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Citation for final published version:

O'Leary, Jade ORCID: <https://orcid.org/0000-0002-1684-9880>, Journeaux, Katie L., Houthuijs, Kas, Engel, Jasper, Sommer, Ulf, Viant, Mark R., Eastwood, Daniel C., Muller, Carsten ORCID: <https://orcid.org/0000-0003-0455-7132> and Boddy, Lynne ORCID: <https://orcid.org/0000-0003-1845-6738> 2021. Space and patchiness affects diversity-function relationships in fungal decay communities. ISME Journal 15 , pp. 720-731. 10.1038/s41396-020-00808-7 file

Publishers page: <https://doi.org/10.1038/s41396-020-00808-7>  
<<https://doi.org/10.1038/s41396-020-00808-7>>

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1 **Space and patchiness affects diversity-function relationships in fungal decay communities**

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3 Running title: Diversity-function relationships

4

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14

15

16 **Abstract**

17 The space in which organisms live determines health and physicality, shaping the way in which they  
18 interact with their peers. Space, therefore, is critically important for species diversity and the  
19 function performed by individuals within mixed communities. The biotic and abiotic factors defined  
20 by the space that organisms occupy are ecologically significant and the difficulty in quantifying  
21 space-defined parameters within complex systems limits the study of ecological processes. Here, we  
22 overcome this problem using a tractable system whereby spatial heterogeneity in interacting fungal  
23 wood decay communities demonstrates that scale and patchiness of territory directly influence  
24 coexistence dynamics. Spatial arrangement in 2- and 3- dimensions resulted in measurable  
25 metabolic differences that provide evidence of a clear biological response to changing landscape  
26 architecture. This is of vital importance to microbial systems in all ecosystems globally, as our results  
27 demonstrate that community function is driven by the effects of spatial dynamics.

28

29

## 30 **Introduction**

31 Space determines the nature of and scale over which individuals meet and interact. The  
32 characteristics of the discrete spatial habitat which an organism occupies affects individual  
33 competitive success with a bottom-up effect on population-wide colonisation, speciation and  
34 extinction [1]. There is a dynamic link between spatial ecology and competitive success where  
35 transitive (where species A outcompetes B, which outcompetes C) communities with a strict  
36 competitive hierarchy become intransitive ( $A > B$ ;  $B > C$ ;  $C > A$ , like the game of rock-paper-scissors)  
37 when competing in a spatially more complex system [2], allowing individuals outcompeted under  
38 some scenarios to coexist with their competitors [3]. Dimensionality of habitat landscapes influences  
39 individual behaviour [4], and stochasticity of species interactions results in changes to the pool of  
40 community-produced metabolites [5], altering individual combative ability, community succession  
41 and structure between 2- and 3-dimensional landscapes [6]. Despite these findings, the mechanisms  
42 that influence stability and succession in the context of how communities occupy and exploit space  
43 are rarely adequately quantified as most ecological study systems are too complex and largely  
44 intractable. A model system is needed that allows such quantification, and understanding of how  
45 altered dynamics, coexistence and community-scale biodiversity in the context of space underpins  
46 changes in community function.

47 The functional diversity-area relationship, i.e. the correlation between increased habitat size and  
48 greater functional diversity, is one theory explaining how space mediates function in biodiverse  
49 communities [7]. However, the model does not account for effects of distributions and patch  
50 dynamics of species within habitats of varied area, yet these factors cause competitive communities  
51 to shift between hierarchical transitive and non-hierarchical intransitive relationship states [2,8].  
52 Non-hierarchical intransitivity is an established mechanism of coexistence [9,10] and is thought to be  
53 associated with an intrinsically related functional-diversity mechanism [11,12]. Wood decay fungi  
54 offer an ideal ecologically relevant model for the study of these processes as individual mycelia

55 occupy columns of decay forming complex 3-dimensional communities in wood and, through their  
56 decomposition activities release carbon and nutrients [13]. They typically form a hierarchical  
57 community structure (tertiary/late stage colonists outcompete secondary colonists which in turn  
58 outcompete primary/earliest colonists) [14] and competitive interactions between species can be  
59 easily observed [15]. In addition to compounds that primarily function in the exploitation and  
60 decomposition of lignocellulose [16,17], these fungi produce a plethora of potentially antagonistic  
61 compounds which function in changing territory, and differ in quantity and identity during  
62 interspecific competition [6,18,19,20].

63 Here, we used a tractable system of wood decay fungi to quantify the impact of space on the  
64 mechanisms of coexistence and community composition, in the context of its occupation and  
65 exploitation. We compared the combative abilities of fungi in linear 2-dimensional systems and  
66 species richer 3-dimensional systems. The study system allowed more detailed analysis of  
67 community dynamics between 3-dimensional systems where fungi were dispersed in evenly spaced  
68 patches and 3-dimensional systems where the weakest member of the community occupied the  
69 same volume but as a larger adjacent patch size. Previously, our data revealed an emergent property  
70 where intransitivity promoted biodiversity in more spatially diverse 3-dimensional systems where  
71 territory was less fragmented [2]. Here, we assess the underlying mechanisms causing community  
72 stability and coexistence dynamics to change. We do this by measuring the metabolic response of an  
73 individual to changing coexistence dynamics across spatial scales. Our large-scale untargeted  
74 metabolomics and other chemical methods analysed a comprehensive network of intracellular,  
75 extracellular and gas-phase metabolic products produced during community interactions. We  
76 hypothesised that stochasticity of species would influence functional biochemical processes, and  
77 that changes to metabolites involved in pathways for resource utilisation and antagonism would  
78 alter coexistence dynamics and community composition. Spatially heterogeneous systems  
79 containing non-hierarchical communities promote biodiversity [2,21], and here we deepen our

80 understanding of this concept with the novel finding that space occupied alters metabolic function  
81 and coexistence, therefore moderating the diversity-function relationship.

82

## 83 **Methods**

### 84 *Experimental design and sampling*

85 We constructed pair-wise interactions of 2 cm<sup>3</sup> *Fagus sylvatica* (beech) blocks that had been  
86 pre-colonised for 12 weeks by placing on agar (5 gL<sup>-1</sup> malt extract, 15 gL<sup>-1</sup> agar; Lab M, UK) cultures  
87 of field isolates (fruit body/wood) of *Vuilleminia comedens* (strain VcWvJH1; a primary coloniser),  
88 *Trametes versicolor* (strain TvCCJH1; a secondary coloniser) and *Hypholoma fasciculare* (strain Hf  
89 DD3; a late secondary coloniser) wood decay fungi (maintained in the Cardiff University fungus  
90 culture collection), which all co-exist in nature, at 20 °C (as in [2]). Interactions were performed in  
91 all combinations including, self-pairings (n = 10). We also constructed 3x3x3 27-block cubes  
92 containing 9 blocks of each species. 27-block cubes were arranged with all three species dispersed  
93 and no two blocks of the same species in contact (n = 10), and also so that all 9-blocks of the  
94 weakest competitor, *V. comedens*, occupied an adjacent volume while the other two fungi were  
95 dispersed (n = 10) (Fig. 1). Plus, entire 27-block assemblages containing each fungus solely were  
96 made [2]. Blocks in pair-wise interactions were arranged with cut vessel ends touching, and in  
97 27-block cubes blocks were joined such that some cut vessel ends were touching but others were  
98 not, but all vessels were parallel (Fig. 1). Fungal species and specific strains were selected based on  
99 their successional order and expected combative hierarchy in the natural environment:  
100 *H. fasciculare* > *T. versicolor* > *V. comedens* [22,23,24] (Supplementary Table 1). Interacting  
101 combinations of wood blocks were incubated individually at 20 °C in 70 ml and 500 ml polypropylene  
102 pots for pair-wise and 27-block cubes respectively, and were laid upon a layer of perlite (20 ml and  
103 85 ml) containing 2 ml and 12 ml of water respectively which was maintained by weekly addition of  
104 water to retain the original water content, as detailed in [2]. After 1 d and 28 d volatile organic

105 compound (VOC) production was measured (described below), and we deconstructed n = 5  
106 systems. Each individual block was split along the grain into quarters, and three of the quarters from  
107 each block were foil wrapped, flash frozen in liquid nitrogen and stored at -80 °C. These were  
108 subsequently analysed for enzyme activity and metabolomics (see below). For the remaining  
109 quarter, two chips (~2 mm<sup>3</sup>) were taken from an inside face and inoculated onto 2 % malt agar,  
110 incubated at 20 °C, then emerging mycelia identified morphologically, to determine which species  
111 occupied the wood (Fig. 2). We removed the chip excised face from the quarter by splitting with a  
112 chisel, and determined final density as dry weight (80 °C for in excess of 72 h) per fresh volume (cm<sup>3</sup>)  
113 (blocks sampled at 28 d only), and the rate of decay estimated by comparison with density of blocks  
114 scarified (n = 10) at the end of the pre-colonisation period.

115

#### 116 *Overview of metabolite analysis*

117 To quantify changes to metabolic function (antagonistic chemicals and compounds for habitat  
118 exploitation/wood decomposition) associated with different spatial dynamics in response to  
119 changing community composition, we extracted and measured: the profile of VOCs from the  
120 headspace of interactions, the activity of 12 targeted enzymes, chosen because they are directly  
121 involved in interspecific competition [6,19]. We also conducted ultra-high performance liquid  
122 chromatography-mass spectrometry (UHPLC-MS) metabolomics analysis. All analyses were  
123 conducted after 1 and 28 d interactions in blocks originally colonised by *T. versicolor* (n = 5), and in  
124 27-block interactions 'pseudo'-replicates representing different spatial locations within  
125 3-dimensional cube arrays were analysed (all n = 5). We chose to target *T. versicolor* since the fungus  
126 has been well characterised in the past [16,19,25], and because it neither dominated systems (as did  
127 *H. fasciculare*) nor was it driven to near extinction (as was *V. comedens*). There were no significant  
128 differences (ANOVA: p > 0.05) in enzyme activity or small metabolite levels between

129 pseudoreplicates of *T. versicolor* in 3-dimensional cubes, so we pooled activities of all  
130 pseudoreplicates for each system (therefore, n = 15).

131

### 132 *VOC extraction and data-pre-processing*

133 We collected VOCs from the headspace of interactions after 1 d and 28 d (n = 3) by inserting pots  
134 individually and lidless into a multi-purpose roasting bag (46 × 56 cm; Lakeland, UK), which was  
135 sealed for 30 min to allow VOCs to equilibrate in the headspace. Then, 500 ml headspace gas was  
136 collected onto thermodesorption (TD) tubes (Tenax TA & Sulficarb, Markes International Ltd.) using  
137 an EasyVOC manual pump (Markes International Ltd., UK).

138 VOCs were desorbed using a TD100 thermodesorption system (Markes International Ltd., UK) with  
139 the following settings: tube desorption 10 min at 280 °C, at a trap flow of 40 ml min<sup>-1</sup>; trap  
140 desorption and transfer 40 °C s<sup>-1</sup> to 300 °C, with a split flow of 20 ml min<sup>-1</sup> into gas chromatograph  
141 (GC; 7890A; Agilent Technologies Inc., USA). VOCs were separated over 60 m, 0.32 mm I.D., 0.5 µm  
142 Rx5ms (Restek, UK) with 2 ml min<sup>-1</sup> helium as carrier gas under constant flow conditions using the  
143 following temperature program: 35 °C for 5 min, 5 °C min<sup>-1</sup> to 100 °C, hold 5 min. Mass spectra were  
144 recorded from *m/z* 30 to 350 on a time-of-flight mass spectrometer (BenchTOF-dx, Markes  
145 International Ltd., UK). C8-C20 alkane standard (0.5 µl, Supelco) was loaded onto a blank  
146 thermodesorption tube as a retention standard and quality control (QC).

147 GC-MS data checked with MSD ChemStation software (E.02.01.1177; Agilent Technologies, Inc) and  
148 chromatograms were deconvoluted and integrated with AMDIS (NIST11) using a custom retention-  
149 indexed mass spectral library. MS spectra from deconvolution were searched against the NIST 2011  
150 library (Software by Stein et al., version 2.0 g, 2011). VOCs scoring > 80% in forward and backward fit  
151 and a retention index (RI) match of ± 15 were included into the custom mass spectral library as  
152 putatively identified VOCs; VOCs scoring > 80% in forward and backward fit and no RI match were

153 included as chemical class, e.g. alkane, alkanol and recurrent components that did not show either  
154 the required mass spectral fit or RI match were added as 'unknown'. Peak list from integration with  
155 AMDIS were aligned using the pivot function in Excel in preparation for subsequent statistical  
156 analysis.

157

158

159

#### 160 *Enzyme assays*

161 For enzyme assays, we freeze dried the *T. versicolor* frozen blocks for 48 h (Edwards Modulyo, UK),  
162 then ground them to sawdust using a spice and coffee grinder (Wahl James Martin, UK). 0.5 g of  
163 sawdust was added to 5 ml of 50 mM sodium acetate buffer and shaken overnight at 4 °C. For pair-  
164 wise interactions, *T. versicolor* blocks from all interactions and one block from its respective  
165 self-pairing were used (n = 5). In 27-block cubes, each fungus occupied 3 different spatial positions in  
166 both of the mixed-species assemblages, and *T. versicolor* occupied 4 spatial positions within the  
167 assemblage which it fully occupied. For each spatial position for *T. versicolor* blocks within 27-block  
168 systems (excluding the central-cube position which *T. versicolor* occupied when it was the sole  
169 occupant, as this was not represented in the mixed-species assemblages), 5 replicates were used for  
170 assays (full details in Supplementary Table 2).

171 The activities of the following terminal hydrolases were measured using 4 methylumbelliferol  
172 (MUF)-based substrates:  $\beta$ -glucosidase (EC 3.2.1.21),  $\alpha$ -glucosidase (EC 3.2.1.20), cellobiohydrolase  
173 (EC 3.2.1.91),  $\beta$ -xylosidase (EC 3.2.1.37), N-acetylglucosaminidase (EC 3.2.1.30), phosphodiesterase  
174 (EC 3.1.4.1), phosphomonoesterase (EC 3.1.3.2) and arylsulfatase (EC 3.1.6.1). Briefly, substrates  
175 (40  $\mu$ l in dimethylsulphoxide) at final concentration of 500 mM were combined with three technical  
176 replicates of 200  $\mu$ L of samples (diluted 1:10) in a 96 well plate. Background fluorescence was

177 determined by combining 200  $\mu$ L sample (diluted 1:10) with 40  $\mu$ L MUF standards. The 96 well plates  
178 were incubated at 40 °C and fluorescence recorded at 5 and 125 min using a Tecan Infinite  
179 microplate reader (Tecan, Switzerland) with an excitation wavelength of 355 nm and an emission  
180 wavelength of 460 nm. Quantitative enzymatic activities were calculated after blank subtraction  
181 based on a standard curve of MUF. One unit of enzyme activity was defined as the amount of  
182 enzyme releasing 1 nmol of MUF min<sup>-1</sup>.

183 Laccase (phenoloxidase; EC 1.10.3.2) activity was determined by monitoring the oxidation of 2,2'-  
184 azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) in citrate phosphate  
185 buffer (100 mM citrate, 200 mM phosphate, pH 5.0), by monitoring the formation of green  
186 colouration spectrophotometrically at 420 nm. Three technical replicates were performed for each  
187 sample.

188 Manganese peroxidase (MnP; EC 1.11.1.13) activity was determined by monitoring  
189 spectrophotometrically at 595 nm the purple colouration from oxidative coupling of  
190 3-methyl-2-benzothialone-hydrazone hydrochloride (MBTH) and 3-(dimethyl amino)-benzoic acid  
191 (DMAB) in succinate-lactate buffer (100 mM, pH 4.5). Three technical replicates were performed for  
192 each sample. The results were corrected by activities of samples without manganese, and with  
193 ethylene diamine tetraacetate (EDTA) to chelate any Mn<sup>2+</sup> present in the samples, allowing  
194 detection of Mn<sup>2+</sup>-independent peroxidases (versatile peroxidase). The results were also corrected  
195 by activities of samples in the absence of H<sub>2</sub>O<sub>2</sub>, allowing detection of oxidase (but not peroxidase)  
196 activity.

197 For each enzyme, n = 3 technical replicates were performed and enzyme activities were normalised  
198 to the protein content of each sample, which was determined using Qubit™ fluorometric assays  
199 (ThermoFisher Scientific Inc., UK).

200

201 *Metabolomics analysis*

202 As for enzyme assays, we selected blocks pre-colonised by *T. versicolor* (n = 5) upon which to  
203 perform UHPLC-MS (Supplementary Table 2). 0.5 g of sawdust was added to 1666 µl of each of H<sub>2</sub>O,  
204 methanol and chloroform, vigorously vortexed and sonicated for 15 min (Elmasonic S150, Singen,  
205 Germany). The extracts were allowed to sit until the polar (containing H<sub>2</sub>O and methanol) and non-  
206 polar (containing mostly chloroform) layers separated, then removed 1500 µl of the upper layer  
207 containing the polar metabolite extracts. The extracts were centrifuged for 5 min at 17,000 x g  
208 (Biofuge, Thermo Fisher Scientific, MA, USA), and 200 µl of supernatant removed and dried *in vacuo*  
209 (Thermo Savant, NY, USA) for ca. 3 h. We then stored extracts at -80 °C until metabolomics analysis.

210 An intrastudy quality control (QC) sample was prepared by pooling small aliquots of all samples, and  
211 the single extract was removed and dried down by centrifugation (20,000 x g for 10 min at 4 °C,  
212 Biofuge). UHPLC-MS based metabolomics was performed (20 µl per sample) on a Thermo Dionex  
213 Ultimate 3000 RS system with a Thermo Scientific Q Exactive Orbitrap mass spectrometer. Samples  
214 (20 µl) were separated over a 100 x 2.1 mm, 1.9 µm particles, C18 column (Thermo Hypersil Gold) at  
215 a flow rate of 400 µl min<sup>-1</sup> using a 14 min linear gradient programme from 0.1 % formic acid in water  
216 to 0.1 % formic acid in methanol. MS acquisition started at 0.1 min, with the flow up to 0.45 min  
217 directed towards waste. We acquired data in positive ion and profile mode from *m/z* 100-1000 at  
218 70,000 resolution. Samples were analysed in a controlled randomised order, with the intrastudy QC  
219 sample repeatedly analysed equidistantly between the biological samples.

220

221 *Metabolomics data processing*

222 To process the UHPLC-MS data, the Thermo.raw data files in profile mode were converted into  
223 mzML format in centroid mode using MSConvert (Proteowizard 3.0.7665). Data were then aligned  
224 using an R (3.2.0) based XCMS and CAMERA script (both R packages [26,27]) which resulted in a csv

225 file intensity matrix (containing 9309 features, i.e. peaks in the mass spectra). This matrix was  
226 imported into MatLab and inserted into a direct infusion mass spectrometry (DIMS) SIMStitch  
227 workflow [28] where a blank filter of  $> 2x$  sample over blank signal was applied, and a sample filter  
228 of peak-presence of at least 50 % of all samples [29]. The matrix was further processed by  
229 probabilistic quotient normalisation (PQN) and subsequently missing values were imputed using  
230 K-nearest neighbour (KNN) with  $k = 5$ . The imputed data matrix was used as an input to univariate  
231 statistics, including calculation of fold-changes. For multivariate statistics, a  $g$ -log transformation of  
232 the imputed data matrix was additionally applied, using an assessment of the technical variance  
233 across the repeated measurements of the intrastudy QC sample [29].

234 We putatively annotated UHPLC-MS features by inputting  $m/z$  values and their associated mean  
235 intensities into MI-Pack software version 2 beta [30], where metabolites were compared against the  
236 Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. A set of highly significant metabolites  
237 (ANOVA:  $p < 0.001$ ) were further searched against the KEGG database to determine possible roles  
238 within metabolic pathways.

239

#### 240 *Statistical analysis*

241 To analyse the rate of decay and progression of interactions, we used R statistical software [31]. For  
242 each interaction, we assigned every species an individual score of combative ability, expressed as the  
243 percentage of the total system that it finally occupied. Briefly, each competitor scored between 0  
244 and 2 for each block within a system (since two regions of every block were isolated; no outgrowth  
245 of a competitor from either isolation point scored 0, outgrowth from one isolation point scored 1,  
246 outgrowth from both isolation points scored 2). Scores for all blocks within a system were combined  
247 for each competitor individually, normalised to the number of replicates and converted to a  
248 percentage of the total system colonised. The data were analysed using a General Linear Model  
249 combined with Tukey *post hoc* tests, with individual block position (i.e. number of faces of that block

250 involved in direct combat) and access to water (water was added to the perlite, as such, in 27-block  
251 interactions the layer laid on the perlite has greatest access to water) factored into the model. The  
252 rate of decay of wood in all interactions was compared using a one-way ANOVA followed by Tukey  
253 *post hoc* tests.

254 For enzyme activities, we used a one-way ANOVA with Tukey *post hoc* tests to compare mean  
255 activity (from five replicates), or Kruskal-Wallis tests followed by a Dunn's test *post hoc* procedure  
256 when data were non-normally distributed, in R statistical software [31].

257 For GC-MS (VOCs) data, the entire data set was analysed by principal components analysis (PCA) to  
258 check for clustering of the QCs and, therefore, robustness of the data set (see Supplementary Fig. 2  
259 for QC clustering), using MetaboAnalyst 3.0 [32]. We then removed QCs from the matrix, and  
260 orthogonal projection to latent structures-discriminant analysis (OPLS-DA) ( $Q^2 = 0.93$ ,  $R^2 = 0.96$ ),  
261 chosen for its cross-validation method which reduces false-positive results [33], was applied to the  
262 standardised binned data to determine the degree of separation between the four major sample  
263 groups: pair-wise samples 1 d and 28 d after interaction set up, and 27-block samples 1 d and 28 d  
264 after interaction set up. Next, we separated the data and applied OPLS-DA to pair-wise ( $Q^2 = 0.57$ ,  $R^2$   
265  $= 0.61$ ) and 27-block ( $Q^2 = 0.37$ ,  $R^2 = 0.55$ ) sample groups separately. The modelled covariance and  
266 correlation were used to identify the features contributing most to the discriminant model  
267 separation, and one-way analysis of variance (ANOVA) with a 5 % Benjamini-Hochberg false  
268 discovery rate (FDR) correction for multiple comparisons [34], and Tukey *post hoc* tests were applied  
269 to those features.

270 Lastly, for UHPLC-MS data we applied PCA to the g-log transformed data to explore the separation  
271 between control samples (*T. versicolor* growing alone), interaction samples and QCs. The median  
272 Relative Standard Deviation (RSD) for the intrastudy QC samples was 11.15 %, indicating that the MS  
273 data were of sufficiently high quality for further statistical analysis (see Supplementary Fig. 3 for QC  
274 clustering). After removing the QCs, analysis of variance simultaneous components analysis (ASCA)

275 was applied, and the model was permutation tested (5000 permutations) to determine the  
276 significance of factors (sample day, and block position within systems) [35,36]. An additional ASCA  
277 model was tested to determine the significance of species distribution patterns within cubes, i.e.  
278 species being dispersed, *V. comedens* occupying a larger adjacent volume, or *T. versicolor* comprising  
279 the entire system, but did not include spatial location within assemblages as a factor. Pair-wise  
280 comparisons using ASCA were carried out for *post hoc* testing of significant effects, and the *p*-values  
281 were adjusted for multiple testing using a 5 % Bonferroni-Hochberg multiple testing correction [37].  
282 Univariate analysis of variance (ANOVA) was applied to the whole normalised matrix with a 5 % FDR  
283 correction [34] to test for significant metabolites. Finally, we determined fold changes (FC) between  
284 significant groups.

285

#### 286 *Network analytics*

287 We investigated the synergy of metabolites produced by *T. versicolor* during all interactions by  
288 creating a co-occurrence Force Atlas2 [38] network analysis plot using Sci2 [39] and Gephi [40]. Data  
289 were filtered such that only significantly abundant features (ANOVA/ASCA: adjusted  $p < 0.05$ )  
290 relative to the baseline were included in the analysis, and abundancies of  $< 10\%$  the maximum were  
291 removed from the matrix, resulting in 908 retained variables. The weighted degree of nodes was  
292 calculated, and nodes were partitioned based on their weighting to facilitate removal of those which  
293 did not cluster into a discrete module. A final network was constructed from the refined dataset,  
294 with edges weighted by count of occurrence and clusters coloured by weighted degree. The average  
295 abundance of all features within a cluster was calculated, and we used a one-way ANOVA with Tukey  
296 *post hoc* tests with a 5 % FDR correction [34] to compare mean abundance between interacting  
297 systems after 28d, in R statistical software [31].

298

299

## 300 **Results**

### 301 *Hierarchy and coexistence dynamics*

302 Typical transitive hierarchy of the focus decay species, *T. versicolor*, was established in paired block  
303 interactions (Fig. 1), i.e. *H. fasciculare* > *T. versicolor*, *H. fasciculare* > *V. comedens* and *T. versicolor* >  
304 *V. comedens* (Fig. 3). This hierarchy reflected the general niche occupied in wood decomposition:  
305 least competitive *V. comedens* an early/primary decay species, *T. versicolor* a secondary colonising  
306 species, and *H. fasciculare* a late/tertiary decay species, the most competitive. Transitivity was also  
307 exhibited when the three fungi were dispersed throughout more spatially heterogeneous  
308 3-dimensional systems (Fig. 1e): 15 % of the original territory (defined as the relative proportion of a  
309 block occupied by a single fungus) of *T. versicolor* was captured by *H. fasciculare*, and 69 % of the  
310 original territory of *V. comedens* was captured by its competitors. However, when spatial dynamics  
311 within the 3-dimensional cubes were changed such that *V. comedens* occupied a larger adjacent, but  
312 same total, volume as its competitors (Fig. 1f), *V. comedens* was displaced from just 6 % of its  
313 original territory, and *T. versicolor* was displaced from 46 % of its original territory. The three species  
314 coexisted without *V. comedens* being driven to near extinction (within a closed community network  
315 loop) as it was when territory was patchy, which is a characteristic of an intransitive relationship (Fig.  
316 3).

317

### 318 *Metabolite network function*

319 In addition to our 12 targeted enzyme assays, our untargeted analyses yielded 67 VOCs, and 2825  
320 LC-MS signals of which 1597 were putatively annotated against existing compound libraries  
321 (Supplementary Table 3). Univariate ANOVA analysis (with false discovery rate correction of  
322 p-values) and ASCA multivariate analyses provided evidence of significant differences in the quantity  
323 of individual functional metabolites from blocks originally colonised by *T. versicolor* between

324 community systems which showed divergent coexistence dynamics. Network analysis of the  
325 detectable metabolome, VOCs and enzymes throughout all interactions (average weighted  
326 degree = 3230), revealed 10 distinct clusters of synergistic metabolites (both antagonistic and  
327 lignocellulose decaying), plus an additional 11 independently functioning compounds with a high  
328 network degree. Namely, toluene (VOC: C66), an undefined oxidase enzyme, manganese peroxidase  
329 (MnP), manganese independent peroxidase (peroxidase), arylsulfatase, phosphodiesterase, and five  
330 unidentified small metabolites (Fig. 4; Cluster Identity Table 1).

331 By comparing the average abundance of individual clusters of metabolites produced in blocks  
332 pre-colonised by *T. versicolor* in pair-wise competitive systems, we found that production of 7  
333 clusters of antifungals was induced in significantly greater quantities when *H. fasciculare* was  
334 *T. versicolor*'s opponent, compared to 3 clusters when it was paired against the weaker *V. comedens*,  
335 relative to single species controls (Fig. 5). This particular response was consistent, and in  
336 3-dimensional community interactions there was a stronger competition response shown in  
337 *T. versicolor* pre-colonised blocks when *V. comedens* was more combative when occupying a larger  
338 adjacent patch size (Clusters 6 and 9 were significantly more abundant (ANOVA:  $p < 0.05$ )),  
339 compared to when all fungi were dispersed in 3-dimensions. Cluster 6 contained the putatively  
340 annotated metabolite swainsonine, which functions in the biosynthesis pathway of piperidine- and  
341 pyridine-based antimicrobial alkaloids, and isolongifolene, a sesquiterpene with known antifungal  
342 properties, featured in Cluster 9 (Table 1). Although only putatively identified, the increased  
343 abundance of these combative/defensive compounds in *T. versicolor*-colonised blocks correlates  
344 with the increased combative ability of competitor *V. comedens* and longer coexistence of the three  
345 fungi within an intransitive relationship loop (Fig. 3).

346 In addition to our findings in competitive systems, we found distinct metabolic differences between  
347 2-dimensional and more spatially complex 3-dimensional controls where *T. versicolor* was the sole  
348 occupant of these resource habitats. For example, the unclustered enzyme arylsulfatase and

349 Cluster 10, which comprised another six similarly functioning enzymes (Table 1), were significantly  
350 more highly abundant (ANOVA:  $p < 0.05$ ) when *T. versicolor* solely occupied a 3-dimensional cube  
351 compared to when it was solely paired in 2-dimensions (Fig. 5).

352 The presence of other species affected metabolic function, as for example Clusters 7 and 8, amongst  
353 others, comprising compounds such as antifungal sesquiterpenes (Table 1), were significantly more  
354 abundant (ANOVA:  $p < 0.05$ ) when *T. versicolor* was paired in an interaction compared to when it  
355 decayed wood alone. Similarly, when we compared whole *T. versicolor* 3-d cubes with 3-d  
356 community interactions, clusters 2, 3 and 4 which contain metabolites such as ankorine and  
357 fortimicin involved in antibiotic biosynthesis pathways, were significantly more abundant  
358 (ANOVA:  $p < 0.001$ ) in the more species-rich systems. Within this experimental time frame the  
359 process of decomposition was not affected by spatial dynamics or species diversity (ANOVA:  $p >$   
360  $0.05$ ; Supplementary Fig. 1).

361

362

### 363 **Discussion**

364 Our results indicated that coexistence dynamics and metabolic function are directly affected by  
365 spatial occupation and patchiness of territory and can be translated into mechanistic functional  
366 processes. Furthermore, we provide evidence of a metabolic response to shifts in community  
367 structure as a result of altered connectivity. Landscape architecture changes combative mechanisms  
368 [2] and consequently may cause variation in expenditure of metabolic products between systems of  
369 varied levels of spatial heterogeneity. Community composition may be altered by a change in  
370 combative mechanisms as a result of landscape structural complexity [2], possibly due to stochastic  
371 effects within community assemblages [3] or different gaseous regimes throughout 3-dimensional  
372 structures and altered edge effects in 2-dimensions vs 3-dimensions. A study in which individuals of

373 a community were paired against each other in artificial media [21], found negligible effects of  
374 species diversity alone on community function, but that a diverse community comprising weak  
375 competitors with high intransitivity exhibited a positive diversity-function relationship, i.e. the  
376 structure of a competitive network impacts community-level function. The more realistic complexity  
377 of the model system used in the present study revealed very different relationships: *T. versicolor*  
378 changed its mechanism of combat in systems where *V. comedens* occupied a larger adjacent volume,  
379 as clusters of metabolites functioning in the biosynthesis pathways of antagonistic antimicrobial  
380 compounds were produced in greater abundance. Presumably this emergent property was as a  
381 result of the greater combative strength of *V. comedens* (i.e. more antifungals were needed to attack  
382 the connected *V. comedens*), or as a result of a change of strategy by *H. fasciculare* which focused its  
383 attention on antagonising the now weakest competitor with whom it shared the most  
384 antagonistic-fronts, *T. versicolor*, which responded to this change with increased antimicrobial  
385 compound production, or, the emergent property could have resulted from both simultaneously.  
386 Additionally, in this scenario *H. fasciculare* was able to capture some of the territory of *T. versicolor*  
387 which would result in a change to species-specific biochemical production. When *V. comedens* was  
388 dispersed it was less stable than when connected, and connectivity of *V. comedens* altered the  
389 community dynamic such that the usually stronger competitor, *T. versicolor*, came under survival  
390 pressure. The change in mechanisms led to coexistence of the three species with more similar  
391 relative abundancies and a closed community network loop (characteristic of intransitivity),  
392 compared to transitive community dynamics when the individuals were dispersed, where  
393 *V. comedens* was outcompeted to near extinction. It is worth noting that in natural dead wood  
394 species diversity would be greater than that presented here, and the presence of other fungal  
395 species as well as bacteria would influence interaction outcomes and, therefore, metabolism [41].  
396 Some fungal-bacterial interactions are mutualistic [42], which could give individual fungal species a  
397 competitive advance against their competitors, and further alter expected community hierarchies.

398 In the present study, production of seven substrate processing enzymes functioning in resource  
399 exploitation was boosted when spatial scale was increased from linear 2-dimensions to more  
400 heterogeneous 3-dimensions. The differences in functional metabolic processes across spatial scales,  
401 and between the more diverse community exhibiting intransitive characteristics and the transitive  
402 community is, therefore, reflective of a relationship between diversity and function that is regulated  
403 by space. The impact of the nature (space occupancy) of communities in directing community  
404 structure should be considered when looking at complex communities where outcomes are less  
405 predictable, and metabolic quantification to potentially inform predicted outcomes is, therefore,  
406 very useful.

407 That species diverse communities promote coexistence and positively impact community function is  
408 well known [21,43,44,45]. Our results confirm this relationship between function and diversity, i.e.  
409 metabolic function changed significantly between systems with different numbers of species (single  
410 species assemblages vs multi-species assemblages), but we also show that spatial scale and  
411 distribution of species (patch dynamics) affect metabolic function as well. While these effects were  
412 not directly translated into ecosystem services (i.e. the rate of wood-decay was not significantly  
413 affected), decay in the natural environment occurs over much larger time-scales than used here [46].  
414 So it might be predicted that given a greater length of time spatial heterogeneity and species  
415 diversity would have resulted in changes to the rate of decomposition, since substrate utilisation  
416 was affected over the short time-scale measured in this study. The decomposition activities of wood  
417 decay fungi determine the rate of nutrient cycling in forest ecosystems which impacts forest  
418 function [47]. The relationship between spatial dynamics, species diversity and function highlighted  
419 in our study is, therefore, a key mechanism in the release of carbon from organic substrates into the  
420 carbon cycle, which drives global change [48]. Microbial communities in every global ecosystem  
421 carry out an array of functions as important as that of wood decayers [49,50,51], but the effects of  
422 spatial dynamics and species diversity on these functions have not previously been measured and  
423 quantified by experimental studies. The ecologically pertinent systems presented here are

424 pioneering in their quantitative capture of 3-dimensional spatial dynamics into the experimental  
425 study of microbial, community and landscape structural ecology, which can be adapted,  
426 reconfigured and reimagined for the study of communities with a range of interaction types (e.g.  
427 neutral, mutualistic, facilitative). Our 3-dimensional experimental design, and the finding that spatial  
428 dynamics directly impact coexistence, diversity and function, are not only translatable to the  
429 understanding of diversity in existing microbiomes but may also provide key insights into extinctions  
430 and predictions of future ecological trends and community-level evolution.

431

432 **Acknowledgements**

433 This work was supported by a Natural Environment Research Council (NERC) GW4+ DTP studentship  
434 (JO'L: NE/L002434/1), and NERC Biomolecular Analysis Facility (R8-H10-61) through an NBAF award  
435 (NBAF-977). We thank Dr Clement Heude for training in small metabolite extraction technique.

436

437 **Author contributions**

438 LB, DCE, CTM and JO'L designed the experiment. JO'L and KJ set up the experiment, KJ, JO'L and CTM  
439 analysed VOCs, JO'L analysed enzymes. JO'L, US and MRV analysed small metabolites (LC-MS). J'OL,  
440 JE and KH contributed to analysis of the untargeted LCMS data, and J'OL analysed the block  
441 interactions data, enzymes data and VOC's data. J'OL, LB, DCE and CTM drafted the paper, and all  
442 authors had editorial input.

443

444 The authors declare no competing interests.

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## Figure Legends

**Fig. 1:** Spatial distribution of species in pair-wise (a-d) and 3-dimensional 27-block (e-g) interactions. a-d: pair-wise interactions in all conceivable combinations, plus *T. versicolor* self-pairing; e: dispersed cube (fungi were dispersed throughout the system and arranged so that no two blocks containing the same species had adjacent faces); f: “wall” distribution cubes (all fungi occupied the same total volume of wood but the adjacent territory occupied by *V. comedens* was larger; the other two competitors were arranged so that no two blocks containing the same species were adjacent); g: single species, *T. versicolor* 27-block cube. Cut vessels in pair-wise interactions and rows within 27-block layers were touching so that the wood grain ran in the same parallel direction as denoted by arrows.

**Fig. 2:** Sampling and deconstruction of wood blocks. Individual blocks were split along the grain into quarters, and three of the quarters from each block were foil wrapped, flash frozen in liquid nitrogen and stored at -80 °C. For the remaining quarter, two chips (~2 mm<sup>3</sup>) were taken from an inside face and inoculated onto 2 % malt agar, then emerging mycelia identified morphologically.

**Fig. 3:** Interaction progression after 28 d in pair-wise interactions (*T. versicolor* (Tv) against *V. comedens* (Vc); *T. versicolor* against *H. fasciculare* (Hf); *H. fasciculare* against *V. comedens*), and 3-dimensional cubes in which all three species were evenly dispersed (Fig. 1e), and where *V. comedens* occupied a larger adjacent volume (Wall; Fig. 1f). Bars show the proportion of territory occupied by each species at the end of the experiment (mean of n = 5, SEM = ± 12.2).

**Fig. 4:** Metabolic network of the full complement of significant compounds produced by *T. versicolor* throughout all interactions. Synergistic clusters of putatively annotated metabolites are clearly visualised and grouped based on their weighted degree (WD) (number of weighted edges, i.e.

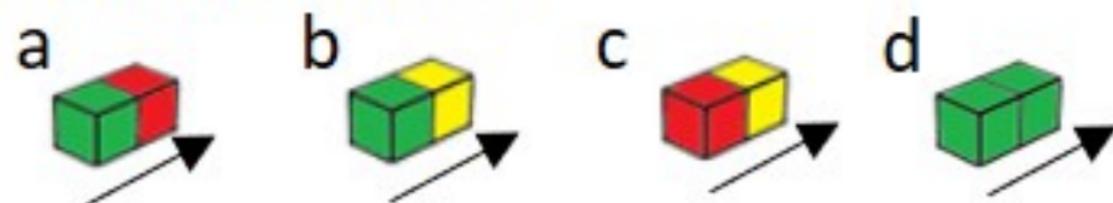
connections to other nodes, for a node. Average WD = 3230) and grey nodes labelled with compound names denote metabolites that did not form clusters (note that unlabelled grey nodes lack putative identifications). Clusters and their WD are detailed in Table 1. Edges are weighted by the count of occurrence of synergistic metabolites within samples, and node sizes and cluster colours represent weighted degree.

**Fig. 5:** Significant differences in the average abundance of metabolic clusters (details of composition of clusters in Table 1) between interactions. Orange denotes cluster abundance is significantly higher (ANOVA:  $p < 0.05$ ) in the interaction on the left of the colon; Blue denotes significantly lower (ANOVA:  $p < 0.05$ ) cluster abundance in the interaction on the left; and no colour denotes no significant difference (ANOVA:  $p > 0.05$ ) between a pair of interactions. Tv, *T. versicolor*; Vc, *V. comedens*; Hf, *H. fasciculare*; Wall, 3-dimensional cube where *V. comedens* occupied a larger adjacent volume; Dispersed, 3-dimensional cube where all three species were dispersed; TvCube, 3-dimensional cube colonised entirely by *T. versicolor*.

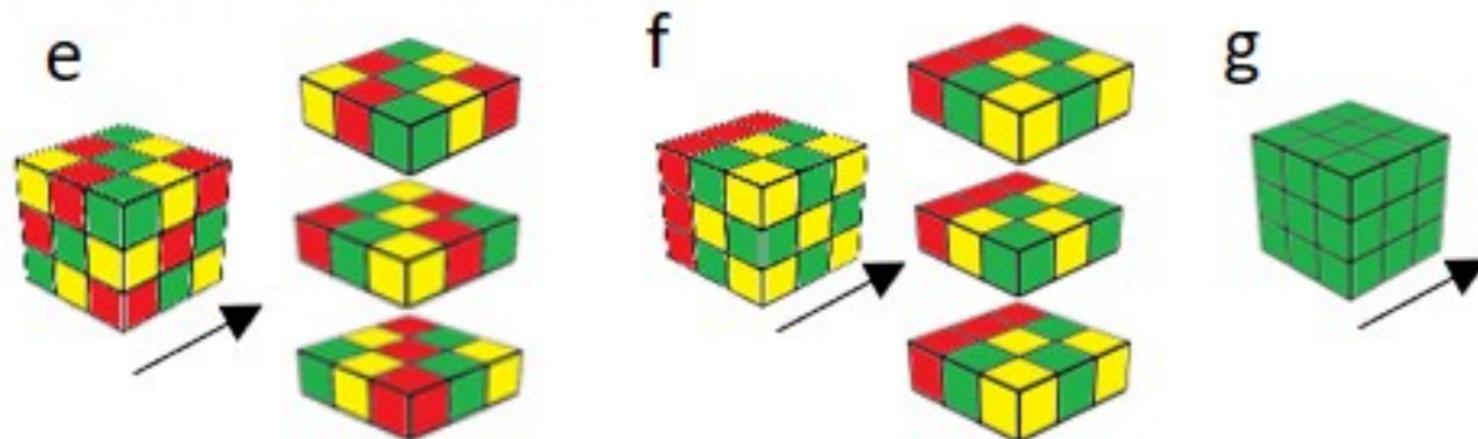
## Table Legends

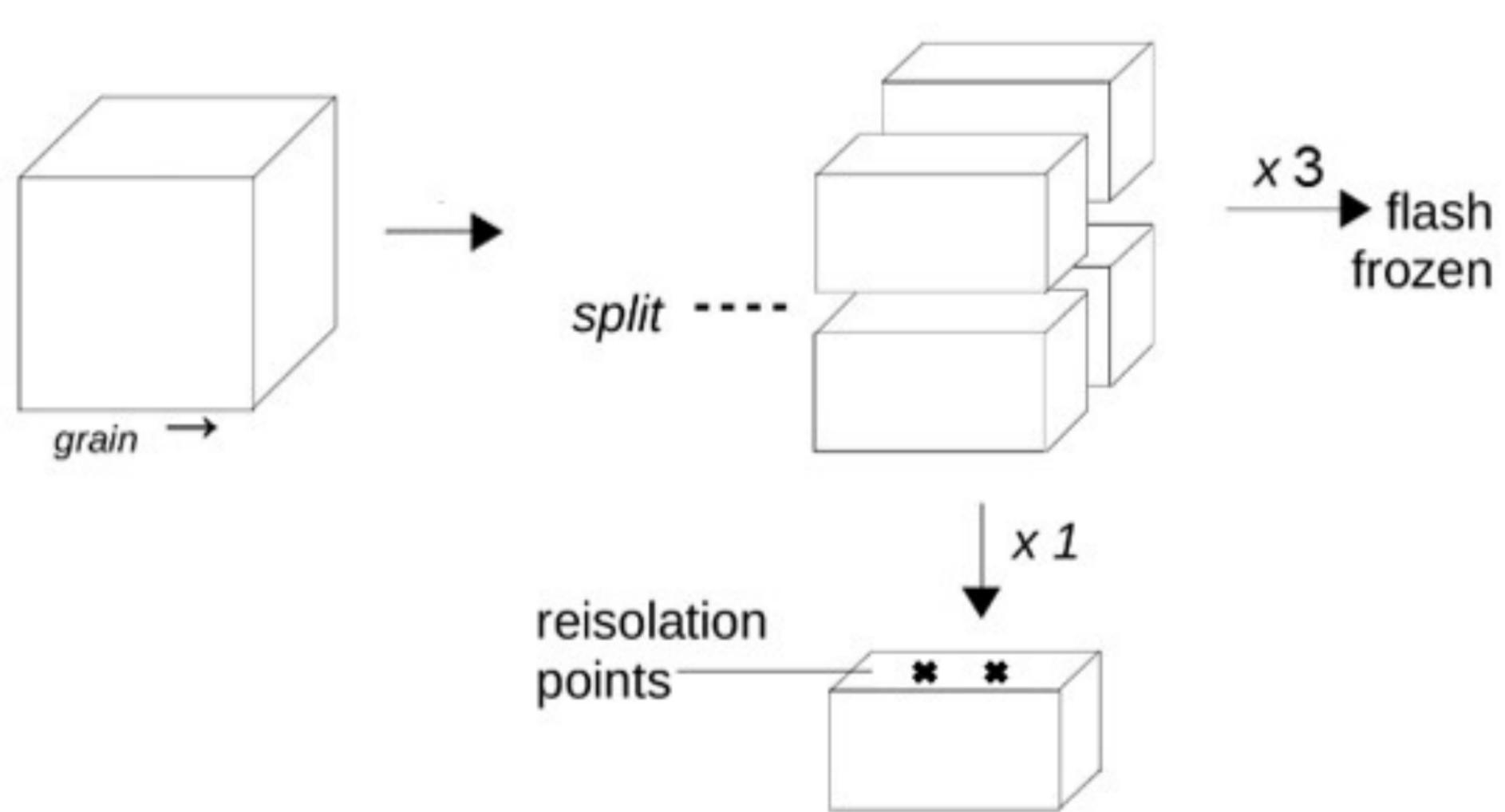
**Table 1:** Details of a subset of putatively annotated metabolites clustered into 10 cluster sets plus unclustered metabolites, with the weighted degree (WD) of clusters given. All putatively identified compounds were searched against the KEGG compound database, and those listed on KEGG with details of known metabolic pathways are given here along with possible function of the pathway. Retention Index (RI) is given for VOCs. See Supplementary Table 3 for full breakdown of compounds within clusters.

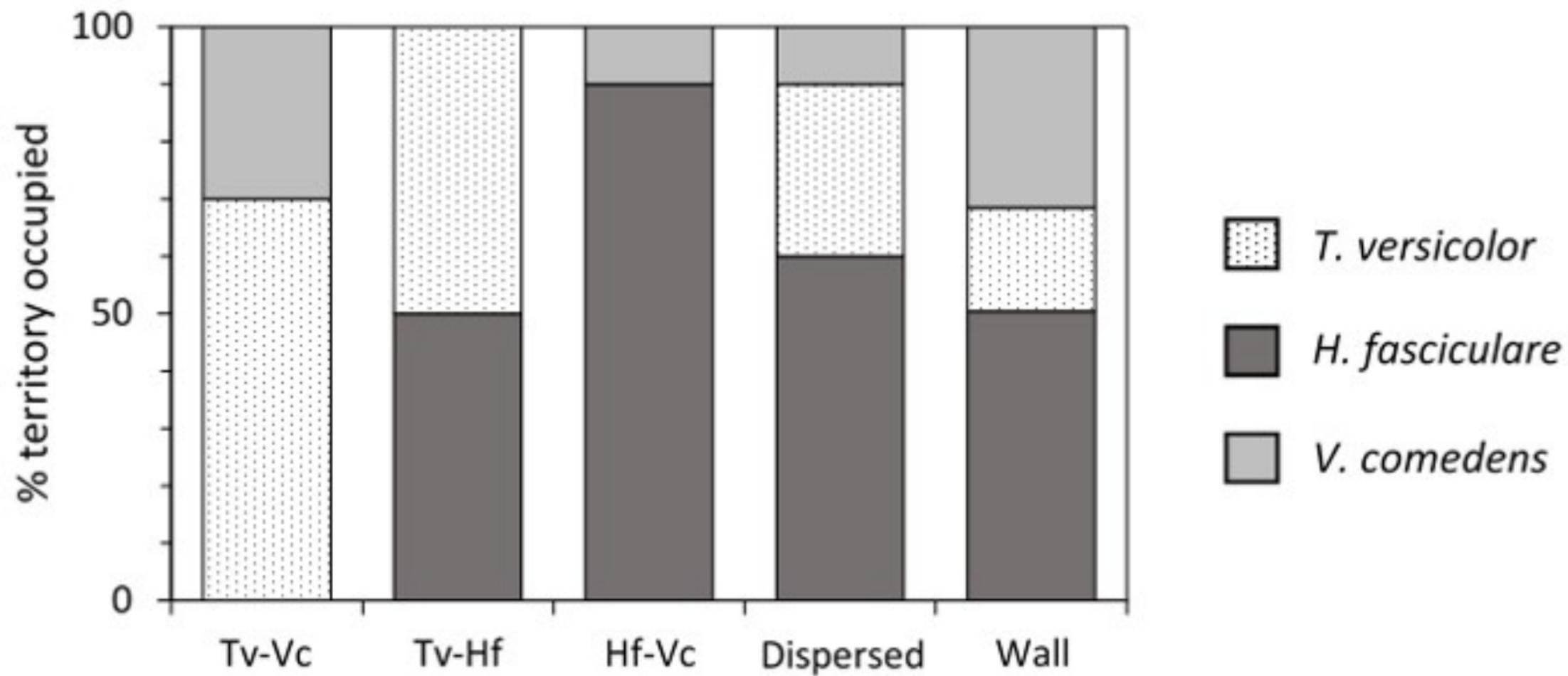
## Pair-wise interactions

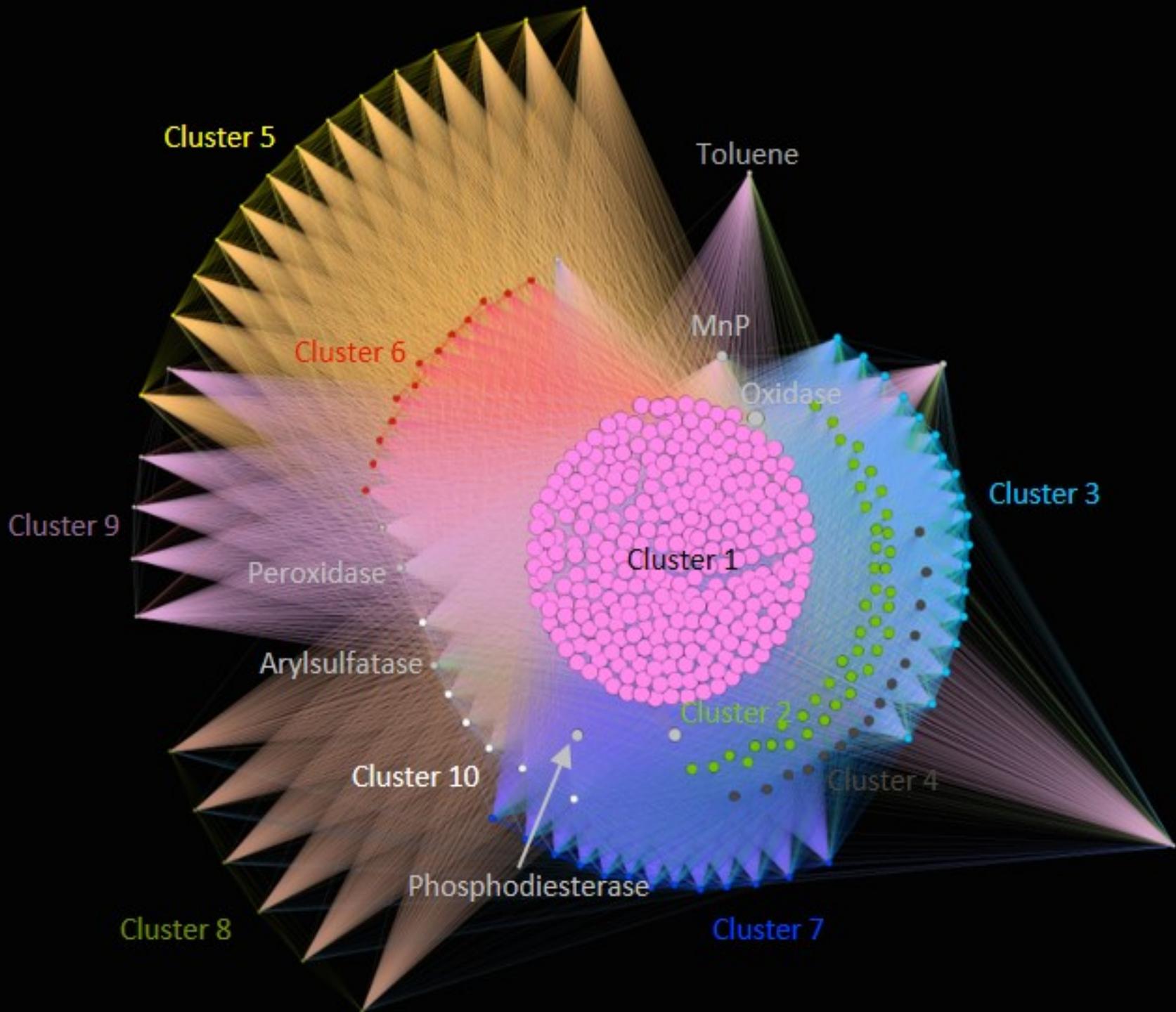


## 27-block interactions









|                    | Cluster1 | Cluster2 | Cluster3 | Cluster4 | Cluster5 | Cluster6 | Cluster7 | Cluster8 | Cluster9 | Cluster10 | M1881  | C66 | M2460  | M2050 | M1214  | MnP    | MUFs | Peroxidase | Oxidase | M1890  | MUFpp  |
|--------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|-----------|--------|-----|--------|-------|--------|--------|------|------------|---------|--------|--------|
| TvTv : TvHf        | Blue     | Yellow   | Yellow   | Yellow   | Blue     | Blue     | Yellow   | Yellow   |          | Blue      | Yellow |     |        | Blue  | Yellow |        | Blue |            |         | Yellow | Blue   |
| TvTv : TvVc        |          |          |          |          |          |          | Yellow   |          |          | Blue      |        |     |        |       |        |        | Blue |            |         |        | Blue   |
| TvVc : TvHf        | Blue     | Yellow   | Yellow   | Yellow   | Blue     | Blue     |          | Yellow   |          |           | Yellow |     | Yellow | Blue  | Yellow |        |      |            |         | Yellow |        |
| TvCube : Dispersed | Blue     | Yellow   | Yellow   | Yellow   | Blue     | Blue     |          | Yellow   | Yellow   | Yellow    | Yellow |     |        |       | Yellow |        |      |            |         | Yellow | Yellow |
| TvCube : Wall      | Blue     | Yellow   | Yellow   | Yellow   | Blue     | Blue     |          | Yellow   |          | Yellow    | Yellow |     |        | Blue  | Yellow |        |      |            |         |        | Yellow |
| Dispersed : Wall   |          |          |          |          |          | Blue     |          |          | Blue     |           |        |     |        |       |        |        |      |            |         |        |        |
| TvTv : TvCube      |          |          |          |          |          |          |          |          |          | Blue      |        |     |        |       |        |        | Blue |            |         |        | Blue   |
| TvVc : Dispersed   |          | Yellow   | Yellow   | Yellow   | Blue     | Blue     |          |          | Blue     | Yellow    | Yellow |     | Blue   | Blue  | Yellow | Yellow |      |            |         | Yellow | Yellow |
| TvVc : Wall        |          | Yellow   | Yellow   | Yellow   | Blue     | Blue     |          |          | Blue     | Yellow    | Yellow |     | Blue   | Blue  | Yellow | Yellow |      |            |         | Yellow | Yellow |
| TvHf : Dispersed   |          |          |          |          | Yellow   | Yellow   |          |          | Yellow   |           |        |     |        |       |        | Yellow |      |            |         | Yellow |        |
| TvHf : Wall        |          |          |          |          | Yellow   | Yellow   |          |          | Yellow   |           |        |     |        |       |        | Yellow |      |            |         | Yellow |        |

**Table 1:** Details of a subset of putatively annotated metabolites clustered into 10 cluster sets plus unclustered metabolites, with the weighted degree (WD) of clusters given. All putatively identified compounds were searched against the KEGG compound database, and those listed on KEGG with details of known metabolic pathways are given here along with possible function of the pathway. Retention Index (RI) is given for VOCs. See Supplementary Table 3 for full

|                    | Putative annotation                                  | RI   | KEGG         | Pathway   | Possible Function                   |
|--------------------|--|------|--------------|---|-------------------------------------|
| <b>Cluster 1</b>   | Methyl farnesoate                                    |      | C16503       | Control of filamentous growth in <i>Candida albicans</i> , particularly in presence of proline and n-acetyl glucosamine | Cell signalling and growth control  |
| <b>WD: 4089</b>    | Xylobiose  |      | C01630       | Hemicellulose depolymerisation  | Decomposition / sugar metabolism    |
|                    | Fructoselysine                                       |      | C16488       | Phosphotransferase system (PTS)   | Carbon metabolism                   |
|                    | Mannopine  |      | C16692       | ABC transporters  | Cellular transport                  |
|                    | Decylubiquinol                                       |      | C15495       | Mitochondrial electron transfer chain; linked to hydroquinone biosynthesis  | Lignocellulose depolymerisation     |
|                    | Homocysteine   |      | C00155       | Cysteine and methionine metabolism; Sulphur metabolism  | Enzyme metabolism; Decomposition    |
| <b>Cluster 2</b>   | (Z)-Phenylacetaldehyde oxime                         |      | C16075       | Microbial metabolism in diverse environments  | Metabolism of enzymes for defence   |
| <b>WD: 2231</b>    | Dethiobiotin   |      | C01909       | Biotin metabolism   | Metabolism of enzymes for defence   |
|                    | alpha-Curcumene                                      |      | C09649       | Sesquiterpene   |                                     |
|                    | alpha-Irone  |      | C09690       | Sesquiterpene   |                                     |
|                    | Pantetheine  |      | C00831       | Carbapenem biosynthesis; Pantothenate and CoA biosynthesis  | Antagonism (antimicrobial activity) |
|                    | Etidocaine   |      | C09943       | Biosynthesis of alkaloids derived from terpenoid and polyketide   | Antagonism (antimicrobial activity) |
|                    | Fortimicin KL1                                       |      | C17973       | Biosynthesis of antibiotics   | Antagonism (antimicrobial activity) |
|                    | Ankorine   |      | C09337       | Isoquinoline alkaloid biosynthesis  | Antagonism (antimicrobial activity) |
|                    | Fortimicin KK1                                       |      | C17974       | Biosynthesis of antibiotics   | Antagonism (antimicrobial activity) |
| <b>Cluster 3</b>   | Guanine  |      | C00242       | Purine metabolism   | Carbon uptake                       |
| <b>WD: 1518</b>    |  |      |              |   |                                     |
| <b>Cluster 4</b>   | D-Glutamine  |      | C00819       | Metabolism of other amino acids   | Defence                             |
| <b>WD: 1892</b>    |  |      |              |   |                                     |
| <b>Cluster 5</b>   | N6-(delta2-Isopentenyl)-adenine                      |      | C04083       | Zeatin biosynthesis   | Antagonism (antimicrobial activity) |
| <b>WD: 331</b>     | 4-Carboxy-2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate |      | C18345       | Aminobenzoate degradation   | Metabolism of enzymes for defence   |
| <b>Cluster 6</b>   | Swainsonine  |      | C10173       | Tropine, piperidine and pyridine alkaloid biosynthesis  | Antagonism (antimicrobial activity) |
| <b>WD: 953</b>     |  |      |              |   |                                     |
| <b>Cluster 7</b>   | Palmitic Acid  | 1983 | C00249       | Fatty acid biosynthesis; Cutin, suberine and wax biosynthesis   |                                     |
| <b>WD: 1074</b>    |  |      |              |   |                                     |
| <b>Cluster 8</b>   | 5'-Phosphoribostamycin                               |      | C18004       | Neomycin, kanamycin and gentamicin biosynthesis   | Antagonism (antimicrobial activity) |
| <b>WD: 314</b>     | Decanal  | 1306 | C12308       | Fatty acid degradation  |                                     |
| <b>Cluster 10</b>  | α-glucosidase  |      | EC 3.2.1.20  | Galactose, starch and sucrose metabolism  | Antagonism (antimicrobial activity) |
| <b>WD: 1465</b>    | Cellobiohydrolase                                    |      | EC 3.2.1.91  | Starch and sucrose metabolism   | Antagonism (antimicrobial activity) |
|                    | β-glucosidase  |      | EC 3.2.1.21  | Cyanoamino acid, starch and sucrose metabolism; Phenylpropanoid biosynthesis  | Antagonism (antimicrobial activity) |
|                    | N-acetylglucosaminidase                              |      | EC 3.2.1.30  | Lipopolysaccharide neomycin, kanamycin and gentamicin biosynthesis; Amino and nucleotide sugar metabolism               | Antagonism (antimicrobial activity) |
|                    | Phosphomonoesterase                                  |      | EC 3.1.3.2   | Thiamine and riboflavin metabolism  | Regulatory processes                |
|                    | β-xylosidase   |      | EC 3.2.1.37  | Amino and nucleotide sugar metabolism   | Antagonism (antimicrobial activity) |
| <b>Unclustered</b> | Toluene  | 781  | C01455       | Degradation of aromatic compounds   | Decomposition & Antagonism          |
|                    | Manganese peroxidase                                 |      | EC 1.11.1.13 | Oxidative degradation of lignin   | Antagonism (antimicrobial activity) |
|                    | Arylsulfatase  |      | EC 3.1.6.1   | Steroid hormone biosynthesis; Sphingolipid metabolism   | Antagonism (antimicrobial activity) |
|                    | Peroxidase   |      | C05785       | Porphyrin and chlorophyll metabolism  | Antagonism (antimicrobial activity) |

breakdown of compounds within clusters.