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**The ratio of Gram-positive to Gram-negative bacterial PLFA markers as an indicator of carbon availability in organic soils**

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1 **Abstract:** Despite recent progress in understanding soil microbial responses to carbon (C)  
2 limitation, the functional shifts in microbial community structure associated with decreasing soil  
3 C availability and changes in organic matter chemistry remain poorly known. It has been proposed  
4 that Gram-negative (GN) bacteria use more plant-derived C sources that are relatively labile, while  
5 Gram-positive (GP) bacteria use C sources derived from soil organic matter that are more  
6 recalcitrant. Because these two groups may differ in how they influence the fate of different C  
7 forms in soils, it is important to understand how they vary across ecosystems that differ in their  
8 vegetation cover and ecosystem productivity or across environmental gradients. In this study, we  
9 used a 19-year plant functional group removal experiment across a long term post-fire  
10 chronosequence to assess how microbial community structure (assessed using phospholipids fatty  
11 acids; PLFAs) and the association of bacterial functional groups (specifically, the GP:GN ratio)  
12 responded to changes in organic matter chemistry (measured *via* nuclear magnetic resonance;  
13 NMR). We found that the GP:GN ratio increased upon removal of shrubs and tree roots and with  
14 decreasing ecosystem productivity along the chronosequence, thus showing the greater dependence  
15 of GN than GP bacteria on more labile plant-derived C. Overall, GN bacteria were associated with  
16 simple C compounds (alkyls) whereas GP bacteria were more strongly associated with more  
17 complex C forms (carbonyls). Therefore, we conclude that the GP:GN ratio has potential as a useful  
18 indicator of the relative C availability for soil bacterial communities in organic soils, and can be  
19 used as a coarse indicator of energy limitation in natural ecosystems.

20

21 **Keywords:** carbon cycle, copiotroph-oligotroph model, energy limitation, Gram-positive bacteria,  
22 Gram-negative bacteria, microbial community structure, phospholipid fatty acids, PLFA, soil  
23 carbon.

24

25 Microbes are important regulators of the terrestrial carbon (C) budget through their impact on  
26 decomposition and incorporation of organic material in soils (Schimel and Schaeffer, 2012;  
27 Cotrufo et al., 2013). It is therefore important to understand how substrate availability controls the  
28 structure and function of soil microbial communities. The addition of fresh organic substrates to  
29 the soil usually stimulates copiotrophic microbial communities with high growth rates and fast  
30 turn-over (Bastian et al., 2009; Pascault et al., 2013). In contrast, decreasing availability of easily  
31 accessible C favors oligotrophic microbial communities characterized by low biomass, and low  
32 enzymatic activities and respiration rates (Potthast et al., 2010; Fanin and Bertrand, 2016). While  
33 analytically distinguishing between copiotrophic and oligotrophic microorganisms is challenging,  
34 Kramer & Gleixner (2008) used stable isotope analyses to propose that Gram-negative (GN)  
35 bacteria use more plant-derived C sources which are generally labile, while Gram-positive (GP)  
36 bacteria use more soil organic matter (SOM)-derived C sources which are more recalcitrant. If  
37 these two groups effectively depend upon different C sources in soils, shifts in their relative  
38 proportion could be used to assess energy limitation in soils. In line with this idea, several studies  
39 have found that the GP:GN ratio increases with decreasing C availability down the soil profile or  
40 after the exhaustion of labile substrates (Fierer et al., 2003; Breulmann et al., 2014; Fanin et al.,  
41 2014). However, whether and how the GP:GN ratio changes across environmental gradients or  
42 with decreasing levels of plant-derived C compounds in natural ecosystems has received little  
43 attention.

44       To understand how GP and GN bacteria vary with soil organic C availability, and how these  
45 groups vary across contrasting natural ecosystems, we used an ongoing biodiversity manipulation  
46 experiment along an island chronosequence in northern Sweden (see Supplementary Methods for  
47 more details). These islands vary greatly in time since their most recent wildfire, with larger islands  
48 being struck by lightning more often and therefore on average having burnt more recently; these

49 islands therefore collectively represent a post-fire chronosequence spanning over 5000 years  
50 (Wardle et al., 2003). We divided the islands into three size classes of ten islands each: large islands  
51 (on average  $\pm 585$  years old), medium islands ( $\pm 2180$  years) and small islands ( $\pm 3250$  years), as  
52 done in numerous previous studies (Gundale et al., 2012; Clemmensen et al., 2013; Kardol et al.,  
53 2018). Previous work in this study system has shown that as the time since fire increases, plant  
54 biomass, net primary productivity and quality of OM inputs (i.e., plant litter and microbial inputs)  
55 decline as a consequence of decreasing soil nutrient availability (Lagerström et al., 2007; Wardle  
56 et al., 2012; Clemmensen et al., 2015) (Appendix 1). On each of these 30 islands, a plant functional  
57 group removal experiment was implemented in 1996 which had been running for 19 years at the  
58 time of measurement: it consists of full factorial removals of tree roots (by trenching), mosses and  
59 dwarf shrubs (by manual removal) (Wardle and Zackrisson, 2005; Fanin et al., 2018) (Appendix  
60 1). Because organic C availability is proposed as a good proxy of bacterial community structure  
61 (Fierer et al., 2007), we hypothesized that plant removals should increase the GP:GN ratio, mainly  
62 because fresh plant-derived C compounds should decline as increasing numbers of plant functional  
63 groups are removed ( $H_1$ ). Further, because plant productivity and OM quality declines as the island  
64 chronosequence proceeds (Wardle et al., 1997; Lagerström et al., 2007; Crutsinger et al., 2008;  
65 Clemmensen et al., 2013), we hypothesized that the GP:GN ratio should increase from early (large  
66 islands) to late (small islands) successional stages ( $H_2$ ).

67         We collected soil samples in each of eight plots on each of the 30 islands, representing a  
68 full factorial combination of removals of each of the three plant functional groups (i.e., tree roots,  
69 shrubs and mosses) between 3 and 15 August 2015 (19 years after experimental set-up). Briefly, 5  
70 soil cores were taken in each plot (in total 240 plots) using a stainless steel cylinder (3 cm diameter)  
71 to a depth of 10 cm (Fanin et al., 2018). One of these soil cores was taken in the center of each plot  
72 for characterization of the organic matter (OM) chemistry *via* mid infra-red (MIR) spectroscopy

73 calibrated against  $^{13}\text{C}$  solid-state nuclear magnetic resonance (NMR) spectroscopy. The other four  
74 cores were collected 20 cm between the center and corner of each plot and bulked for measurements  
75 of phospholipid fatty acids (PLFAs), which we used to quantify GP and GN bacteria (see  
76 Supplementary Methods).

77 We found that the GP:GN ratio was strongly affected by plant functional group removals;  
78 the ratio increased significantly after shrub removal, and to a lesser extent after tree roots removal  
79 (Table 1). In partial agreement with our first hypothesis, this suggests that tree and shrub removals  
80 (but not mosses) led to an increase in the GP:GN ratio (Figure 1). The observed changes in the  
81 GP:GN ratio were due to a larger increase in GP bacteria compared with GN bacteria (Appendix  
82 2). This is likely because removing tree roots and shrubs reduced both the quality and quantity of  
83 fresh plant-derived compounds entering the soil. In contrast, poor-quality litter produced by mosses  
84 could represent a different C source that decomposes more slowly than that of the vascular plant  
85 species present in the system (Lindo et al., 2013).

86 The GP:GN ratio also varied greatly among island size classes; the ratio increased from  
87 large (early successional stage) to small islands (late successional stage), independent of the plant  
88 functional group removals (Table 1). In agreement with our second hypothesis, this underlines that  
89 decreasing plant productivity and OM quality as succession proceeds will significantly increase the  
90 GP:GN ratio. This supports the idea that GN bacteria are more dependent of plant biomass and C  
91 inputs than GP bacteria. However, we cannot exclude the possibility that simultaneous changes in  
92 plant community composition (i.e., from resource-acquisitive towards resource-conservative  
93 species) and changes in soil physicochemical conditions (i.e., decrease in nutrient availability) may  
94 also have influenced the GP:GN ratio along the environmental gradient, especially because GP  
95 bacteria are thought to be more resistant to stress and harsh conditions than are GN bacteria (De  
96 Vries and Shade, 2013).

97           When investigating the relationships between SOM chemistry and microbial groups across  
98 all plots on all islands (Figure 2a), we found that GN bacteria were positively associated with alkyl  
99 and N-alkyl compounds (representing lipids and other aliphatics) while GP bacteria were  
100 associated with carbonyl, aryl, and ketone compounds (representing more complex and chemically  
101 recalcitrant C compounds). This result indicates that these two bacterial guilds are associated with  
102 different C forms in soils, and suggests that they may play different functional roles in the  
103 decomposition of SOM (Kramer and Gleixner, 2008), particularly in organic soils where mineral  
104 protection mechanisms are minimal (Cotrufo et al., 2013). However, the strength of relationships  
105 between GP (or GN) bacteria and specific NMR regions varied with island size (Figure 2b). For  
106 instance, the slopes between GP bacteria and carbonyls differed between large and medium or  
107 small islands, while those between GN bacteria and alkyls differed between large and small islands  
108 (Figure 2b). This highlights that GN and GP bacteria may each show contrasting relationships with  
109 SOM functional groups across different stages of boreal forest succession. This is likely because  
110 decreasing nutrient availability as the island chronosequence proceeds may increasingly constrain  
111 the abundance of GN bacteria as islands become smaller (Fanin et al., 2015), or because the high  
112 availability of easily accessible C compounds on the more productive large islands may prime the  
113 degradation of SOM, thus reducing the amounts of recalcitrant material and thereby weakening the  
114 relationship between GP and recalcitrant C (Bird et al., 2011; Creamer et al., 2015).

115           By using a long-term vegetation manipulation along a post-fire chronosequence, we sought  
116 to test whether the GP:GN ratio varied consistently with C availability across contrasting  
117 ecosystems. We found that the GP:GN ratio increased due to shrub and tree root removals, but also  
118 with decreasing ecosystem productivity across contrasting ecosystems. These results coupled to  
119 our NMR analyses support the idea that GN bacteria are more dependent on simple C compounds  
120 derived from plants whereas GP bacteria are linked to more complex C compounds in organic soils.

121 These results highlight that the GP:GN ratio may therefore be used as a coarse indicator of the  
122 relative C availability of bacterial communities when comparing among contrasting natural  
123 ecosystems. We conclude that understanding functional shifts in bacterial community structure  
124 could be used as a useful way to assess energy limitation in natural ecosystems.

125

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130

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203 ecosystem properties. *Science* 277, 1296-1299.

204 **Table 1** Results from a full factorial mixed linear model to test for the effects of plant functional  
 205 group removals [Trees (T), Shrubs (S), and Mosses (M)], island size (IS) class and their interactions  
 206 on the ratio between Gram-positive to Gram-negative bacteria.  
 207

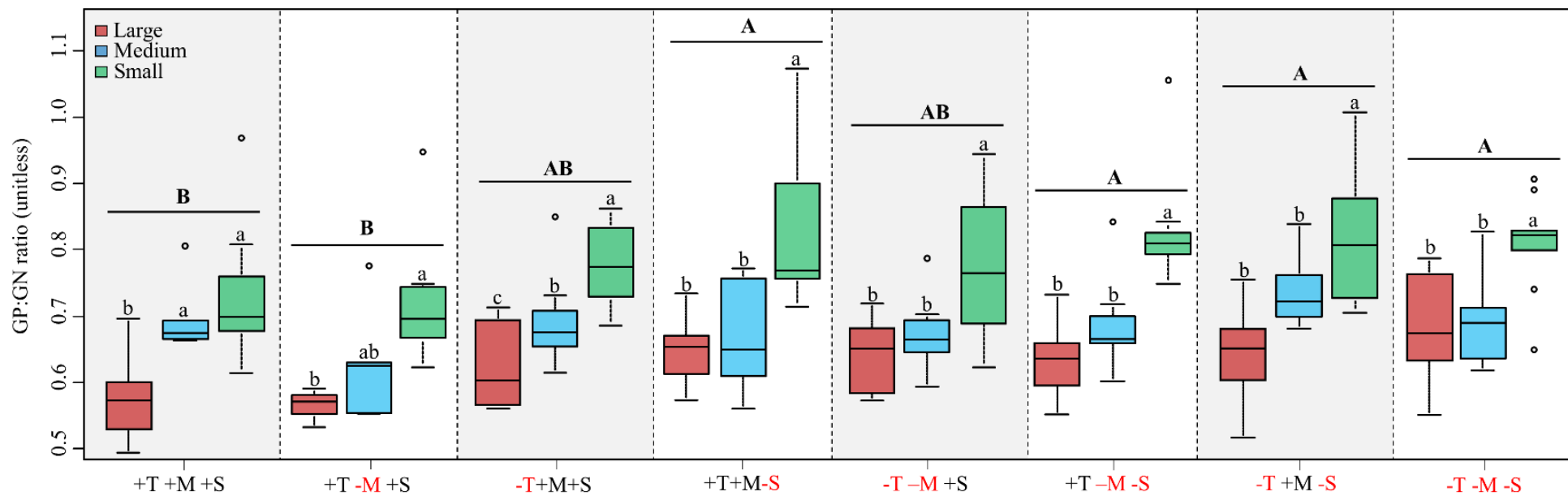
Variable	Numerator DF	Denominator DF	F-value	P-value
Island Size (IS)	2	27	21.1	<b>&lt;0.0001</b>
Tree root removal (T)	1	186	9.7	<b>0.002</b>
Moss removal (M)	1	186	0.0	0.89
Shrub removal (S)	1	186	33.8	<b>&lt;0.0001</b>
IS × T	2	186	0.4	0.69
IS × M	2	186	1.4	0.25
T × M	1	186	0.0	0.95
IS × S	2	186	2.3	0.10
T × S	1	186	2.4	0.12
M × S	1	186	0.1	0.81
IS × T × M	2	186	1.9	0.16
IS × T × S	2	186	1.1	0.33
IS × M × S	2	186	0.1	0.93
T × M × S	1	186	0.8	0.36
IS × T × M × S	2	186	0.6	0.57

208 **Fig. 1.** The effects of plant functional group removals [Trees (-T), Shrubs (-S), and Mosses (-M)]  
209 across contrasting island size classes [Large (red), Medium (blue), and Small (green)] on the ratio  
210 between Gram-positive (GP) and Gram-negative (GN) bacteria. Boxes represent the central 50% of  
211 the data and the whiskers on the boxes represent the 95% quantiles. Different lowercase letters  
212 indicate significant differences in GP:GN ratio among island size classes within each of the eight  
213 plant functional removal combinations, while uppercase letters indicate significant differences  
214 among the 8 removal combinations (Tukey's HSD test,  $\alpha = 0.05$ ).

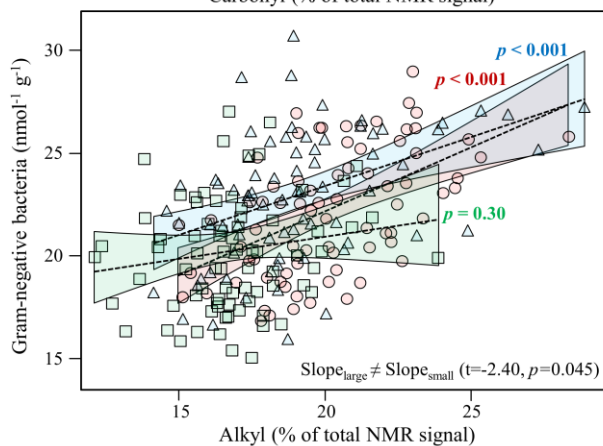
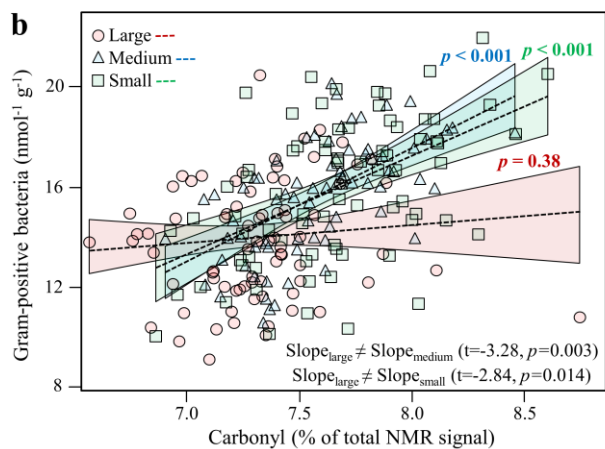
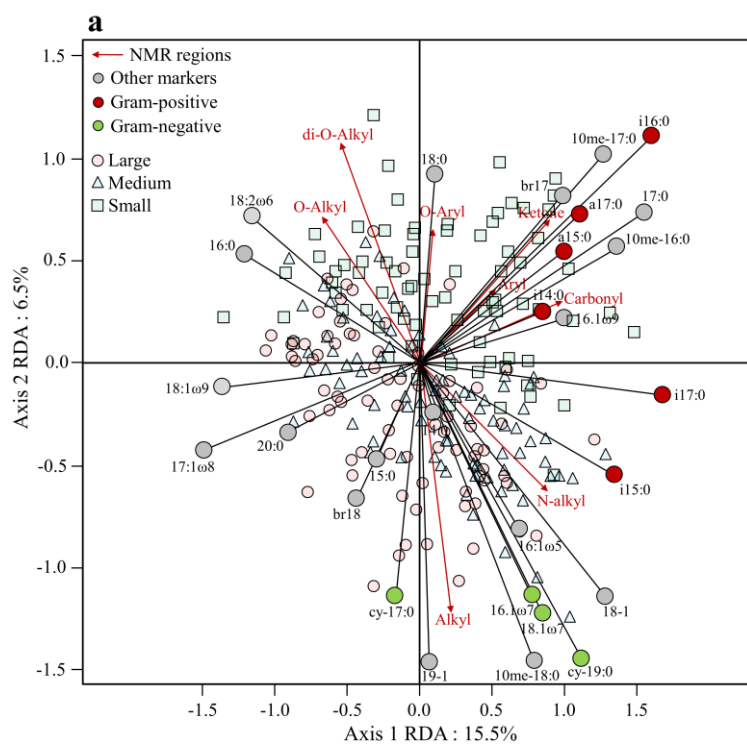
215

216 **Fig. 2.** (a) Ordination plot from redundancy analysis (RDA) of organic matter chemistry and the  
217 relative abundance of the 28 individual PLFAs that represent Gram-positive bacteria (GP) (red),  
218 Gram-negative bacteria (GN) (green) and other PLFA markers (grey) across plant functional group  
219 removal treatments and island size classes. Red arrows indicate the projection of the different NMR  
220 regions according to the two first RDA axes. (b) Relationships of GP and GN bacteria with  
221 Carbonyl and Alkyl regions, respectively, for each island size class. Statistically significant  
222 differences in slopes among island size classes at  $\alpha = 0.05$  are indicated in the right bottom corner  
223 of each panel with the t-scores and associated *p*-values. *P*-values presented next to each of the three  
224 regression lines in each panel indicate if the slope of the relationship is significantly different from  
225 0. In both panels A and B, island size classes are indicated by different symbols [Large (circle),  
226 Medium (triangle), and Small (square)].

227 Fig. 1.



228 Fig. 2.



229 **Supplementary methods**

230

231 *1. Experimental design*

232 The study was conducted on a group of forested islands that collectively represent a  
233 chronosequence situated in the two adjacent Swedish lakes, Lake Hornavan and Lake Uddjaure  
234 (65°55' N to 66°09' N; 17°43' E to 17°55' E). The islands were formed after the most recent  
235 glaciation and are subjected to the same climatic conditions (Wardle et al., 1997; Wardle et al.,  
236 2003). The mean annual precipitation is 750 mm and the monthly mean temperature varies between  
237 -14°C in January to 13°C in July. The only major extrinsic factor that differs among islands is the  
238 history of lightning-ignited wildfire, with larger islands having burned more frequently than  
239 smaller islands because of their larger area to intercept lightning (Wardle et al., 1997); several large  
240 islands have burned in the past century, whereas some small islands have not burned in the past  
241 5000 years. The 30 islands include three classes of each of ten islands (hereafter 'island size class'):  
242 10 'large' islands (>1.0 ha; on average, mean time since fire 585 years), 10 medium islands (0.1 to  
243 1.0 ha, 2180 years), and 10 small islands (<0.1 ha, 3250 years) (Appendix 1). All major plant  
244 species occur on all islands but their relative abundance varies from large to small islands (Wardle  
245 et al., 2012). As such, the islands enter a state of ecosystem retrogression and as time since fire  
246 increases, net primary productivity, soil fertility and nutrient recycling decrease (Wardle et al.,  
247 2003), leading to humus layers that can exceed 1 m in depth on the smallest islands due to  
248 accumulation of organic matter (Appendix 1). The average SOC accumulated belowground is of  
249 6.2, 11.2 and 22.5 kg C m<sup>-2</sup> in large, medium and small islands, respectively (Clemmensen et al., 2013).

250 A plant removal experiment was established on each of these islands in 1996 and is still  
251 ongoing; it is the longest running across-ecosystem biodiversity manipulation experiment in  
252 existence (Fanin et al., 2018, Kardol et al. 2018). The experiment consists of a full factorial removal

253 combination of three different plant functional group removals (eight treatments in total); that is,  
254 tree root removal (performed by root trenching), ericaceous shrub removal (performed manually)  
255 and feather moss removal (performed manually), yielding a total of 240 plots. The three plant  
256 functional groups represent > 99% of all plant biomass present on the islands. This design allows  
257 assessment of the contributions of all possible interactions among functional groups to ecosystem  
258 functioning at local spatial scales across the island size gradient. Removal treatments are  
259 implemented on each plot each year (in its nineteenth year on the sampling date), and we have  
260 shown that the legacy effects of removal disturbances had mostly ceased by 2003 (that is, by the  
261 seventh year) (Wardle and Zackrisson, 2005). All plots are 55 cm × 55 cm, but only the inner 45  
262 cm × 45 cm area is measured; plots are located at similar distances from the island shore  
263 irrespective of island size, to minimize the potential of confounding factors covarying with island  
264 size, such as edge effects and macroclimate (Wardle et al., 2012).

265

## 266 *2. Microbial community structure*

267 After freeze-drying, phospholipid fatty acids (PLFAs) were extracted from each soil subsample  
268 according to the Bligh and Dyer method as modified by White et al. (Bligh & Dyer 1959; White et  
269 al. 1979). The abundance of PLFAs was quantified using a Perkin–Elmer Clarus gas  
270 chromatograph equipped with a flame ionisation detector (Waltham, MA, USA) and was converted  
271 to nmol PLFA per gram of organic matter using conventional nomenclature. A total of 28 different  
272 PLFAs were identified. Among them, the branched and saturated PLFAs i-14:0, i-15:0, a-15:0, i-  
273 16:0, i-17:0, and a-17:0 were assigned to Gram-positive bacteria while the mono-unsaturated and  
274 cyclopropyl PLFAs 16:1 $\omega$ 7c, cy-17:0, 18:1 $\omega$ 7c, and cy-19:0 were assigned to Gram-negative  
275 bacteria (Frostegård and Bååth 1996; Zelles 1999).

276



277 3. Organic matter chemistry

278 Organic matter (OM) chemistry was quantified by a combination of solid-state cross-polarization  
279 magic-angle spinning (CP/MAS)  $^{13}\text{C}$  nuclear magnetic resonance (NMR) spectroscopy and mid  
280 infra-red (MIR) spectroscopy. First, 60 soil samples were selected to provide a calibration set of  
281 solid-state  $^{13}\text{C}$  NMR spectra; these samples were chosen to provide a strong contrast across the  
282 entire dataset (i.e., samples from the control plots with no vegetation removal and the treatment  
283 plots with all functional groups removed, for each of the 30 islands), using a Bruker 200 Avance  
284 spectrometer (Bruker Corporation, Billerica, MA, USA) equipped with a 4.7 T, 25 wide-bore  
285 superconducting magnet operating at a resonance frequency of 50.33 MHz. Operating conditions  
286 were identical to those reported in Baldock et al. (2013) with the exception that only 5000 scans  
287 were obtained for each sample due to the higher C content of the soils in the present study. The  
288 proportional allocation of signal intensity to the following eight chemical shift regions (and the C  
289 compounds they represent) was determined: 0-45 (Alkyl), 45-60 (N-Alkyl/Methoxyl), 60-95 (O-  
290 Alkyl), 95-110 (di-O-Alkyl), 110-145 (Aryl), 145-165 (O-Aryl), 165-190 (Amide/Carboxyl) and  
291 190-215 (Ketone) (Baldock et al. 2004). Diffuse reflectance mid-infrared (MIR) spectra were  
292 acquired for all 240 samples using a Thermo Fisher Nicolet 6700 spectrometer (Waltham, MA)  
293 equipped with a KBr beam-splitter, a DTGS detector and an automated diffuse reflectance  
294 accessory (Pike Technologies, Madison, WI). For each sample, five replicate subsamples were  
295 analyzed by MIR and averaged. Partial least squares regression (PLSR) analyses with full cross  
296 validation were used to derive algorithms for predicting the proportional allocation of sample C to  
297 the various NMR spectral regions using the data acquired for the 60 samples on which NMR  
298 analyses were performed (Baldock et al. 2013). The respective  $r^2$  and root mean standard error of  
299 prediction (MSE) values derived for each prediction algorithm were: alkyl ( $r^2 = 0.89$ , MSE =  
300 1.164), N-alkyl ( $r^2 = 0.62$ , MSE = 0.264), O-alkyl ( $r^2 = 0.87$ , MSE = 1.129), di-O-alkyl ( $r^2 = 0.92$ ,

301 MSE = 0.254), aryl ( $r^2 = 0.71$ , MSE = 0.715), O-aryl ( $r^2 = 0.71$ , MSE = 0.409), carbonyl ( $r^2 = 0.38$ ,  
302 MSE = 0.407) and ketone ( $r^2 = 0.25$ , MSE = 0.164). The MIR/PLSR prediction algorithms were  
303 then applied to all 240 samples to provide estimates of the allocation of C to each NMR spectral  
304 region and thus functional group of C.

305

#### 306 4. *Statistical analysis*

307 We used linear mixed models to assess the effects of island size class, tree removal, shrub removal,  
308 moss removal and their interactions on the GP:GN ratio. Island identity was used as a random  
309 factor in this model. We then applied contrasts and performed post-hoc tests (Tukey's HSD test,  $\alpha$   
310 = 0.05) to assess significant differences in GP:GN ratio among island size or among removal  
311 combinations. We employed redundancy analysis (RDA) on centered and normalized data to test  
312 the relationships between microbial community composition (PLFAs) and soil organic matter  
313 chemistry. The RDA axes were constrained to be linear combinations of the 28 PLFAs and the 8  
314 NMR regions identified. Finally, we used correlation analyses to assess relationships of the GP and  
315 GN bacteria with the most important NMR region associated with these groups (defined by random  
316 forest analysis) (Cutler et al., 2007). We assessed adjusted  $R$ -squared and associated  $p$ -values for  
317 testing the significance of the relationships for each island size class separately, and multiple  
318 comparison tests were applied to test differences in slopes among island size classes. All statistical  
319 analyses were done with R version 3.1.1 (R Development Core Team 2009).

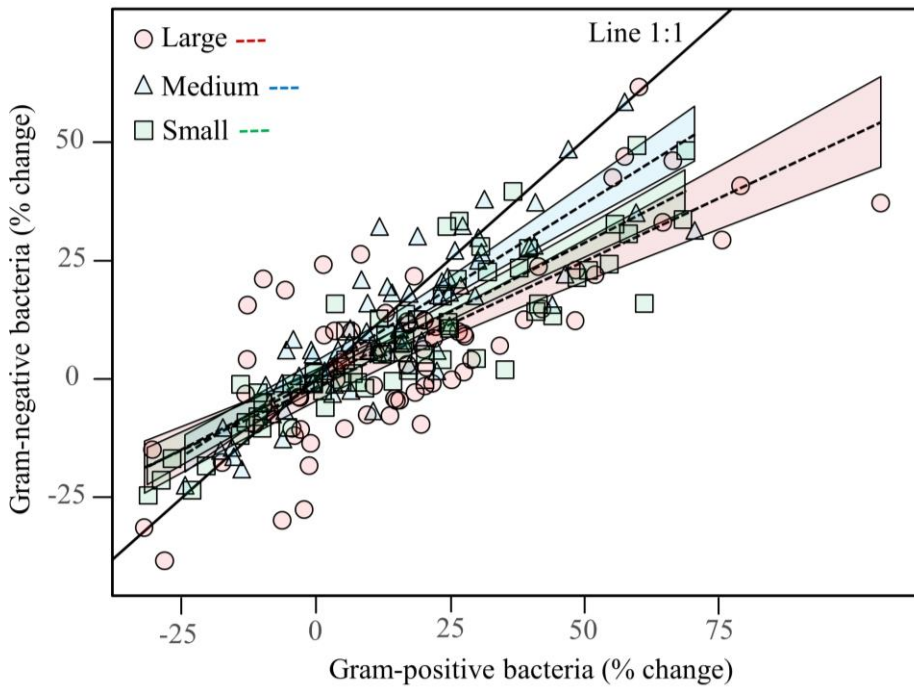
320 **Appendix 1** Variability in soil and vegetation parameters (mean  $\pm$  SE) across the island size classes  
 321 (Wardle et al., 1997; Wardle et al., 2003; Lagerström et al., 2009; Wardle et al., 2012; Clemmensen  
 322 et al., 2013). Within each row, numbers followed by different lowercase letters indicate significant  
 323 differences (Tukey's HSD test,  $\alpha = 0.05$ ).

324

Ecosystem properties	Island size		
	Large	Medium	Small
Time since last fire (years)	585 $\pm$ 233 c	2180 $\pm$ 385 b	3250 $\pm$ 439 a
Net primary productivity (g m <sup>2</sup> yr <sup>-1</sup> )	256 $\pm$ 14 a	247 $\pm$ 12 a	159 $\pm$ 18 b
Standing plant biomass (g m <sup>2</sup> )	9349 $\pm$ 485 a	8340 $\pm$ 877 a	3470 $\pm$ 470 b
Vascular plant species richness	6.6 $\pm$ 0.6 c	8.6 $\pm$ 0.4 b	10.6 $\pm$ 0.6 a
Humus C to N ratio	40.4 $\pm$ 1.18 a	36.0 $\pm$ 1.17 ab	32.9 $\pm$ 0.79 b
Humus C to P ratio	623 $\pm$ 20 b	687 $\pm$ 36 ab	759 $\pm$ 30 a
Humus N to P ratio	15.4 $\pm$ 0.5 c	19.1 $\pm$ 0.9 b	23.3 $\pm$ 1.1 a
Mineral N (MIN) ( $\mu$ g N g <sup>-1</sup> )	38.2 $\pm$ 14.4 ab	58.1 $\pm$ 9.2 a	25.3 $\pm$ 8.0 b
Dissolved org N (DON) ( $\mu$ g N g <sup>-1</sup> )	39.1 $\pm$ 7.2 b	50.7 $\pm$ 5.5 a	40.3 $\pm$ 4.6 b
MIN/(MIN+DON)	0.49 $\pm$ 0.04 a	0.53 $\pm$ 0.05 a	0.39 $\pm$ 0.03 b
Mineral P ( $\mu$ g P g <sup>-1</sup> )	43.6 $\pm$ 4.9 a	37.7 $\pm$ 4.3 a	24.4 $\pm$ 2.3 b
Membrane-extractable P (mmol kg <sup>-1</sup> )	5.9 $\pm$ 0.7 ab	6.5 $\pm$ 0.4 a	4.9 $\pm$ 0.3 b
Light transmission (%)	55.8 $\pm$ 4.5 ab	47.1 $\pm$ 3.7 b	68.6 $\pm$ 2.6 a
pH	3.51 $\pm$ 0.029 a	3.42 $\pm$ 0.027 ab	3.38 $\pm$ 0.039 b

325 **Appendix 2** Relationship between the percentage change in Gram-negative (GN) and Gram-  
326 positive (GP) bacteria after plant functional group removals for each of the three island size classes  
327 (large, medium and small). The percentage change in removal plots was calculated relative to non-  
328 removal plots separately for each island, and compared to the line 1:1. This shows that changes in  
329 the GP:GN ratio after plant functional group removals were due to a higher increase in GP than in  
330 GN. Bands around the regression lines represent 95% confidence intervals.

331



332 **References for Supplementary Methods**

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