymes in Soil A, which cannot be related to the bacterial biomass in our microcosms as measured by qPCR, may be rather related to the different microbial taxa settling onto both soils, but also to its lower resource content compared to Soil B. Soil A had a lower organic matter, nitrogen and phosphorus content (Table 1), which may all limit the production of soil enzymes. Alternatively, potential reductions in the amount of soil carbon after six month of incubation might have limited the microbial activity in microcosms from soil A, to the an extent, potentially, that enzyme activity is no longer detectable. The lack of resources would probably result in a high level of dormancy within the community, explaining the low activity but similar biomass level suggested by qPCR.

Altogether, our work provides an experimental proof of concept of the microbial species-area relationship, providing empirical support to future studies aimed at understand

this type of relationship under real world conditions. Additionally, our results support other evidence that resources and microbial diversity play important roles in driving ecosystem functioning, which is particularly relevant for understanding how ongoing climate change might affect ecosystem functioning in terrestrial ecosystems.

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Data accessibility

Data associated with this paper has been deposited in figshare:
https://figshare.com/s/d993311f1aa8f64f79e9 (10.6084/m9.figshare.5785605).

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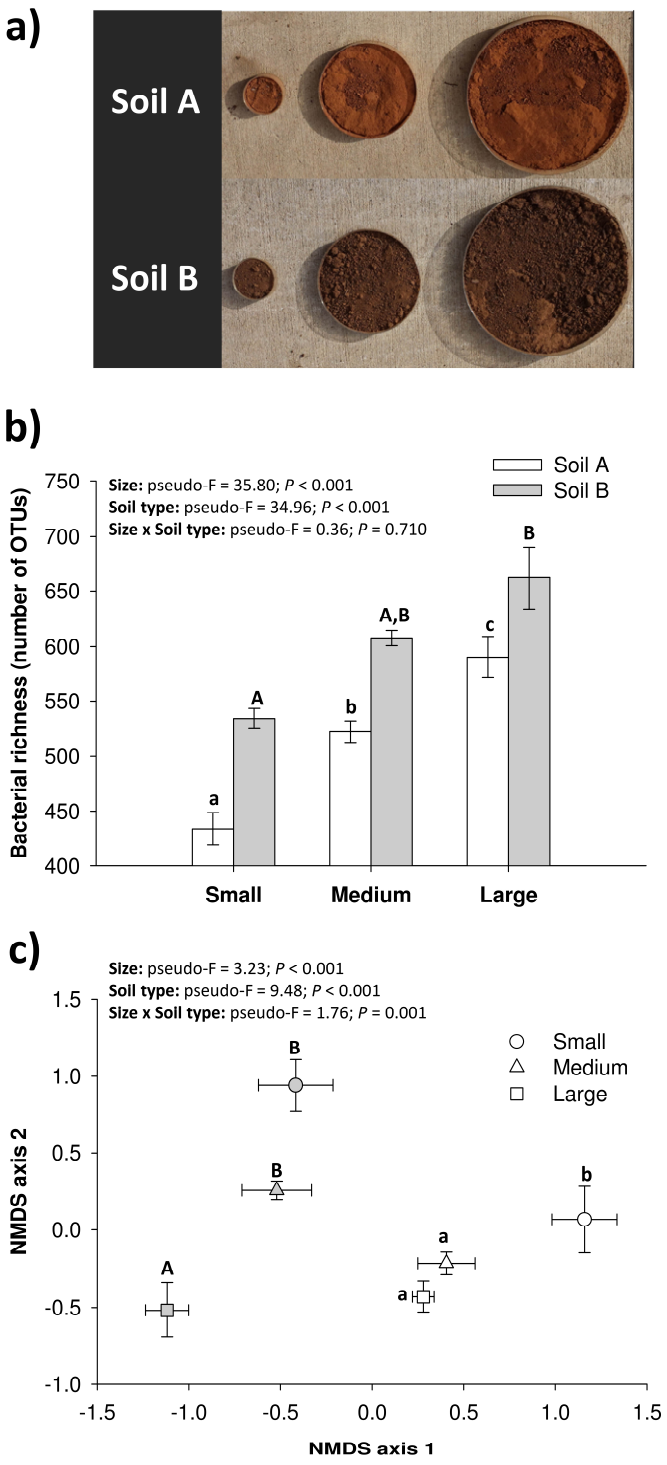

Figure 1. Habitat area effects on the diversity and composition of bacteria. Panel (a) represents an example of the different island sizes used in this study for Soils A and B. Panel (b) presents mean values (± SE) for bacterial richness across different island sizes and soil types. Panel (c) presents results from a nMDS (mean ± SE) showing shifts in microbial composition at the OTU level across different island sizes and soil types. Different lower and upper-case letters indicate significant differences after post-hoc Tukey tests (only when applicable) for soils A and B, respectively.

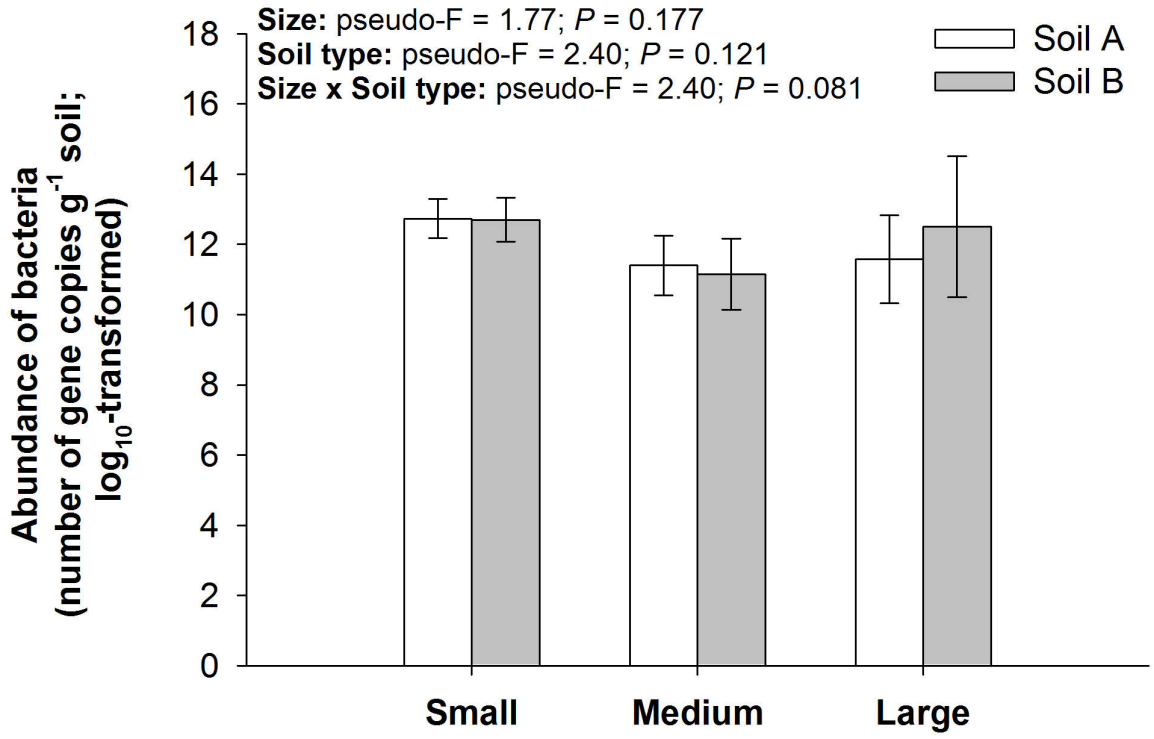

Figure 2. Habitat-area effects on total bacterial abundance (qPCR) for Soils A and B.

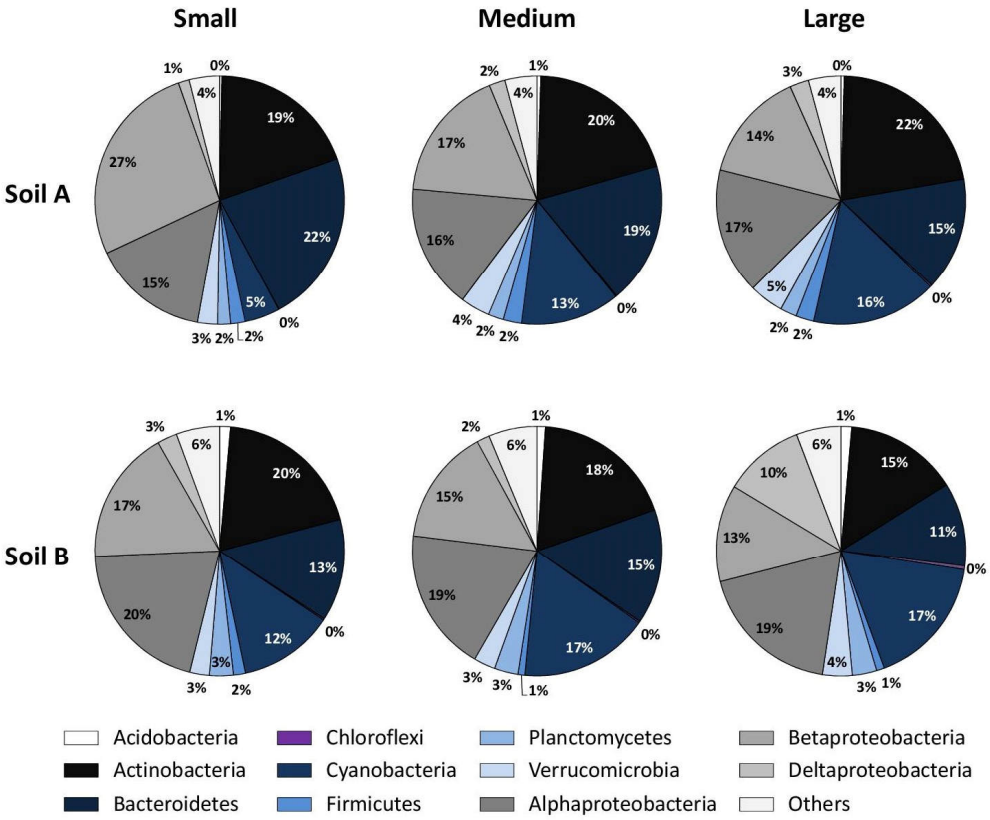

Figure 3. Bacterial composition (i.e. relative abundance of main bacterial taxa) across different island sizes and soil types.

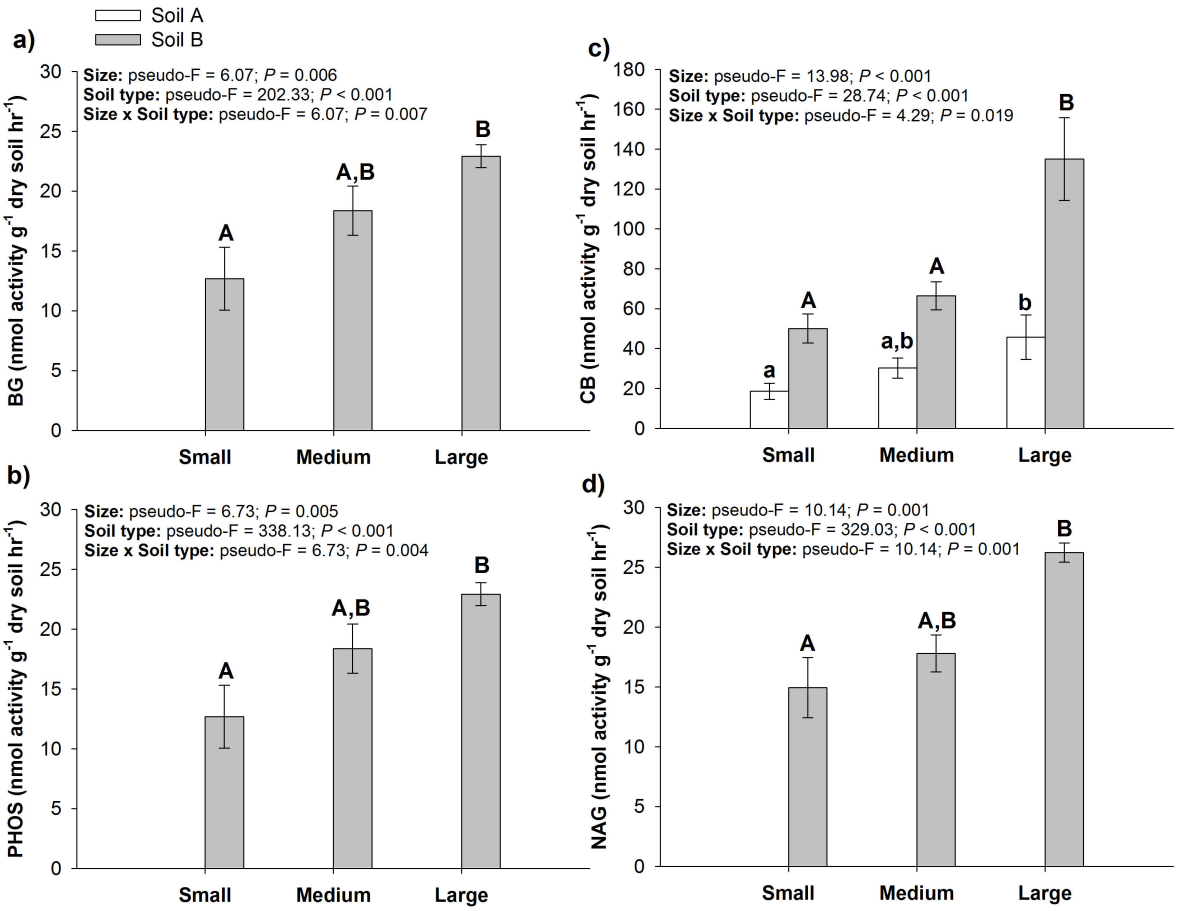

Figure 4. Habitat-area effects on soil function (i.e. extracellular enzyme activities) (mean ± SE) for Soils A and B. Note that we were unable to detect the activity of BG, PHOS and NAG in Soil A. Different lower and upper-case letters indicate significant differences after post-hoc Tukey tests (only when applicable) for soils A and B, respectively.

505 **Table 1.** Location, climate and main soil properties for Soils A and B.

	Soil A	Soil B
Location (°)	-34.00, 145.73	-33.73, 148.20
Mean annual temperature (°)	17	16
Annual precipitation (mm)	418	656
Altitude (m)	113	335
pH	6.36	7.35
Clay (%)	33	37
Bulk density (g cm^{-3})	1.43	1.17
Organic matter (%)	5.21	8.16
Dissolved organic N (mg N kg^{-1} soil)	0.00	40.48
NH_4^+ (mg N kg^{-1} soil)	2.99	6.40
Available P (mg P kg^{-1} soil)	2.18	11.23

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Table 2. Correlation (Pearson) between island size and bacterial diversity, abundance, composition and functions. Significance levels of each predictor are *P < 0.05, **P < 0.01, [a]P < 0.10. BG = β-glucosidase; CB = β-D-cellobiosidase; PHOS = Phosphatase; NAG = N-acetyl-β-D-glucosaminidase. ND = these enzymes could not be detected in soil A. Blank cells = no correlation was detected.

	Variable	Soil A	Soil B
Biodiversity	**Richness**	0.880**	0.814**
Abundance	**Number of gene copies**		
Composition	**Acidobacteria**		
	Actinobacteria		-0.661*
	Bacteroidetes	-0.747**	
	Chloroflexi	0.794**	0.820**
	Cyanobacteria	0.657**	
	Firmicutes		
	Planctomycetes		
	Verrucomicrobia	0.624*	0.529[a]
	α-Proteobacteria		
	β-Proteobacteria	-0.667**	-0.658*
	δ-Proteobacteria	0.457[a]	0.548[a]
Soil functions	**BG**	ND	0.707**
	CB	0.606*	0.846**
	PHOS	ND	0.741**
	NAG	ND	0.804**

Supplementary Materials

Table S1. Correlation (Pearson) between island size and the diversity (richness) of main bacterial taxa. Significance levels of each predictor are *P < 0.05, **P < 0.01, [a]P < 0.10. Blank cells = no correlation was detected.

Variable	Soil A	Soil B
Acidobacteria	0.572*	0.500[a]
Actinobacteria	0.551*	0.685**
Alphaproteobacteria	0.746**	0.697**
Bacteroidetes	0.853**	0.504[a]
Betaproteobacteria	0.568*	0.527[a]
Chloroflexi	0.728**	0.728**
Cyanobacteria	0.821**	
Deltaproteobacteria	0.892**	
Firmicutes	0.499	0.662*
Planctomycetes	0.718**	0.783**
Verrucomicrobia	0.778**	0.751**

Table S2. Correlation (Pearson) between bacterial richness, abundance and composition and soil functions. Significance levels of each predictor are *P < 0.05, **P < 0.01, [a]P < 0.10. BG = β-glucosidase; CB = β-D-cellobiosidase; PHOS = Phosphatase; NAG = N-acetyl-β-D-glucosaminidase. Blank cells = no correlation was detected.

		Soil A	Soil B			
		CB	BG	CB	PHOS	NAG
Biodiversity	**Richness**	0.457[a]	0.593*	0.743**	0.531[a]	0.547[a]
Abundance	**Number of gene copies**					
Composition	**Acidobacteria**					
	Actinobacteria			-0.575*		-0.547[a]
	Bacteroidetes					
	Chloroflexi		0.585*	0.707**		0.648*
	Cyanobacteria	0.811**				
	Firmicutes					
	Planctomycetes					
	Verrucomicrobia			0.512[a]		
	α-Proteobacteria					
	β-Proteobacteria	-0.623*	-0.499[a]	-0.649*	-0.497[a]	-0.53[a]
	δ-Proteobacteria					

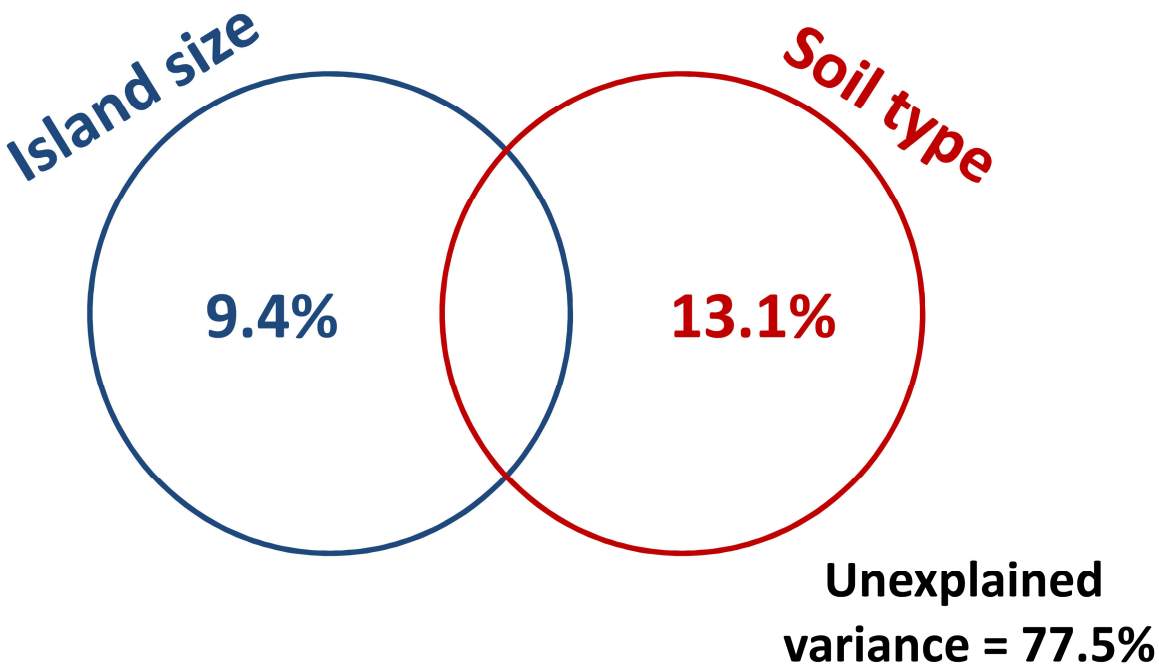

Figure S1. Variation partitioning modelling aiming to identify the % variance of bacterial composition at the OTU level explained by island size and soil type. Shared effects of these variable groups are indicated by the overlap of circles.