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Isolation studies reveal a shift in the microbiome of oak affected with Acute Oak Decline

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Running title: AOD microbiome shift

1 **Abstract**

2 Acute Oak Decline is a syndrome within the Oak Decline complex in Britain. Profuse stem
3 bleeding and larval galleries of the native buprestid, *Agrilus biguttatus* characterize the
4 disease. A systematic study comparing healthy with diseased trees was undertaken. This
5 work reports the result of isolations from healthy trees, diseased and non-symptomatic
6 tissue within AOD affected trees, at five sites in England. Bacteria and fungi were identified
7 using the DNA gyrase B gene, or ITS 1 sequencing. A significantly higher proportion of
8 diseased tissues (82%) yielded bacteria than either healthy (18%) or non-symptomatic tissue
9 in diseased trees (33%). Overall bacterial community compositions varied at each site, but
10 significant similarities were evident in diseased tissues at all sites. *Enterobacteriaceae*
11 dominated in diseased trees whereas *Pseudomonadaceae* dominated healthy trees.
12 Significant associations between diseased tissues and certain bacterial species occurred,
13 implying that the cause of tissue necrosis was not due to random microbiota. *Brenneria*
14 *goodwinii* and *Gibbsiella quercinecans* were key species consistently isolated from diseased
15 tissue; *Rahnella victoriana* and an un-named *Pseudomonas* taxon were also frequently
16 isolated from both healthy and diseased trees. Most fungi isolated were from the outer bark
17 and had no significant association with tree health status. It was concluded that there was a
18 shift in the bacterial microbiome of diseased trees, with *Enterobacteriaceae* strongly
19 represented in symptomatic but not healthy tissues. No single species dominated the
20 isolations from diseased tissues and the tissue degradation in AOD is therefore likely to have
21 a polymicrobial cause.

22

23

24 **Keywords**

25 Acute Oak Decline, bacterial microbiome, *Brenneria goodwinii*, *Enterobacteriaceae*,
26 *Gibbsiella quercinecans*, oak, polymicrobial disease, *Rahnella victoriana*

27

28 **Introduction**

29 Acute Oak Decline (AOD) has recently been described as a distinctive disease on native oak
30 species, *Quercus robur* L. (pedunculate oak) and *Q. petraea* (Matt.) Liebl. (sessile oak) within
31 the broader Oak Decline syndrome in Britain [1,2]. Affected trees are characterized by
32 symptoms of bark cracking, dark fluid seeping from the cracks, macerated, decaying tissues

33 underlying the weeping patches, and the presence of larval galleries of the native buprestid
34 *Agrilus biguttatus* (Coleoptera: Buprestidae) (commonly called the two spotted oak borer,
35 or TSOB). AOD occurs throughout southern and midland England extending no further north
36 than the Manchester area, and west along the Welsh borders including its recent discovery
37 in Newport, Wales [2; <http://www.forestry.gov.uk/fr/infid-7ul9xr>]. Reports from landowners
38 suggest that many thousands of trees are affected, and on monitored sites more than 1.3%
39 of symptomatic trees die every year, but a good proportion (40%) of affected trees appear
40 to go into remission with lesions callusing over, but it is unknown whether this process leads
41 to full recovery [1,2]. Although the cause of death is likely attributable to multiple factors
42 that could include any combination of biotic and abiotic stressors, e.g. plant available water,
43 root health, insect attack and host primary and secondary metabolic status, the cause of
44 tissue degradation and stem weeping characteristic of AOD is yet to be fully elucidated.

45

46 Declining oak trees with symptoms similar to those that characterize AOD have also been
47 reported in continental Europe [1,3,4,5,6,7,8,9] where mostly, the disease was attributed to
48 TSOB attack, but some reports implicated a bacterial component [4,5,9]. In the UK, the inner
49 bark lesions formed as part of the tissue degradation process show expansion, suggesting a
50 microbial as opposed to insect or abiotic origin [1]. If either insect or abiotic damage was
51 suspected as the primary cause of the lesions, they would not have expanding margins and
52 would heal quickly as wounds caused by injury or pest attack tend to heal soon after being
53 inflicted [1,10].

54

55 Initial diagnoses of AOD cases reported to Forest Research (FR) through citizen science
56 channels, revealed a high incidence of isolation of bacteria, and more varied appearances of
57 fungal species, many of which were known generalist saprophytes. To facilitate rapid, cost
58 effective diagnosis for a large volume of samples submitted, rapid diagnostics were needed
59 [11]. However, many of the isolated bacteria appeared to be previously unknown or un-
60 named species. Thus as a first step to identifying putative biotic causal agents, a
61 concentrated effort on formally identifying and describing a number of the commonly
62 occurring bacterial taxa was made. Thirteen novel species and two new genera have been
63 created to accommodate frequently detected species [12,13,14,15,16,17]. Amongst these

64 were *Brenneria goodwinii*, *Gibbsiella quercinecans*, *Lonsdalea britannica* ssp. *britannica*,
65 *Rahnella victoriana* and *R. variigena*.

66

67 The next step towards resolving the cause of tissue necrosis was to select species that were
68 likely to play a role in tissue decay. However, the necrogenic ability of these novel bacteria is
69 unknown, and no single bacterial species had dominated the isolation efforts at FR, which
70 would give clues as to causal agents. Furthermore, it was apparent that very little was
71 known about the composition of microbial communities of native oak species in Britain, and
72 so comparative information on microbial assemblages of healthy trees was missing. A
73 systematic study examining statistical associations of microbial species with tree health
74 status would help to identify micro-organisms that might contribute to the AOD stem lesion
75 pathosystem, and would give confidence in selecting organisms for further testing to
76 determine a potential role in causing tissue (phloem and sapwood) necrosis. A number of
77 approaches could be taken to obtain an overview of the composition of the microbiome,
78 including metabarcoding (454 pyrosequencing, see [18]), metagenomics and
79 metatranscriptomics. However, conventional isolation methods were applied here as one of
80 the main purposes of the current study was to obtain cultures to further study the biology,
81 phenotype and genomic aspects so that appropriate selections for Koch's postulates testing
82 could be made. The hypothesis behind this study states that there are similarities in the
83 composition of the microbiomes of the diseased tissues of trees affected by AOD.

84

85 The aims of the study were thus to: isolate bacteria and fungi from the stems of healthy and
86 diseased trees, and determine similarities and differences in the composition of bacterial
87 and fungal communities at the site level, as well as at the tree health status (healthy vs
88 diseased) and tissue type levels.

89

90

91 **Materials and methods**

92

93 **Field Sites**

94 Five sites were selected based on representative spatial distribution of symptomatic trees,
95 and willingness of the landowners / managers to donate trees for research since destructive

96 sampling was used. The number of diseased trees sampled per site varied because some
97 owners were only willing to allow sampling of one healthy and one diseased tree. The sites
98 chosen were: Attingham (OS Eastings 356033, Northings 310372), Bisham Wood (OS
99 Eastings 485362, Northings 184100), Great Monks Wood (OS Eastings 582100, Northings
100 225300); Stratfield Brake (OS Eastings 449400, Northings 211900) and Runswood OS
101 Eastings 563207, Northings 310858) (Figure 1).

102

103 Sample taking

104 Sampling was carried out as described by Denman *et al.* (2014). Tree location was recorded
105 using GPS (Garmin GPSmap 60CSx - Global Positioning Systems, UK). Diameter at breast
106 height (DBH i.e. 1.3 m) was measured using a Forestry Suppliers metric diameter tape. A
107 sharp, surface disinfected chisel was used to remove a fully barked 20 cm x 15 cm (LxB)
108 panel, 5-7 cm in depth. Between samples, equipment was thoroughly surface disinfected by
109 dousing with industrial methylated spirits (IMS) for 2 mins. Panels included one or more
110 bleed points. On each of the symptomatic trees a non-symptomatic panel was cut some
111 distance from the panel with symptoms. Healthy tree panels and non-symptomatic panels in
112 diseased trees did not have stems bleeds. Once cut, panels were placed separately in clean
113 polythene bags, kept cool and taken to the laboratory for processing. Material to be
114 processed was kept in a cold room at 4°C overnight and was processed over the next few
115 days. Tissues were manually separated into outer bark, inner bark, sapwood and heartwood
116 by clamping the panel in a vice grip and prising the tissues apart using a disinfected chisel.
117 Tissues were then prepared for isolation and plated onto various culture media as described
118 below.

119

120 Sample demographics

121 Samples from both healthy and diseased trees from five representative sites in England
122 were collected. One healthy tree from each of the five sites (n=5) was sampled, and ten
123 symptomatic trees, one tree each from Bisham Woods and Stratfield Brake (n=2); two trees
124 from Great Monks Wood (n=2) and three trees each from Attingham and Runswood (n=6),
125 were sampled. In total five healthy trees and ten symptomatic trees were sampled (n=15).
126 Tree health statuses were thus categorized as healthy or diseased and in total fifteen trees
127 were sampled.

128

129 Tissue condition within healthy trees was considered healthy, but in the diseased trees,
130 tissue condition was either non-symptomatic or symptomatic. The symptomatic tissues
131 consisted of lesions – particularly the dead-live-junction (dlj) area at the advancing margins
132 of the lesions, and galleries of TSOB larvae; whereas the non-symptomatic tissue group
133 consisted of apparently healthy tissue adjacent to, but some distance from the lesions in the
134 diseased trees.

135

136 Four tissue types were sampled: Outer bark, inner bark, sapwood and heartwood. Where
137 possible the number of tissue pieces per tissue type per tree was the same (10 pieces per
138 tissue type), but this sometimes varied if, for example, the necrotic area was large. A total of
139 4262 pieces of tissue were processed for isolations (Table 1).

140

141 In the study there were 109 site vs. tissue condition vs. tissue type vs. unique tree
142 combinations (hereafter called sampling combinations), of which 21 produced no bacterial
143 growth, and 42 produced no fungal growth.

144

145 Isolation

146 Once the samples had been manually separated into outer bark, inner bark, sapwood and
147 heartwood tissues, they were divided in half so that one half could be analysed using DNA
148 metabarcoding [18]. The remaining half was processed for classical isolation. Material was
149 surface sterilized by submerging in 70% ethanol for 1 min, dipping in 1% sodium
150 hypochlorite (NaOCl) for 1 min, transferring to 70% ethanol for 1 min and rinsing in distilled
151 water for 1 min. Tissue pieces were air dried for 30 mins and small chips of tissue (3-5 mm x
152 3-5 mm) were cut from the lesion margin and plated onto peptone yeast glucose agar
153 (PYGA) bacterial culture medium (yeast extract 5 g, proteose peptone 5 g, glucose 10 g, agar
154 technical no.3 – 15g, tap water 1000 ml) for bacteria isolation, a non-selective fungal culture
155 medium, malt agar (MA+S) made according to the manufacturer's instructions and amended
156 with streptomycin sulphate (0.05 gL⁻¹), and a selective MAT (malt-antibiotic-thiabendazole)
157 culture medium (malt extract, 10 gL⁻¹; agar, 15 gL⁻¹; Penicillin-G, 0.05 gL⁻¹; Streptomycin
158 sulphate 0.05 gL⁻¹; Polymyxin 0.025 gL⁻¹; Thiabendazole lactate (23 %) 1 mL⁻¹) which was
159 autoclaved for 15 mins at 121 p.s.i., cooled to 50°C then dispensed in 20 ml aliquots into

160 disposable 9 cm diameter Petri plates, and Synthetic Mucor Agar (SMA, a selective medium
161 for *Phytophthora* species [19]). In general, ten pieces of tissue per tree part, per medium
162 were plated but in the inner bark and sapwood areas more tissue pieces were often
163 processed, as the symptoms were most prevalent in these tissues, while in the heartwood
164 areas where there were usually no symptoms, fewer pieces were analysed. Samples from
165 Attingham and Runswood had 1240 pieces plated in total (310 pieces per medium type),
166 Bisham 555 pieces (156 pieces on PYGA and 133 pieces each on the other culture media),
167 Great Monks 904 pieces (226 per medium type), and Stratfield Brake (323 pieces, with 174
168 of those on PYGA). Isolations were incubated under aerobic conditions at room temperature
169 for two to three weeks, but examined every 2-3 days, and when present, bacteria on PYGA
170 or fungi on the other culture media were sub-cultured by transferring to nutrient agar (NA)
171 for bacteria, or MA for fungi. Bacterial subcultures were later streaked onto nutrient agar
172 and processed to obtain single colony cultures [20]. Fungal cultures were incubated under
173 black light at 25°C until colonies had developed, after which they were processed for DNA
174 extraction and sequencing.

175

176 PCR amplification and marker gene sequencing

177 All single colony bacterial strains were KOH tested to infer Gram reaction grouping as this
178 was the quickest method [21]. Strains from single colonies were transferred to nutrient agar
179 or 5 ml of LB broth using either a sterile pipette tip or inoculating loop. Cultures were
180 incubated in a shaking (or stationary) incubator (28-30°C, 250 rpm) overnight (>16 h). PCR
181 was carried out using primers for the DNA gyrase B gene (*gyrB*) or the 16S rRNA gene as
182 described in [22]. DNA was purified using DNA Clean and Concentrator™- 5 (Zymo
183 Research) before being sent for Sanger sequencing. Sequence analysis was performed using
184 Sequencher 5.3 sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA.
185 All sequences were identified using NCBI-BLAST.

186

187 For fungal identification, if colonies had sporulated, a slide was made to get an initial
188 identification based on morphology and colonies were subsequently produced from single-
189 spores. If no sporulation had taken place, subcultures were produced from hyphal tips. For
190 DNA extraction, single-spore or hyphal tip isolates were cultured on sterile squares of clear
191 cellophane (5 cm x 5 cm) placed on MA and incubated at 25°C in the dark until sufficient

192 mycelial growth was obtained and cultures could easily be scraped off the cellophane.
193 Genomic DNA was extracted using FASTDNA[®] Spin Kit system (MP Biomedicals, USA)
194 following the manufacturer's instructions. After incubation for 10 min at 55°C in a water
195 bath, samples were centrifuged at 14,000 x g for 1 min, eluted DNA was placed in clean
196 catch tubes. DNA was stored at -20°C until required. Sequences of the ITS regions were
197 obtained using primer pair ITS-1/ITS-4 (White *et al.*, 1990). PCR reactions were carried out
198 using a HotStarTaq[®] DNA Polymerase kit (Qiagen, UK). PCR mixtures comprised 10x PCR
199 buffer, dNTPs (2mM each), primers (50 µM each), 0.5 µl HotStarTaq DNA Polymerase (2.5 U
200 reaction⁻¹), 1 µl template DNA (20 – 100 ng) and 19 µl sterile MilliQ water. PCR was
201 performed using a Mastercycler[®] pro Vapo protect (Eppendorf, USA). PCR amplification
202 cycles were: 5 min initial denaturation at 95°C, 30 cycles of 1 min denaturation at 95°C,
203 primer annealing for 30 s at 55°C and extension for 1 min at 72°C, a final extension for 5 min
204 at 72°C was included to complete the reaction. PCR products were visualised after
205 electrophoresis on 1.5% agarose gel (Sigma, USA), stained with GelRed (Biotium cat: 41003).
206 PCR products with the expected size were purified using DNA Clean & Concentrator kits
207 (Zymo Research, USA) and the amplicons were sequenced in both directions at Source
208 BioScience, Cambridge, UK. Sequences were inspected and assembled using Sequencher
209 5.2.4 software (Genecodes, USA).

210

211 Data processing

212 The NCBI Blast identities, accession numbers, % coverage and % similarity were entered into
213 a spreadsheet together with site, tree reference, tree health status and tissue type. Repeat
214 identities from the same chip of tissue were removed so that only presence or absence data
215 per taxon, per tissue piece was recorded.

216

217 Statistical analyses

218 Bacterial or fungal growth from tissue pieces on agar was recorded. Differences in
219 percentage yield (calculated as pieces of tissue having bacterial growth, out of pieces
220 plated), between healthy, non-symptomatic and symptomatic tissues were tested using a
221 Generalised Linear Model with binomial error distribution and logit link function. Least
222 square means of the percentage yields were estimated to take into account the imbalanced
223 sampling of diseased trees across sites.

224

225 Multivariate analyses of the bacterial and fungal communities were analysed at the species
226 level using detrended correspondence analysis (DCA). This is a similar type of multivariate
227 analysis to the NDMS analysis of [18] where Operational Taxonomic Units (OTUs) were used
228 to describe community structure. DCA was considered appropriate in these analyses as data
229 comprised counts of positively identified bacterial species relating to individual pieces of
230 tissue. Analysis was undertaken using the CANOCO ordination package and effects of
231 combinations of site/tissue condition/tissue types/tree (hereafter called sampling
232 combinations) were tested for significance using permutation tests included within the
233 software.

234

235 **Results and discussion**

236

237 **Sample demographics and yield**

238 The number of symptomatic trees sampled per site varied, but in total 109 combinations of
239 site vs. tissue condition vs. tissue types vs. tree (sampling combinations) were studied (Table
240 1).

241

242 Bacteria were isolated from 88 and fungi from 67 of the sampling combinations.

243

244 Table 1. Sampling combination^a totals for bacteria and fungi at the site level, tissue condition level and tissue type level.

245 ^a Sampling combinations comprise: site vs. tissue condition vs. tissue type vs. unique tree

| | | For Bacteria | | | | For Fungi | | | |
|------------------|--|---|---------------------------------|---|--|---------------------------------|--|---|--|
| Level | Site Name or Tissue condition or Tissue type | Number of trees or tissue types sampled | Number of tissue pieces sampled | Number of sampling combinations with bacterial growth | Number of sampling combinations without bacterial growth | Number of tissue pieces sampled | Number of sampling combinations with fungal growth | Number of sampling combinations without fungal growth | |
| Site | Attingham | 4 | 310 | 29 | 2 | 930 | 23 | 8 | |
| | Bisham | 2 | 156 | 10 | 3 | 399 | 5 | 8 | |
| | Great Monks | 3 | 226 | 16 | 5 | 678 | 12 | 9 | |
| | Runs Wood | 4 | 310 | 23 | 8 | 930 | 20 | 11 | |
| | Stratfield Brake | 2 | 154 | 10 | 3 | 169 | 7 | 6 | |
| | Total | 15 | 1156 | 88 | 21 | 3106 | 67 | 42 | |
| Tissue Condition | Healthy | 1 | 242 | 13 | 7 | 546 | 11 | 9 | |
| | Non-symptomatic | 1 | 364 | 28 | 12 | 1092 | 24 | 16 | |
| | Symptomatic | 1 | 550 | 47 | 2 | 1468 | 32 | 17 | |
| | Total | 3 | 1156 | 88 | 21 | 3106 | 67 | 42 | |
| Tissue type | Outer bark | 1 | 262 | 17 | 8 | 696 | 24 | 1 | |
| | Inner bark | 1 | 278 | 20 | 5 | 786 | 15 | 10 | |
| | Sapwood | 1 | 276 | 24 | 1 | 735 | 15 | 10 | |
| | Heartwood | 1 | 241 | 18 | 7 | 646 | 10 | 15 | |
| | Gallery | 1 | 99 | 9 | 0 | 243 | 3 | 6 | |
| | Total | 5 | 1156 | 88 | 21 | 3106 | 67 | 42 | |

246

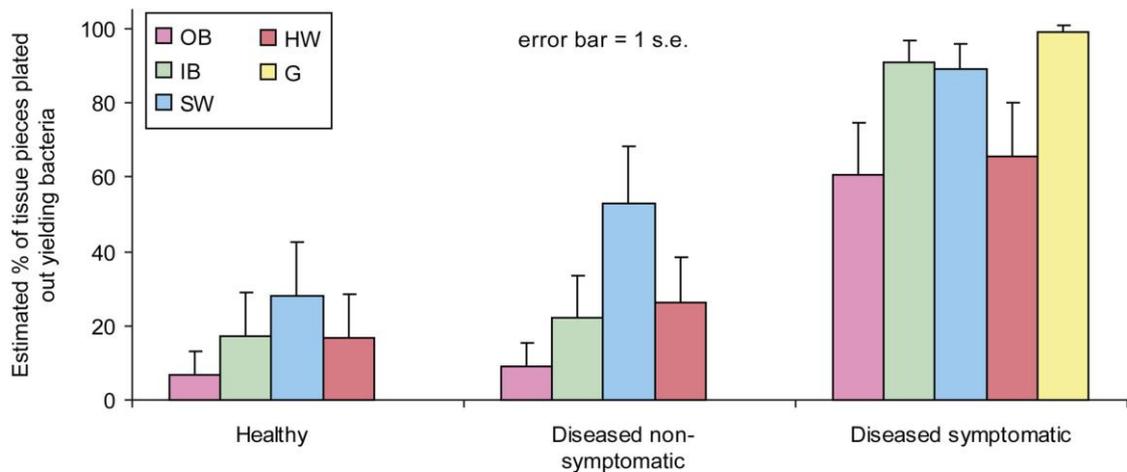
247 Yield of bacteria and fungi from tissue pieces

248 Bacteria: Bacteria were isolated from 65% (13/20) of healthy sampling combinations, 70%
249 (28/40) of diseased non-symptomatic sampling combinations and 96% (47/49) of diseased
250 symptomatic sampling combinations. There were highly significant differences in the
251 percentage of pieces yielding bacterial growth according to tissue condition ($p < 0.001$). A
252 higher proportion of symptomatic tissue pieces from diseased trees yielded bacterial growth
253 than either the non-symptomatic or healthy tissue pieces (Figure 2). There were also
254 significant differences between tissue type ($p < 0.01$), with highest yields from galleries and
255 the sapwood compared with the outer bark, but the inner bark and heartwood not
256 significantly different to either.

257

258 Figure 2. Estimated bacterial yield (% tissue pieces yielding bacteria) of different tissue types
259 in healthy and diseased trees

260



261 Tissue condition and tissue types: Healthy tissue types from healthy trees; non-symptomatic tissue
262 in diseased trees; symptomatic tissue including the larval galleries of *Agilus biguttatus* in diseased trees

261

262

263 OB = Outer bark, IB = Inner bark, SW = Sapwood, HW = Heartwood, G = Galleries

264

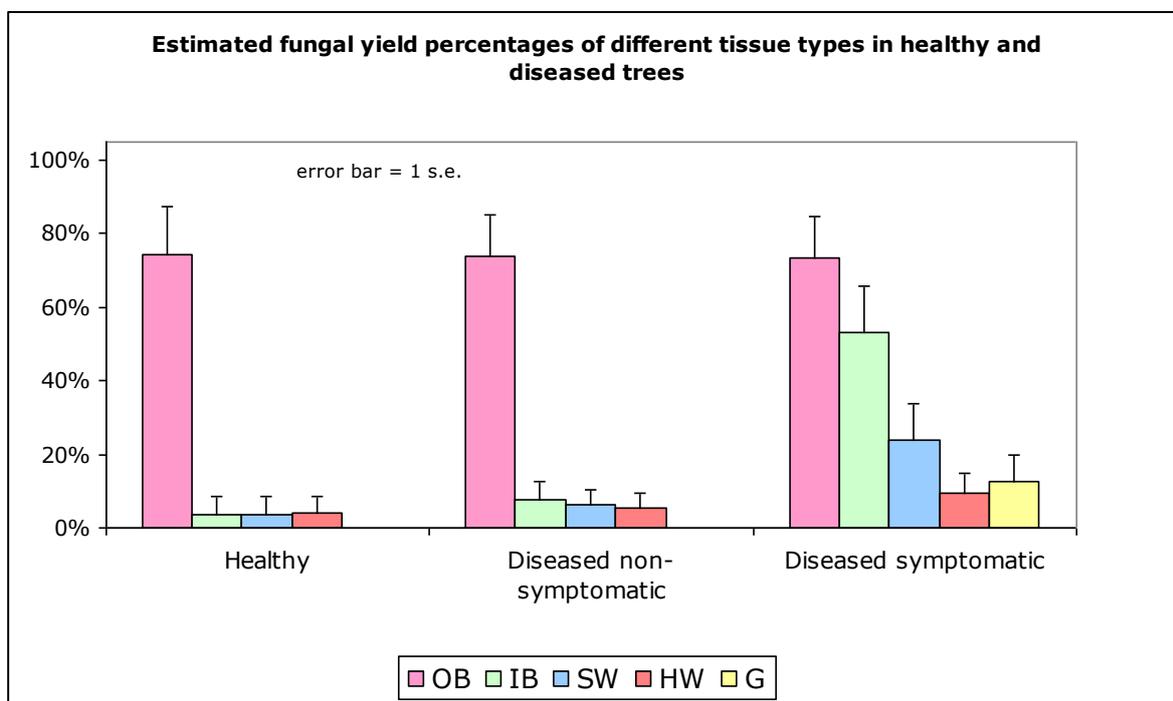
265 Fungi: Percentage fungal yields much lower than bacterial yields. Fungi were isolated from
266 55% (11/20) of healthy sampling combinations, 52% (21/40) of diseased non-symptomatic
267 sampling combinations and 67% (32/48) of diseased symptomatic sampling combinations.
268 No *Phytophthora* (or other fungi) was isolated from SMA, very few fungi were obtained on
269 MAT, but significant differences in the percentage yield of fungi occurred when using MA+S.

270 An increased percentage yield was observed from the outer bark of all 3 tissue conditions
 271 (healthy, non-symptomatic and symptomatic) ($p < 0.001$). A significant interaction was also
 272 identified, with higher yield rates for inner bark, sapwood and heartwood on diseased
 273 symptomatic tissue ($p < 0.05$) than on either healthy or non-symptomatic. It appears that
 274 fungi are more able to penetrate diseased symptomatic tissue than the others.

275

276 Figure 3. Estimated fungal yield (% tissue pieces yielding fungi) of different tissue types in
 277 healthy and diseased trees

278



279

280 OB = Outer bark, IB = Inner bark, SW = Sapwood, HW = Heartwood, G = Galleries

281

282 Overview of the Oak Microbiome

283 Bacteria: From the tissue pieces plated on PYGA, 841 isolates were obtained representing 86
 284 bacterial species or taxa. NCBI-BLAST species identity was only accepted if there was >98%
 285 gene coverage and >99% sequence similarity. If these conditions were not met,
 286 identification as a taxon at Genus level was used.

287

288 There was variation in consistency of occurrence. Eight bacterial species occurred in more
289 than 10% of the sampling combinations, but on the other hand, in one sampling
290 combination, 40 bacterial taxa had a single occurrence.

291

292 The main bacterial families present in oak included: *Alcaligenaceae*, *Bacillaceae*,
293 *Brucellaceae*, *Burkholderiaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Hyphomicrobiaceae*,
294 *Moraxellaceae*, *Nocaridaceae*, *Paenibacillaceae*, *Pseudomonadaceae*, *Rhizobiaceae* and
295 *Xanthomonadaceae*.

296

297 Fungi: There was variation in the incidence and occurrence of fungi, as the relationship
298 shows some interactions it was not clarified by producing a DCA.

299 The fungal families present on oak included: *Botryosphaeriaceae* (Botryosphaeriales),
300 *Chaetomiaceae* (Sordariales), *Bionectraceae* and *Nectraceae* (Hypocreales), *Cladosporiaceae*
301 (Capnodiales), *Dermateaceae* and *Vibrisseaceae* (Helotiales), *Didymellaceae*, *Dothioraceae*
302 (Dothideales), *Leptosphaeriaceae* (Pleosporales), *Meruliaceae* (Helotiales), *Microascaceae*
303 (Microascales), *Trichocomaceae* (Eurotiales), *Trichosporonaceae* (Tremellales), *Stereaceae*
304 (Russulales), *Umbelopsidaceae* (Mucorales), and a few un-named taxa in the Helotiales,
305 Hypocreales, Saccharomycetales, Sordariales.

306

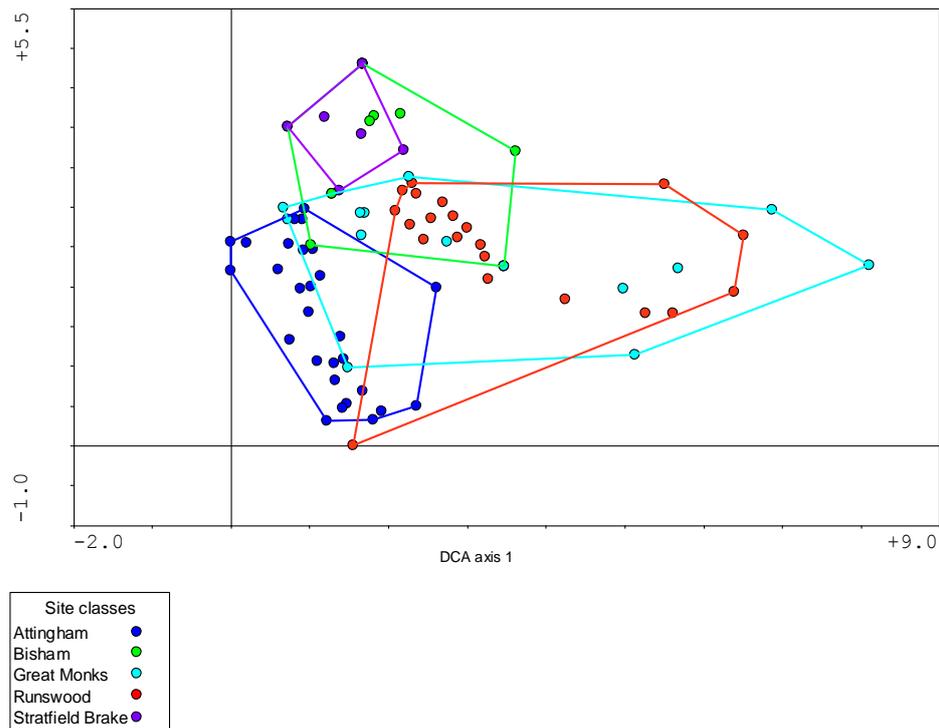
307 Community structure in relation to site

308 Bacteria: There were highly significant differences in the species compositions of entire
309 bacterial communities between sites ($p < 0.001$) as illustrated in the DCA, Figure 4. However,
310 there is a degree of overlap in the composition of communities at the different sites and this
311 can be explained by the tree health status and tissue condition (shown in Figure 5).

312

313 Figure 4. Detrended correspondence analysis of bacterial communities classified by site
314 showing differences in community composition at each site

315



316

317 Samples located close together tended to have the same bacterial communities. Conversely
 318 samples located far apart will tend to have totally different bacterial communities. There is a
 319 degree of overlap. The overlap is clarified in Figure 5.

320

321 Fungi: There were differences in fungal community composition and structure at the site
 322 level, with interactions occurring according to tree health status and tissue condition status
 323 ($p < 0.001$). The interactions are discussed in more detail in the section dealing with tree
 324 health and tissue condition status.

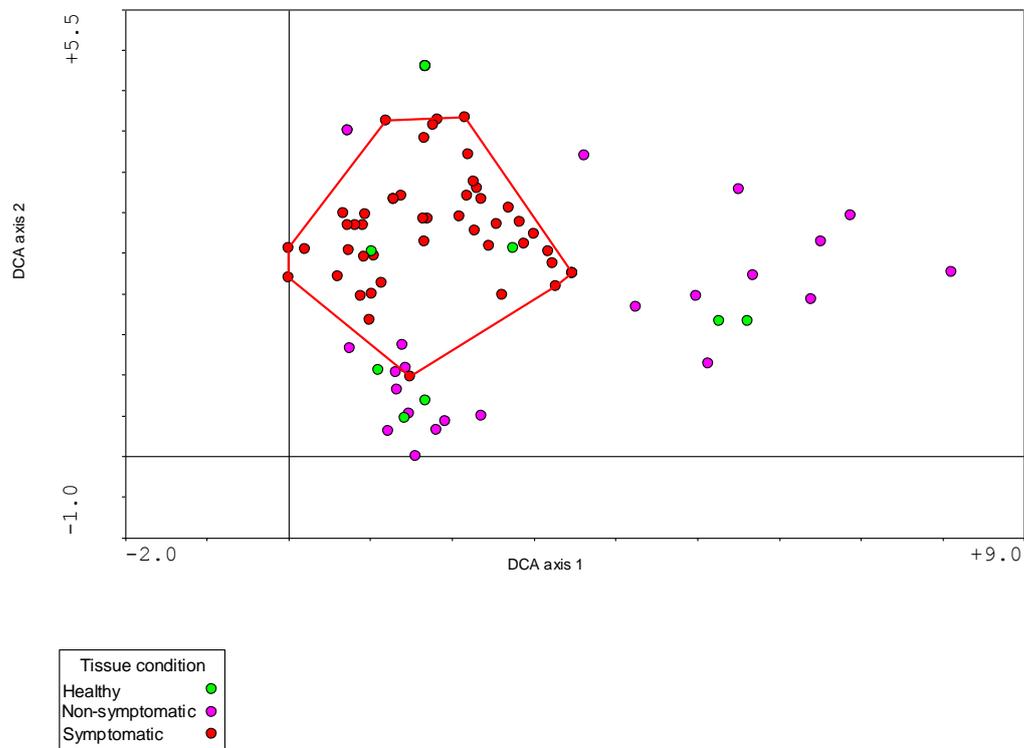
325

326 Community structure in relation to tree health status and tissue condition

327 Bacteria: Having explained bacterial community differences due to site, analyses showed
 328 that there was an additional significant clustering of species composition according to tissue
 329 condition across all sites ($p < 0.001$). Compositionally, bacterial communities in symptomatic
 330 trees are similar, but different to those in healthy or non-symptomatic tissues.

331

332 Figure 5. Detrended correspondence analysis of bacterial communities classified by tissue
333 condition



334

335 The bacterial communities in symptomatic tissues cluster strongly, indicating a high level of
336 similarity, where-as there is a low level of similarity, in healthy and non-symptomatic
337 tissues.

338 Fungi: Analysis of fungal communities identified significant effects of site, tissue condition
339 and tissue type ($p < 0.001$), but not tree health status. Outer bark was the most colonised
340 irrespective of tree health status (Figure 3); inner tissues of healthy and non-symptomatic
341 tissues were rarely colonised, but inner symptomatic tissues of diseased trees were more
342 heavily colonised and frequently with weak or opportunist pathogens such as
343 *Botryosphaeria*, *Fusarium solani*, *Neonectria* and yeasts. There were differences in
344 community compositions at the different sites. Fungi found at Great Monks differed from
345 the other four sites. There was marked species diversity amongst the fungi at Great Monks
346 Wood in both healthy and diseased trees. At Attingham, healthy trees were characterised by
347 *Phialocephala* spp. and an unknown *Helotiales* sp., while diseased trees also had *Helotiales*
348 sp(p)., but in addition were colonised by known canker pathogens such as *Botryosphaeria*

349 and *Pezicula* sp(p), and opportunists such as *Penicillium* spp. and *Trichosporon*. At Bisham,
350 *Penicillium* spp. were obtained from healthy trees and an unknown *Helotiales* from diseased
351 trees. At Runswood healthy trees were colonised by a *Pezicula* sp., but diseased trees, while
352 having the *Pezicula* sp., were also colonised by other pathogens such as *Botryosphaeria*,
353 *Fusarium solani* and *Neonectria*. Diseased trees at Stratfield Brake were also colonised by
354 *Fusarium solani*. At Great Monks the fungal communities on healthy trees were very similar
355 to those on diseased trees. Representatives from *Illyonectria*, *Phialocephala*, *Clonostachys*,
356 *Penicillium* and *Candida* spp. were present. *Fusarium solani* and *Candida* spp. were only
357 found in diseased symptomatic tissue (2 of the 5 sites – Great Monks Wood and Stratfield
358 Brake).

359

360 Differences were evident incidence of some species for example *Pezicula* was abundant and
361 prominent at both Attingham and Runswood, but absent or only had a single occurrence at
362 Bisham, Great Monks and Stratfield.

363

364 In summary:

- 365 • In healthy tissue at all five sites, fungi are predominantly found only on the outer
366 bark.
- 367 • A similar set of fungi species is found on the outer bark of non-symptomatic tissue of
368 diseased trees.
- 369 • Low numbers of fungal isolates were obtained from in the inner bark, sapwood or
370 heartwood of healthy and non-symptomatic tissues.
- 371 • In symptomatic tissue, the outer bark was colonised by similar fungal species to the
372 healthy and non-symptomatic samples, furthermore, the outer bark of the
373 symptomatic samples was more abundantly colonised than inner tissues.
- 374 • Some fungal species were unique to symptomatic tissue (*Candida* sp(p), *Fusarium*
375 *solani*, *Neonectria coccinea*). However, unlike specific bacterial species (e.g. *B.*
376 *goodwinii* and *G. quercinecans*) that were found on symptomatic tissue across all
377 sites, these three fungal species were only found on one or two sites and not
378 consistently across the majority of sites.

379

380 Microbial Community Diversity (Species richness and abundance)
 381 Bacteria: The diversity of the bacterial communities was analysed using the Shannon
 382 diversity index^b. This identified a significant difference in species compositional diversity
 383 between tissue conditions. Diseased symptomatic tissue had higher bacterial diversity in
 384 comparison to healthy tissue (p<0.05). There was no significant effect of tissue type.

385
 386 ^b[Note – a degree of caution in the diversity analysis is required as varying numbers of tissue
 387 replicates were taken across sites, tissue condition and tissue type. Diversity will to a certain
 388 extent be driven by the number of tissue replicates taken for each sampling combination
 389 and although the majority of samples were based on 10 replicates this wasn't the case for
 390 all sampling combinations.]

391
 392 Healthy and non-symptomatic tissues were dominated by *Pseudomonadaceae* but
 393 symptomatic tissue was dominated by *Enterobacteriaceae*. The most abundant bacterial
 394 species isolated in relation to tree health status and tissue condition are listed in Table 2.

395
 396 **Table 2.** Frequency of isolation of most commonly occurring bacterial species and
 397 occurrence on sites, tree health status and tissue condition.

398

| Identity of bacterial taxa | Number of sites where present | Tree health status | Tissue condition | Number of sampling combinations where present |
|----------------------------|-------------------------------|--------------------|------------------|---|
| <i>Brenneria goodwinii</i> | All five sites | Healthy | Healthy | 1 |
| | | Diseased | Non-symptomatic | 4 |
| | | Diseased | Symptomatic | 22 |
| <i>Erwinia billingiae</i> | Three sites | Diseased | Symptomatic | 8 |

| | | | | |
|-------------------------------------|----------------|----------|-----------------|----|
| <i>Gibbsiella quercinecans</i> | All five sites | Healthy | Healthy | 1 |
| | | Diseased | Non-symptomatic | 1 |
| | | Diseased | Symptomatic | 21 |
| <i>Pseudomonas taxon fulva-like</i> | Four sites | Diseased | Non-symptomatic | 1 |
| | | Diseased | Symptomatic | 18 |
| <i>Rahnella victoriana</i> | Three sites | Diseased | Non-symptomatic | 1 |
| | | Diseased | Symptomatic | 15 |

399

400

401

402 Fungi: Diversity indices of the fungal communities were not significantly different across
403 tissue condition or tissue type.

404

405 **Discussion**

406 The aims of the present study were to isolate bacteria and fungi from the stems of healthy
407 and diseased trees, to determine the similarities and differences in the composition of
408 bacterial and fungal communities at the site level, as well as for the different tree health
409 statuses and tissue conditions. High yields of bacteria were isolated confirming their
410 abundance in diseased tissue, thus isolation from chips of plant material on PYGA was a
411 successful method for isolating bacteria from oak and obtaining pure cultures. Cultures of
412 fungi were also obtained, and MA+S proved the most effective fungal culture medium.
413 Having cultures in hand allowed identification to species level of a great many taxa, but
414 certain commonly occurring taxa, particularly in the *Pseudomonadaceae* (bacteria), as well
415 as fungi in the *Helotiales*, *Pezizula* and *Phialocephala* still require identification to species
416 level.

417

418 The study showed that there were significant differences in the composition of the overall
419 bacterial community at a landscape scale (sites). This is expected considering the different
420 environments the trees were located in and the different genotypes that were represented,
421 as the trees were derived from seedstock. Site effects were less evident in fungal
422 community composition, where only one site (Great Monks Wood) was different to the
423 others.

424

425 In primary disease situations there is usually dominance of the causal agent at all scales, but
426 if the agent of disease is unknown and no one species dominates, it is difficult to determine
427 which organisms could be putative pathogens. Comparison of microbiomes of healthy vs
428 diseased tissues is one way of filtering potential candidates. The present study clearly
429 demonstrated highly significant similarities in species composition of symptomatic tissues
430 compared with those in non-symptomatic and healthy tree tissues irrespective of site.
431 Compositionally, the bacterial assemblage in non-symptomatic tissues in diseased trees was
432 more similar to those in healthy trees but did appear to be in a transitional phase, carrying
433 at low levels some elements that characterized the diseased tissue. As the tissue under
434 attack decays, the tree's transport system becomes more 'leaky' and the bacteria could be
435 transported to other parts of the tree. Further research is required to gain an understanding
436 and explanation of this occurrence, however based on results of this study it can be
437 concluded that there is a shift in the bacterial microbiome in healthy trees and non-
438 symptomatic tissues vs symptomatic tissues in diseased trees.

439

440 By contrast, there were no significant differences in fungal community makeup in healthy
441 compared with diseased trees and fungi are therefore not a main consideration in the quest
442 to identify potential candidates having major roles in causing characteristic AOD tissue
443 necrosis.

444

445 In a recent parallel metabarcoding study which used 454 pyrosequencing to characterize the
446 bacterial communities on the same oak trees used in this study, community differences at
447 the site level were also significant [18], corroborating the present results. Additionally it was
448 noted that there was a degree of community similarity at the tree health status level but it
449 was weaker than than that found in this study ($p=0.09$) [18]. This could be attributed in part,

450 to less effective species resolution based on partial 16S rRNA gene sequencing, but also
451 because communities from non-symptomatic and symptomatic tissue in diseased trees
452 were not analysed separately [18], instead results of the two tissue types from diseased
453 trees were pooled, having a diluting effect on significance.

454

455 There were highly significant associations of certain bacterial species with diseased tissues
456 indicating that a particular suite of organisms is consistently isolated from diseased tissues
457 but not healthy tissues, and implying that necrosis is not caused by randomly occurring
458 opportunistic organisms, but that there is a pattern to the bacterial community associated
459 with the diseased tissues. The high incidence of *B. goodwinii* and *G. quercinecans* in AOD
460 tissue is consistent with previous reports [1,12,17,18]. It is therefore reasonable to test
461 these species for necrogenic potential.

462

463 Causes of stem bleeding and tissue necrosis similar to AOD, on oak in continental Europe
464 have recently been reviewed [1,2] and as bacteria dominate in this study it is helpful to
465 consider bacterial research findings of others. In Spain on holm oak (*Q. ilex*) and Pyrenean
466 oak (*Q. pyrenaica*), bacteria in the *Enterobacteriaceae* (*Lonsdalea quercina* ssp. *Iberica* (syn.
467 *B. quercina*) [13]; and *G. quercinecans*, as *Serratia* sp. [12]) caused acorn gummosis and
468 stem tissue necrosis [Poza-Carrion et al, 2008]. The pathogenicity of both these bacterial
469 species on both Mediterranean oak species has therefore been proven [23], but it remains
470 to be shown whether *G. quercinecans* behaves in the same way on *Q. robur* and *Q. petraea*.

471

472 The occurrence of both *G. quercinecans* and *B. goodwinii* from a chip of tissue from the
473 heartwood of a healthy tree at Great Monks Wood, appears anomalous, but should not be
474 overlooked entirely. It is possible that both bacterial species exist as benign symbionts, at
475 very low levels in oak stems or different parts of the tree, so that they are seldom detected,
476 but when trees become stressed and predisposed to attack by other organisms, these
477 bacteria bloom and contribute to causing disease at particular points along the stem. To
478 consider if this is plausible and worthy of further investigation, disease symptomology needs
479 to be reconciled with tree physiology to offer a feasible explanation for the pattern of
480 disease expression and arrival of these species at the disease expression sites. Another
481 explanation for the occurrence of these two species in the healthy heartwood at Great

482 Monks Wood is that some years ago both bacterial species attempted invasion of the tree,
483 but were unable to get a foot hold, and remained locked in a quiescent state in the
484 heartwood instead. Finally, human error or accidental contamination could be another
485 explanation for this finding. Wider metagenomic studies should shed more light on the
486 occurrence of these bacteria in healthy oak or indeed other tree species, and where these
487 species exist in the environment. To date it is known that *G. quercinecans* has been isolated
488 from *Aesculus hippocastanum* (horse chestnut) with bleeding canker primarily caused by
489 *Pseudomonas syringiae* pv *aesculi* (Drs. Bridget Laue and Sarah Green, FR Northern Research
490 Station, Roslin, UK, pers. comm.), apple and pear with twig and stem cankers (as *Gibbsiella*
491 *acetica*) [24], and Spanish oak trees (*Q. pyrenaica* and *Q. ilex*) with bleeding cankers and
492 acorn gummosis [4,23], but also from the gut of *Anoplophora chinensis* [25] and from a
493 female *A. biguttatus* beetle ovipositing on pedunculate oak [26]. To the authors' knowledge
494 the occurrence of *B. goodwinii* on other plant hosts has not been reported, however, it was
495 detected using q-PCR, in bleeding stem lesions on a lime tree (*Tilia tomentosa* Moench.) in
496 the UK (S. Denman, pers. obs.). Thus both bacterial species appear to be detected on trees
497 with bleeding cankers, which leads to interesting considerations about their association and
498 the role they may play in the AOD pathosystem.

499

500 In this study, levels of disease severity were not distinguished because on standing trees it
501 was difficult to recognise whether the disease was in development or recovery stages. The
502 microbiomes of the two scenarios are likely to have different components and emphases in
503 species composition as species succession of the lesion niche progresses. However, the
504 proportionate dominance of the different players and sequence of their participation could
505 be important as was elegantly demonstrated in the development of olive knot on *Olea*
506 *europaea* L. where interaction between two species of bacteria and proportionate
507 dominance was a necessity for disease development [27]. As *G. quercinecans* and *B.*
508 *goodwinii* are often isolated together, it is interesting to consider their relationship and how
509 (or whether) they purposely maintain presence together.

510

511 Fungi were isolated chiefly from the outer bark irrespective of tree health status, and there
512 was no consistency of community composition over sites and there were different
513 compositional patterns on healthy and non-symptomatic vs diseased tissues. It can be

514 concluded that fungi are not primary agents in decay of stem tissue in AOD although certain
515 species may contribute to the degradation process when present. It was interesting to note
516 that *Phytophthora* spp. were not isolated in this study and do not contribute directly to the
517 lesions that characterize AOD, however, they may have a role in predisposing trees to AOD
518 in the broader process of Decline and this is an important line of enquiry [E.g. 28].

519

520 For the first time, insights into elements of the bacterial microbiomes of both healthy and
521 AOD affected native oak in the UK have been obtained and a culture collection amassed for
522 further study. In light of this it would be interesting to find out whether similar statistical
523 associations are evident in an even bigger sample, not only from the UK but from
524 continental Europe as well, where similar disease symptoms have been recorded on oak.
525 Testing is now required to understand the ecological role these organisms play in oak health
526 and their interactions with each other as well as with insects and the host.

527

528 **Conclusions**

- 529 • This study has clearly shown that there is a shift in the bacterial microbiome from
530 healthy to diseased trees.
- 531 • Members of the *Enterobacteriaceae* strongly represented in symptomatic tissues but
532 not in the healthy tissues, which are dominated by *Pseudomonadaceae*.
- 533 • Highly significant statistical associations of certain species of bacteria with necrotic
534 tissues occur, and different species associations with healthy tissue were clear.
- 535 • The microbiome of diseased tissues was not dominated by a single taxon.
- 536 • *Brenneria goodwinii* and *Gibbsiella quercinecans* were the most frequently isolated
537 bacteria in diseased tissues and should be included in pathogenicity tests.
- 538 • *Rahnella victoriana* and a *Pseudomonas* sp. occurred frequently and should also be
539 considered in further testing.
- 540 • There were no significant effects of tree healthy status on fungal communities and it
541 can be concluded that specific fungi do not play a role in causing AOD inner bark
542 necrosis.
- 543 • It appears that the lesions that characterize AOD are not caused by a single organism
544 and are likely to have a polymicrobial cause.

545

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550

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552

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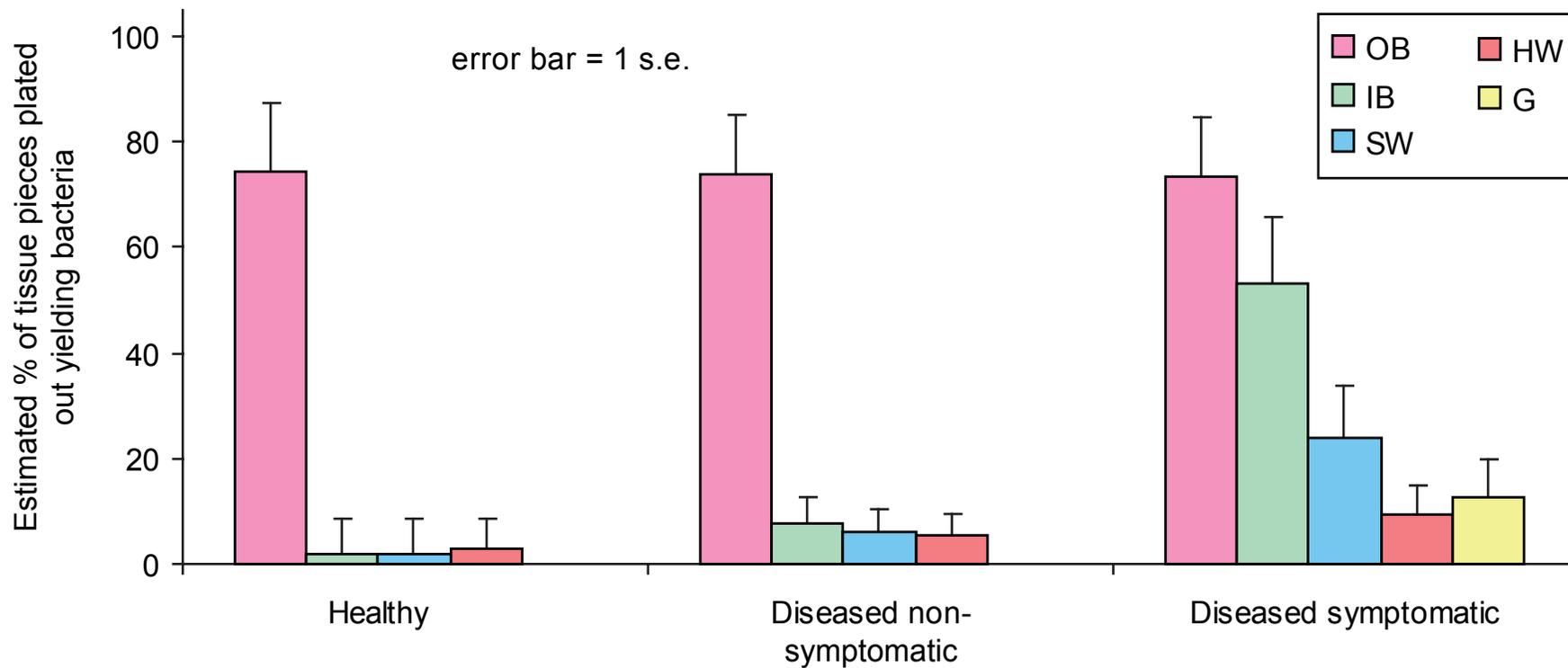
Attingham

Runs Wood

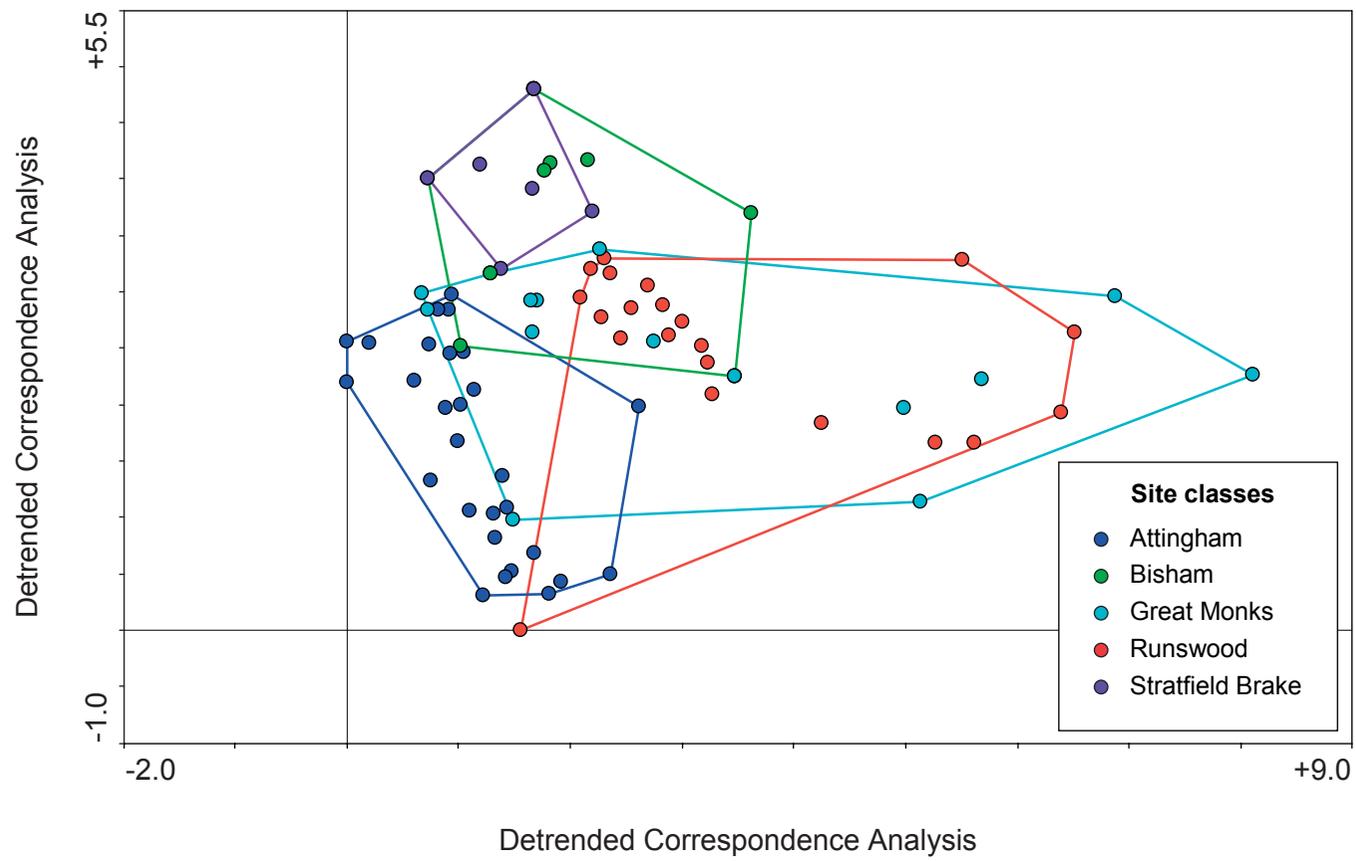
Great Monks

Stratfield Brake

Bisham Woods



Estimated fungal yield percentages of different tissue types in healthy and diseased trees



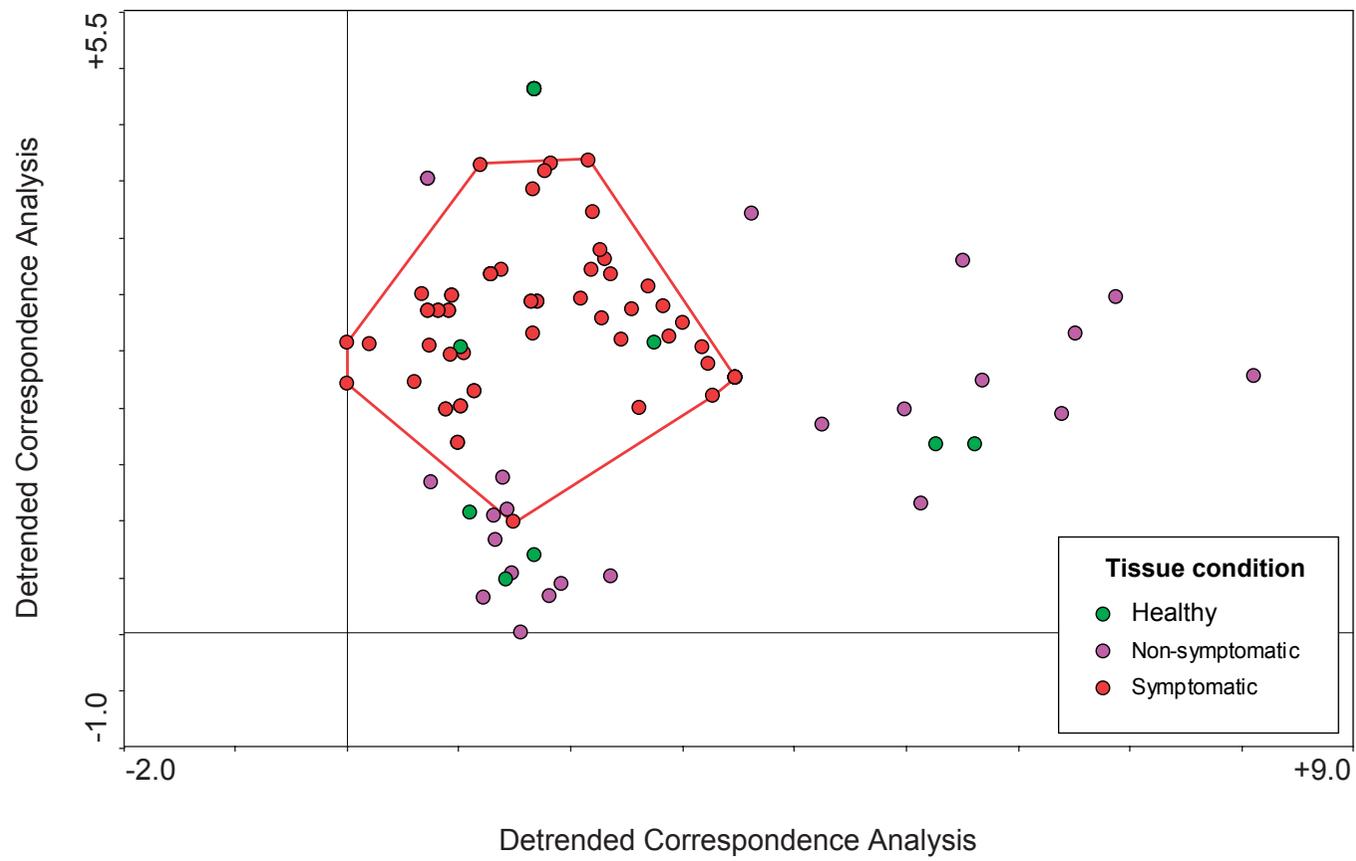


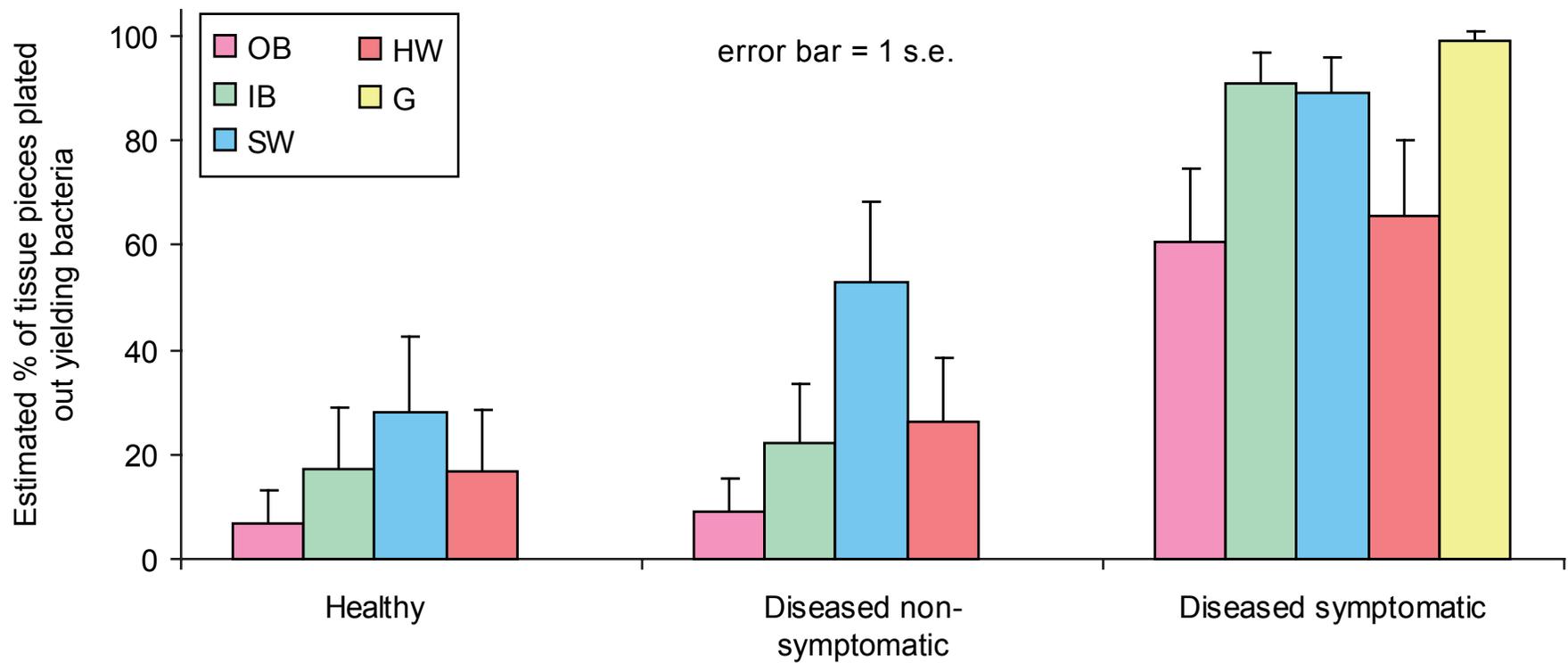
Table 1. Sampling combination^a totals for bacteria and fungi at the site level, tissue condition level and tissue type level.

^a Sampling combinations comprise: site vs. tissue condition vs. tissue type vs. unique tree

| | | For Bacteria | | | | For Fungi | | | |
|------------------|--|---|---------------------------------|---|--|---------------------------------|--|---|--|
| Level | Site Name or Tissue condition or Tissue type | Number of trees or tissue types sampled | Number of tissue pieces sampled | Number of sampling combinations with bacterial growth | Number of sampling combinations without bacterial growth | Number of tissue pieces sampled | Number of sampling combinations with fungal growth | Number of sampling combinations without fungal growth | |
| Site | Attingham | 4 | 310 | 29 | 2 | 930 | 23 | 8 | |
| | Bisham | 2 | 156 | 10 | 3 | 399 | 5 | 8 | |
| | Great Monks | 3 | 226 | 16 | 5 | 678 | 12 | 9 | |
| | Runs Wood | 4 | 310 | 23 | 8 | 930 | 20 | 11 | |
| | Stratfield Brake | 2 | 154 | 10 | 3 | 169 | 7 | 6 | |
| | Total | 15 | 1156 | 88 | 21 | 3106 | 67 | 42 | |
| Tissue Condition | Healthy | 1 | 242 | 13 | 7 | 546 | 11 | 9 | |
| | Non-symptomatic | 1 | 364 | 28 | 12 | 1092 | 24 | 16 | |
| | Symptomatic | 1 | 550 | 47 | 2 | 1468 | 32 | 17 | |
| | Total | 3 | 1156 | 88 | 21 | 3106 | 67 | 42 | |
| Tissue type | Outer bark | 1 | 262 | 17 | 8 | 696 | 24 | 1 | |
| | Inner bark | 1 | 278 | 20 | 5 | 786 | 15 | 10 | |
| | Sapwood | 1 | 276 | 24 | 1 | 735 | 15 | 10 | |
| | Heartwood | 1 | 241 | 18 | 7 | 646 | 10 | 15 | |
| | Gallery | 1 | 99 | 9 | 0 | 243 | 3 | 6 | |
| | Total | 5 | 1156 | 88 | 21 | 3106 | 67 | 42 | |

Table 2. Frequency of isolation of most commonly occurring bacterial species and occurrence on sites, tree health status and tissue condition.

| Identity of bacterial taxa | Number of sites where present | Tree health status | Tissue condition | Number of sampling combinations where present |
|-------------------------------------|--------------------------------------|---------------------------|-------------------------|--|
| <i>Brenneria goodwinii</i> | All five sites | Healthy | Healthy | 1 |
| | | Diseased | Non-symptomatic | 4 |
| | | Diseased | Symptomatic | 22 |
| <i>Erwinia billingiae</i> | Three sites | Diseased | Symptomatic | 8 |
| <i>Gibbsiella quercinecans</i> | All five sites | Healthy | Healthy | 1 |
| | | Diseased | Non-symptomatic | 1 |
| | | Diseased | Symptomatic | 21 |
| <i>Pseudomonas taxon fulva-like</i> | Four sites | Diseased | Non-symptomatic | 1 |
| | | Diseased | Symptomatic | 18 |
| <i>Rahnella victoriana</i> | Three sites | Diseased | Non-symptomatic | 1 |
| | | Diseased | Symptomatic | 15 |



Tissue condition and tissue types: Healthy tissue types from healthy trees; non-symptomatic tissue in diseased trees; symptomatic tissue including the larval galleries of *Agilus biguttatus* in diseased trees