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Abundance determines the functional role of bacterial phlotypes in complex communities

Damian W. Rivett^{1,2} and Thomas Bell^{1*}

¹Department of Life Sciences, Silwood Park Campus, Imperial College London, Buckhurst Road, Ascot, SL5 7PY

²Current address: Division of Biology and Conservation Ecology, School of Science and the Environment, Manchester Metropolitan University, Manchester, UK

*correspondence: tbell@ic.ac.uk

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19 **Bacterial communities are essential for the functioning of the Earth's ecosystems¹. A key**
20 **challenge is to quantify the functional roles of bacterial taxa in nature to understand how the**
21 **properties of ecosystems change over time or under different environmental conditions². Such**
22 **knowledge could be used, for example, to understand how bacteria modulate biogeochemical**
23 **cycles³, and to engineer bacterial communities to optimise desirable functional processes⁴.**
24 **Communities of bacteria are, however, extraordinarily complex with hundreds of interacting**
25 **taxa in every gram of soil and every millilitre of pond water⁵. Little is known about how the**
26 **tangled interactions within natural bacterial communities mediate ecosystem functioning, but**
27 **high levels of bacterial diversity have led to the assumption that many taxa are functionally**
28 **redundant⁶. Here, we pinpointed the bacterial taxa associated with keystone functional roles,**
29 **and show that rare and common bacteria are implicated in fundamentally different types of**
30 **ecosystem functioning. By growing hundreds of bacterial communities collected from natural**
31 **aquatic environment (rainwater-filled tree holes) under the same environmental conditions, we**
32 **show that negative statistical interactions among abundant phylotypes drove variation in broad**
33 **functional measures (respiration, metabolic potential, cell yield), while positive interactions**
34 **between rare phylotypes influenced narrow functional measures (the capacity of the**
35 **communities to degrade specific substrates). The results alter our understanding of bacterial**
36 **ecology by demonstrating that unique components of complex communities are associated with**
37 **different types of ecosystem functioning.**

38 The functional roles of bacterial taxa within communities can be estimated using
39 manipulative experiments that build communities from pure cultures or that remove taxa from intact
40 communities⁷. There are many difficulties with experiments using pure cultures: most bacteria cannot
41 be isolated in pure culture, synthetic communities constructed from pure cultures might not represent
42 any natural community, and there are no general methods for knocking-out specific taxa from natural
43 communities. The alternative has been to use observational methods to look for correlated changes in
44 taxa abundance and ecosystem functioning in nature, or to infer which functional processes are
45 important from metagenomic or metatranscriptomic data⁸⁻¹⁰. Observational approaches also have
46 significant weaknesses because abiotic conditions (e.g. pH, temperature) can impact both ecosystem

47 functioning and community structure, making it difficult to infer causal relationships. We developed
48 an alternative experimental approach that exploits the natural variation in bacterial community
49 composition, comparable to the microbiome association studies that have been proposed for inferring
50 causal relationships between human health and microbiome community composition¹¹ (Fig. 1, steps 1
51 to 6).

52 We sampled 753 aquatic microbial communities, which were taken from a natural micro-
53 ecosystem (rainwater pools in the buttressing of beech trees)^{12,13}. The bacterial cells were separated
54 from the surrounding environmental matrix by filtration and then stored (frozen) so that they could be
55 revived (thawed) for repeatable experiments. Each bacterial community was revived and placed in
56 laboratory microcosms containing a sterile beech leaf medium, which simulated some of the
57 environmental conditions in the natural system. We quantified 7 measures of ecosystem functioning
58 associated with leaf litter degradation. Since the communities were assayed in a common
59 environment, variation in ecosystem functioning was due to the initial differences in community
60 composition. Correlations between the initial absolute abundance of each phylotype and ecosystem
61 functioning allowed us to obtain community-wide estimates of the phylotypes that were associated
62 with changes in functioning¹⁴, which could reflect their impact on functioning in nature. While the
63 approach requires growing the communities in simplified microcosms, there is no need to isolate
64 individual taxa. The approach therefore lies between the complexity of natural ecosystems and the
65 artificiality of synthetic communities.

66 We searched for associations between the abundance of each phylotype and the functional
67 measurements, analogous to a genome-wide association scan in genetics¹⁵. We found 182 significant
68 associations between phylotype abundance and the functional measurements, involving 112 of the 522
69 phylotypes used in the analysis. The associations were approximately equally balanced between
70 negative (96 significant associations) and positive (86 significant associations), but differed
71 substantially among the functional measurements (Supplementary Figure 1). We therefore divided the
72 functional measurements into two categories^{16,17}. First: respiration, cell yield, and metabolic potential
73 (ATP; adenosine triphosphate concentration) of the community were categorised as 'broad' ecosystem
74 functions because they amalgamate many activities and are therefore performed by most community

75 members. We expected widespread functional redundancy of taxa within the broad functional
76 measures, which should result in few significant associations. Second: the cleavage rates of 4
77 substrates added to the microcosms were categorised as 'narrow' ecosystem functions because they
78 encompass fewer activities and were therefore likely to be impacted by a relatively limited set of
79 phylotypes. In contrast with the broad functional measures, we expected the rise and fall in abundance
80 of particular niche specialists would be associated with the rise and fall of the narrow functional
81 measurements¹⁸.

82 Contrasting strongly with our predictions, significant associations were largely confined to
83 the broad functional measures. For the broad functional measures, 90% of the 174 significant
84 associations were 'common' phylotypes (phylotypes that exceeded the median phylotype abundance)
85 (Fig. 2, top panel). For example, the two phylotypes with the highest overall abundance across all
86 communities (*Serratia fonticola* and *Klebsiella pneumonia*) also had among the strongest positive
87 associations with respiration and cell yield. By contrast, only 8 significant associations were found
88 between phylotype abundances and the narrow functional measurements (Fig. 2, right panel). The
89 associations imply that the abundance of individual phylotypes play a role in the broad functional
90 measures in ways that are not compensated by fully-redundant phylotypes. There were linear
91 increases in the number of significant associations with increasing sampling effort (number of
92 communities) (Supplementary Figure 2), showing that there are opportunities to uncover many more
93 significant associations.

94 We expected the impact of each bacterial phylotype on the functional measurements to be
95 mediated by the hundreds of phylotypes that surrounded them. We used the same experiment to
96 characterise 'functional interactions': whether the associations between the abundance of each
97 phylotype and the functional measurements were altered by the abundance of any of the other
98 phylotypes in the community (Fig. 1, steps 5 to 6). A positive functional interaction indicated that
99 ecosystem functioning tended to be elevated when both phylotypes had high abundance, whereas a
100 negative functional interaction indicated that ecosystem functioning was lower when both phylotypes
101 had high abundance. Functional interactions might result from direct (i.e. biological) interactions
102 among taxa, but such direct interaction would need to be verified (see Validation Experiment). The

103 method allowed us to reconstruct a complete portrait of functional interactions within a diverse
104 assemblage (Fig. 2, central panel).

105 Theoretical studies have predicted that direct inter-specific interactions (e.g. competition,
106 mutualism) should be negative¹⁹ and weak²⁰, but it is unclear whether these predictions extend to
107 functional interactions. We found that negative and positive functional interactions tended to occur
108 between common phylotypes for the broad functional measurements. In principle, all of the
109 phylotypes contributed to the broad functional measurements, so it was unsurprising that interactions
110 among the most common phylotypes had the largest influence. For the narrow functional
111 measurements, functional interactions were between rare phylotypes, consistent with the idea that
112 narrow functional measurements were driven by niche specialists (Fig. 2, central panel). The approach
113 therefore generated predictions of which phylotypes in the 'rare biosphere' facilitate specific
114 pathways²¹.

115 The large number of significant functional interactions among phylotypes (364 of 368
116 significant interactions) suggested that specific metabolic pathways were maintained by
117 collaborations among rare phylotypes¹⁸. Positive interactions (e.g. cross-feeding) have been known to
118 emerge over short evolutionary timescales in simplified communities²². The narrow functions used
119 here measure the capacity of the communities to produce secreted exo-enzymes that are publicly
120 available, and which might provide a similar mechanism for positive interactions²³. Positive
121 functional interactions were less common for the broad functional measurements (131 of 265
122 significant interactions), with negative interactions concentrated among the most common phylotypes
123 (Fig. 2, central panel). This result is compatible with culture-based studies using isolated bacteria:
124 isolates obtained from the same study system showed a strong tendency for negative functional
125 interactions, and were also common phylotypes in our 16S gene libraries^{24,25}. Within the limitations of
126 the approach, the large number of positive interactions among rare phylotypes have not previously
127 been documented, and could play a key role in generating hypotheses about how rare taxa impact
128 functional processes.

129 The functional interactions uncovered here are correlations, and should be viewed as
130 hypotheses that require independent validation using experiments that isolate each pairwise

131 interaction. In practice, validation experiments face significant challenges because many of the
132 functional interactions are between phylotypes that have not previously been isolated in pure culture,
133 and because the functional interactions might be contingent on the surrounding community. An
134 advantage of using frozen, archived communities is that it is possible to perform *post hoc* 'community
135 mixture' experiments to validate the correlational results. We mixed communities in microcosms in
136 order to place potentially interacting phylotypes in contact. We determined whether the functional
137 interactions we observed (Figure 2) had a biological basis by mixing communities that each contained
138 one member of the interacting pair. Mixtures of communities that placed interacting phylotypes
139 together should increase (positive interaction) or decrease (negative interaction) the functional
140 measurement relative to the mean functional measurement associated with each of the individual
141 communities.

142 We resuscitated 12 communities to validate the positive functional interactions that we
143 observed in the production of hemicellulase. We measured hemicellulase production in microcosms
144 containing each of the communities on its own and all pairwise mixtures of the communities. Under a
145 scenario of no interactions, the hemicellulase production would simply be the average of the
146 constituent communities. We found that the results were consistent with the functional interactions we
147 observed in Figure 2: community mixtures that placed positively-interacting phylotypes together
148 showed a significantly elevated hemicellulase activity (Figure 3). The result implies that the
149 functional interactions (Figure 2) resulted from direct (causal) interactions, but further work would be
150 needed to identify mechanisms.

151 There are important constraints to the approach described here. Notably, all communities
152 must be tested in a common environment. Since the associations and functional interactions likely
153 depend on environmental conditions (e.g. the abundance and complexity of the available resources),
154 the associations between the phylotypes and the functional measurements in the microcosms might
155 differ from those in nature. Although we found a good correspondence between community
156 composition in the microcosms and in the natural community (Supplementary Figure 3), important
157 changes to community structure also arose due to the experimental manipulations (freezing, fungicide
158 addition). The simple analyses presented here could also be improved and extended. For example, we

159 used linear relationships, but non-linear relationships are common. We also applied a conservative p-
160 value correction, likely resulting in many false negatives. We also tested associations for every
161 phylotype, whereas dimensionality reduction (e.g. by clustering phylotypes with similar abundance
162 profiles across the communities) followed by multivariate analysis could yield models that better
163 predict functioning. Here we have presented the simplest possible analysis, and expect that further
164 studies using more complex analyses may uncover further interesting biology.

165 The results here are among the first to document strong relationships between structure and
166 function in complex, non-synthetic communities under controlled conditions. Natural bacterial
167 communities have previously been painted as world of vast functional redundancy, where high levels
168 of niche overlap among phylotypes buffer ecosystem functioning against extinction²⁶. It is clear from
169 our results that the abundance of phylotypes is significantly associated with a range of functional
170 measurements, both through their direct effects and through their interactions with other phylotypes.
171 In both environmental microbiology and medical microbiology, there is a recognition that even those
172 diseases or phenotypes that are caused by individual bacterial strains are mediated by complex
173 interactions with many other taxa²⁷. Application of the common garden method can provide an
174 important window on the functional role phylotypes, and could in the future provide a method for
175 unravelling the complex interactions among the thousands of phylotypes that inhabit natural
176 environments.

177

178 **Correspondence.** Thomas Bell, thomas.bell@imperial.ac.uk

179

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183

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185 DWR. Analysis and writing by TB and DWR.

186

187 **Competing interests.** The authors declare no competing interests.

188

189 **Additional information.** Supplementary information is available for this paper.

190

191 **Methods**

192 **Microbiome collection.** We sampled 753 beech (*Fagus* sp.) rainwater-filled tree-holes during August
193 2013 to April 2014 from locations across the south of England, primarily located in the Chilterns west
194 of London. These miniature aquatic habitats have been used extensively as 'natural microcosms'²⁸ that
195 house diverse and accessible microbial communities. The water from each tree-hole was homogenised
196 by stirring, after which we collected a 1 ml sample, which was kept at ambient temperature until the
197 samples were returned to the laboratory (<24 hours). Each sample was diluted 1:4 in sterile phosphate
198 buffered saline (pH 7.0, Sigma-Aldrich) prior to filtration (pore size 20-22 µm, Whatman 4 filter
199 paper) to remove debris and large organisms. The filtrate containing the communities was
200 used to inoculate 5 ml into a sterile beech leaf medium supplemented with 200 µg ml⁻¹ cyclohexamide
201 (Sigma-Aldrich) to inhibit fungi. Although fungi were excluded here in order to simplify the
202 communities, fungi are likely to be important decomposers in this ecosystem, and would therefore be
203 useful additions to future studies. Beech leaf medium was created by autoclaving 50 g of dried beech
204 leaves in 500 ml of PBS²⁹, which gave a concentrated stock after filtration of coarse particles. Beech
205 leaf medium is composed of a complex array of carbon sources, which are exploited to differing
206 degrees by bacteria isolated from the tree holes²⁹. Each microcosm (polypropylene centrifuge tube)
207 was incubated at 22°C under static conditions for one week to allow communities to reach stationary
208 phase (Supplementary Figure S4). Each regrown community was stored at -80°C after addition of
209 freezing solution (final concentration 30% v/v glycerol and 0.85% w/v NaCl). Communities were
210 stored frozen so as to allow repeatable experiments using the same starting community compositions.
211 Community composition of the frozen communities was assessed using Illumina MiSeq (250bp-
212 paired end) sequencing performed by Molecular Research DNA (www.mrdnlab.com). The V4
213 region of the 16S rRNA gene was amplified, using primers 515f/806r with the forward primer
214 barcoded. Sequences were curated using a propriety analysis pipeline by Molecular Research DNA;

215 any sequences <150bp and those with ambiguous base calls removed, prior to denoising and editing
216 for chimeras. Operational taxonomic units were specified at a 97% similarity cut-off, which we refer
217 to as 'phylotypes' in the text³⁰. We randomly sampled 15,000 sequences per sample to normalise
218 sequencing effort. We used the number of reads per phylotype as a measure of their relative
219 abundance in the community. Although many biases are introduced during the DNA extraction and
220 PCR steps, these biases would be applied equally across the experiment. Rarefaction curves indicated
221 that we had sampled most of the diversity going into the microcosms (Supplementary Figure S5), and
222 extrapolating to 20,000 sequencing reads³¹ (number of cells inoculated into each microcosm)
223 indicated that we captured on average 95% of the total phylotype richness in the samples. We used a
224 microbial community standard (Zymo Research) as a positive control to assess the degree to which
225 our 16S rRNA gene amplicon sequencing produced biased estimates of abundance. We found good
226 correspondence between observed and expected abundances for most classes of bacteria, but
227 Enterococcaceae and Listeriaceae were considerably under-represented, while over-
228 Enterobacteriaceae was over-represented (Supplementary Figure S6).

229
230 **Common garden experiment.** Microcosms were established in 1.2 ml deep-96-well plates containing
231 840 µl sterile beech leaf medium and inoculated with 40 µl of each revived (thawed) community (4
232 replicates per community, 3172 microcosms in total). Cell densities within the communities were
233 consistent across samples (mean 4.9×10^5 cells ml⁻¹ \pm 2.1×10^4 s.e.m), thus each microcosm was
234 initiated with on average 19,600 cells. The sequencing effort (15,000 reads per sample) was similar to
235 the number of cells used to initiate the microcosms, so we assumed the communities were almost fully
236 characterised. We multiplied the relative abundances obtained from the sequencing by the initial cell
237 numbers to obtain an estimate of absolute abundance of each phylotype at the beginning of the
238 experiment. Although there are known biases in using amplicon sequencing data to measure relative
239 abundance (e.g. due to PCR conditions or DNA extraction methods), we expected those biases to be
240 the same across the microcosms. The microcosms were incubated under static conditions at 22°C for
241 7 days, after which we quantified each of the measurements of ecosystem functioning. Our intention

242 was not to mimic the precise conditions of their native environment since those conditions differed
243 among the communities. However, the microcosms successfully re-created an environment that
244 produced both a standing density (mean 1.7×10^5 cells ml⁻¹ \pm 6.9×10^3 s.e.m) and communities
245 (Supplementary Figure S4) that were similar to the native environment. We also tracked cell densities
246 over 12 days in 32 randomly chosen communities (Supplementary Figure S4). These data showed that
247 all of the communities had reached carrying capacity well before 7 days, implying that there would
248 have been competition both for labile substrates (typically used during growth phase) and for more
249 recalcitrant substrates (typically important later in succession).

250 We categorised community respiration, cell yield, and metabolic potential as 'broad'
251 functional measurements. Bacterial respiration was measured using the MicroResp CO₂ detection
252 system (www.microresp.com) according to the manufacturer instructions with absorbance readings
253 converted to weight of CO₂ using a linear log-log relationship ($R^2 = 0.965$; Supplementary Figure S7).
254 Respiration measurements were taken as the cumulative respiration of the whole community over the
255 7-day incubation period. Yield was the final abundance of all bacterial cells in the community, which
256 was quantified by staining the cells with thiazole orange (42 nM, Sigma-Aldrich) followed by
257 obtaining absolute counts using a C6 Accuri flow cytometer (size threshold of 8000 FSC-H), with
258 cells gated on the SSC-A and FL1-A (533/30) channels. We used a threshold of 800 fluorescence
259 units to distinguish cells from detritus. Potential metabolic activity was measured as the adenosine
260 triphosphate (ATP) concentration within the community, measured using a Biotek Synergy 2
261 multimode plate reader and the BacTitr-Glo Cell Viability assay (Promega). A linear relationship was
262 observed between concentration and luminescence ($R^2 = 0.998$), therefore values were converted to
263 nM ATP.

264 We categorised the degradation of specific substrates as 'narrow' functional measurements.
265 We measured the breakdown of substrates labelled with 4-methylumbelliferone (MUB)³². Production
266 of the appropriate exo-enzyme within the community induces a fluorescent signal that was quantified
267 on a Biotek Synergy 2 multimode reader (Ex/Em: 365 nm/ 445 nm). Samples were incubated with 40
268 μ M of the substrates (100 μ l total volume) and incubated in the dark under the same conditions as the

269 microcosms (static, 22°C) for 60 minutes. After the incubation, 10 µl of 1M NaOH was added and the
270 fluorescence measured over four minutes with the maximum value recorded. Fluorescent values were
271 converted to nM MUB after establishing a linear relationship between MUB concentration and
272 fluorescence ($R^2 = 0.996$) and using negative controls to account for any auto-fluorescence in the
273 medium. The assays quantified the capacity of the communities to degrade the substrates within the
274 microcosms rather than the actual rates at which the substrates were being cleaved *in situ*. Since the
275 medium was derived from beech leaf litter, we selected substrates that were targeted by enzymes
276 associated with organic matter degradation, including xylosidase (cleaves the labile substrate xylose, a
277 monomer prevalent in hemicellulose), β -chitinase (breaks down chitin, which is the main component
278 of arthropod exoskeletons and fungal cell walls), β -glucosidase (break down cellulose, the structural
279 component of plants), and phosphatase (break down organic monoesters for the mineralisation and
280 acquisition of phosphorus).

281

282 **Common garden analysis.** We used linear regressions to relate the functional measurements to
283 phylotype abundance across the sites. For simplicity, we averaged the functional measurements across
284 the 4 replicates, which yielded functional measurements from 753 communities. We excluded
285 phylotypes from the analysis that were rare (<100 individuals across all samples) or that only occurred
286 (abundance > 0) in <10 samples, which reduced the number of phylotypes in the analysis from 1341
287 to 522. These rarest phylotypes were excluded because individual data points frequently had high
288 leverage in the regressions, and because pairwise interactions were undefined because there was no
289 covariation in abundances between rare phylotypes. *One-way associations:* We performed linear
290 regressions between the initial absolute abundance of every phylotype and every functional
291 measurement: $y = b_0 + b_1 \log_{10}(s_1 + 1)$, where y is the functional measurement, b_0 is the intercept, b_1 is
292 the slope associated with phylotype 1, and s_1 is the absolute abundance (number of cells per
293 microcosm) of phylotype 1 at the start of the experiment. We used the significance of the slope as an
294 indication of whether the phylotype was associated with the functional measurement. P-values were
295 corrected for multiple testing across all of the one-way-analyses using a Bonferroni correction,
296 yielding a threshold p-value of 1.4×10^{-5} . *Two-way functional interactions:* We performed linear

297 regressions that related the functional measurements to every pair of phylotypes: $y = b_0 + b_1s_1 + b_2s_2 +$
298 $b_3(s_1 \times s_2)$, where y is the functional measurement, b_0 is the intercept, b_1 and b_2 are the slopes
299 (coefficients) associated with phylotype 1 (s_1) and phylotype 2 (s_2), and b_3 is the coefficient associated
300 with the interaction between phylotype 1 and phylotype 2 ($s_1 \times s_2$). We used b_3 as an estimate of the
301 'functional interaction' between each pair of phylotypes. P-values associated with b_3 were corrected
302 for multiple testing using a Bonferroni correction, yielding a significance threshold of 5.3×10^{-8} .
303 Significant functional interactions imply that correlated changes in pairs of phylotype abundance are
304 directly linked to changes in functioning, but such causal links would need to be verified with
305 experiments.

306

307 **Validation experiment.** We revived (thawed) 12 communities to validate the functional interactions.
308 We replicated the common garden experiment (above), by dispensing 40 μ l of community in to 840 μ l
309 of sterile beech leaf medium. The 40 μ l of community contained either a single community, or a
310 50:50 mixture of two communities. When mixing communities, many phylotypes would be
311 simultaneously put into contact. This was particularly problematic for the broad functions, since our
312 results indicated that community mixtures would bring together both positively- and negatively-
313 interacting phylotypes. We therefore focused on hemicellulase production because our analysis
314 indicated that all of the significant functional interactions were positive, making qualitative
315 predictions straightforward. Communities were incubated at 22°C for 7 days after which the
316 hemicellulase activity was recorded as described above. We categorised the communities according
317 to whether they contained none, one, or both interacting phylotypes from among any of the
318 significantly interacting phylotype pairs (Fig. 2, central panel). Mixed communities that placed two
319 interacting phylotypes together that were not found together in either of the constituent communities
320 would be expected to 'realise' their functional interaction. For the communities we examined, the
321 following significantly-interacting phylotypes pairs were combined: *Deinococcus hohokamensis* x
322 Acidimicrobiales spp., *Solibacter* spp. x *Aurantimonas manganoxydans*, *Solibacter* spp. x *Legionella*
323 spp., *Deinococcus hohokamensis* x *Leptospirillum* spp., *Anaerospobacter* spp. x

324 *Pseudoclavibacter* spp, *Epilithonimonas lactis* x *Pseudoclavibacter* spp. The phylotype pairs have not
325 previously been implicated in hemicellulose degradation so far as we are aware.

326 There were 9 of 65 community mixtures that placed interacting phlotypes into contact
327 ("Interactions") whereas the remainder did not place interacting phlotypes into contact ("No
328 interactions"). We tested the hypothesis that hemicellulase production exceeded what would be
329 expected under a null model. We assumed that, in the absence of any new interactions, mixed
330 communities should simply be the mean of the communities in the mixture. For all of the community
331 mixtures, we therefore subtracted the mean hemicellulase production in each constituent single
332 phylotype community from the observed hemicellulase production in the mixture. We conducted a t-
333 test to determine whether the deviation of hemicellulase production from this null expectation was
334 higher in mixed communities with "Interactions" than communities with "No interactions".

335

336 **Data availability**

337 The data used in the analysis are available in the FigShare repository with digital object identifiers
338 [10.6084/m9.figshare.6100181](https://doi.org/10.6084/m9.figshare.6100181) (phylotype table) and [10.6084/m9.figshare.6100340](https://doi.org/10.6084/m9.figshare.6100340) (functional data).
339 Sequence data that support the findings of this study have been deposited in NCBI Short Read
340 Archive with accession number SRP145037.

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407 **Figures**

408

409 **Figure 1. Illustration of the workflow for microbiome association studies in common gardens.**

410 **(Step 1)** Samples were collected from rainwater-filled tree holes, and **(Step 2)** returned to the lab,
411 where **(Step 3)** they were archived at -80C and a subsample was sequenced to identify community
412 composition. Sequencing revealed 5 phylotypes (A to E) were present in the communities. **(Step 4)**
413 Frozen microbiomes were resuscitated and grown in a standardised beech leaf medium while
414 measuring ecosystem functioning. **(Step 5, 6)** A systematic search for associations between the
415 abundance of each phylotype (A to E, from Step 3) and the functional measurements (from Step 4)
416 identified a significant positive association between functioning and the abundance of Phylotype C, as
417 well as a significant functional interaction between Phylotype C and Phylotype D. Broad functional
418 interactions are above the diagonal (purple), while narrow functional interactions are below the
419 diagonal (blue). **(Step 7)** To validate the interaction, we searched for communities where Phylotype C
420 and D did not co-occur (C only, D only), or where they co-occurred (Both) or were both absent
421 (None). **(Step 8)** The positive functional interaction between Phylotype C and D (identified in step 6)
422 was tested by looking for an increase in functioning in mixtures that combined C-only and D-only
423 communities (red box). **(Step 9)** The large increase in ecosystem functioning when mixing C-only and
424 D-only communities (red point), and the lack of similar result for the other mixtures (green points),
425 confirmed the functional interaction identified in step 6.

426

427 **Figure 2. Associations between bacterial phylotypes and the functional measurements. Left**

428 **panel:** each of the phylotypes were ranked according to their relative abundance (%N) across all 753
429 communities. **Bottom panel:** the range of each phylotype, which is the proportion of samples where
430 each phylotype was present. **Top panel:** association between every phylotype and each of the broad
431 functional measurements. Each datapoint is the $\log_{10}(\text{p-value})$ of the slope of the linear regression
432 between the abundance of each phylotype and the functional measurements. Grey datapoints are non-
433 significant, black datapoints are significant following a Bonferonni correction. Negative values
434 indicate a negative association (the functional measurement declines with increased phylotype

435 abundance) while positive values indicate a positive association. Symbols correspond to the broad
436 functional measurements shown in the upper triangle of the central panel. **Right panel:** association
437 between each of the phylotypes and the narrow functional measurements. Pale blue datapoints are
438 non-significant, and black datapoints are significant following a Bonferonni correction. Negative
439 values indicate a negative association (the functional measurement declines with increased phylotype
440 abundance) while positive values indicate a positive association. Symbols correspond to the narrow
441 functional measurements in the lower triangle of the central panel. **Central panel:** Functional
442 interactions between all pairwise combinations of phylotypes for the broad (above diagonal) and
443 narrow (below diagonal) functional measurements. Significant positive (red) and negative (blue)
444 functional interactions are shown as symbols, with larger symbols being more significant (see key).
445 Symbol definitions are given for broad functions (above diagonal, top left) and narrow functions
446 (below diagonal, bottom right). The violet (above diagonal) or teal (below diagonal) background
447 indicates non-significant interactions, while a white background indicates an interaction that cannot be
448 estimated (e.g. because the phylotypes never co-occur). Phylotypes are increasingly abundant from
449 bottom-to-top and from left-to-right within the central panel. Relationships were analysed using
450 simple- or multiple regression. All datapoints indicated as significant are at a Bonferreonni-adjusted
451 p-value threshold. P-values for the top and left panels are given in Supplementary Table 1, and p-
452 values for the central panel are given in Supplementary Table 2.

453

454 **Figure 3. Validation of the functional interactions using community mixture experiments.** When
455 communities were mixed together, hemicellulase activity was expected to be the mean activity of the
456 two communities in the mixture. The y-axis is the deviation from this expectation, with positive
457 values indicating that hemicellulase activity in the mixed communities exceeded the mean of the two
458 constituent communities. Hemicellulase activity was elevated in pairwise mixtures of communities
459 that placed interacting phylotypes together ("Interactions", n = 9 mixtures) but not in communities
460 that did not place interacting phylotypes together ("No interactions", n = 56 mixtures) (analysis-of-
461 variance, $F_{1,63} = 5.1$, $p = 0.027$). The boxes show the data quartiles, with the dark grey line indicating

462 the mean. Data points are individual mixtures average across $n = 4$ replicates. Mixture were created
463 from 12 communities.