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

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**Systematic and Applied Microbiology**

Published: 01/10/2016

Peer reviewed version

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

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Running title: AOD microbiome shift

**Abstract**

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

**Keywords**

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

**Introduction**

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

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

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

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

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

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

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

## Materials and methods

### Field Sites

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

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

Sample taking

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

Sample demographics

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

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

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

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

Isolation

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

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

PCR amplification and marker gene sequencing

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

For fungal identification, if colonies had sporulated, a slide was made to get an initial identification based on morphology and colonies were subsequently produced from single-spores. If no sporulation had taken place, subcultures were produced from hyphal tips. For DNA extraction, single-spore or hyphal tip isolates were cultured on sterile squares of clear 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.

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

## Results and discussion

Sample demographics and yield

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

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

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

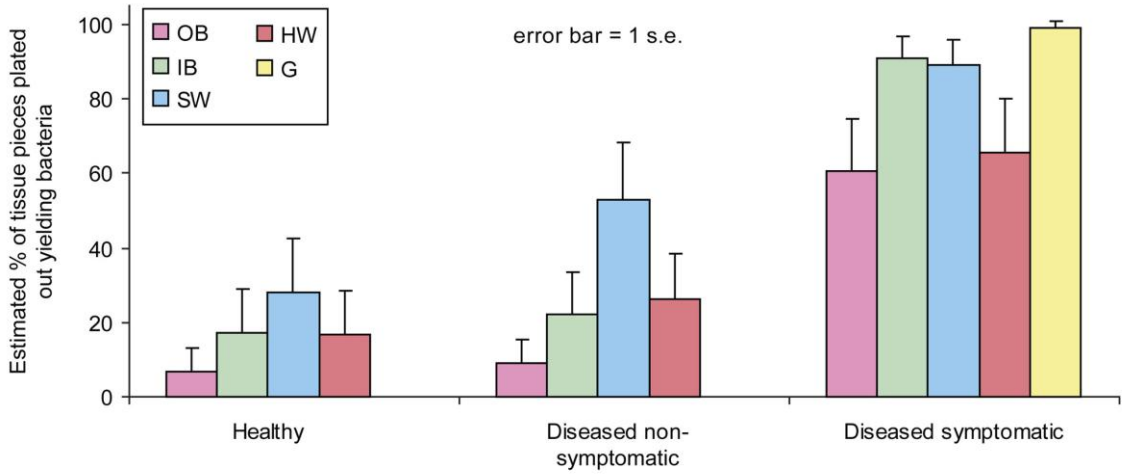
[a] Sampling combinations comprise: site vs. tissue condition vs. tissue type vs. unique tree

| | | 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** |

Yield of bacteria and fungi from tissue pieces

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

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



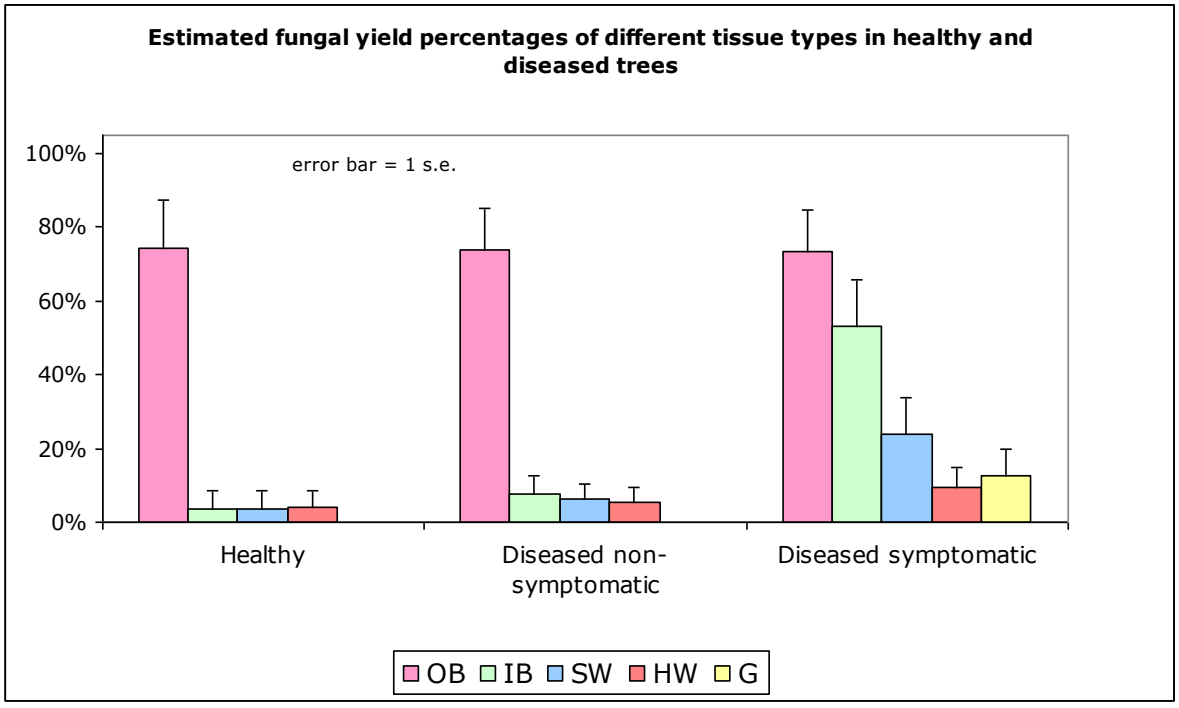

Tissue condition and tissue types: Healthy tissue types from healthy trees; non-symptomatic tissue in diseased trees; symptomatic tissue including the larval galleries of *Agrilus biguttatus* in diseased trees

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

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

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

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




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

Overview of the Oak Microbiome

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

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

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

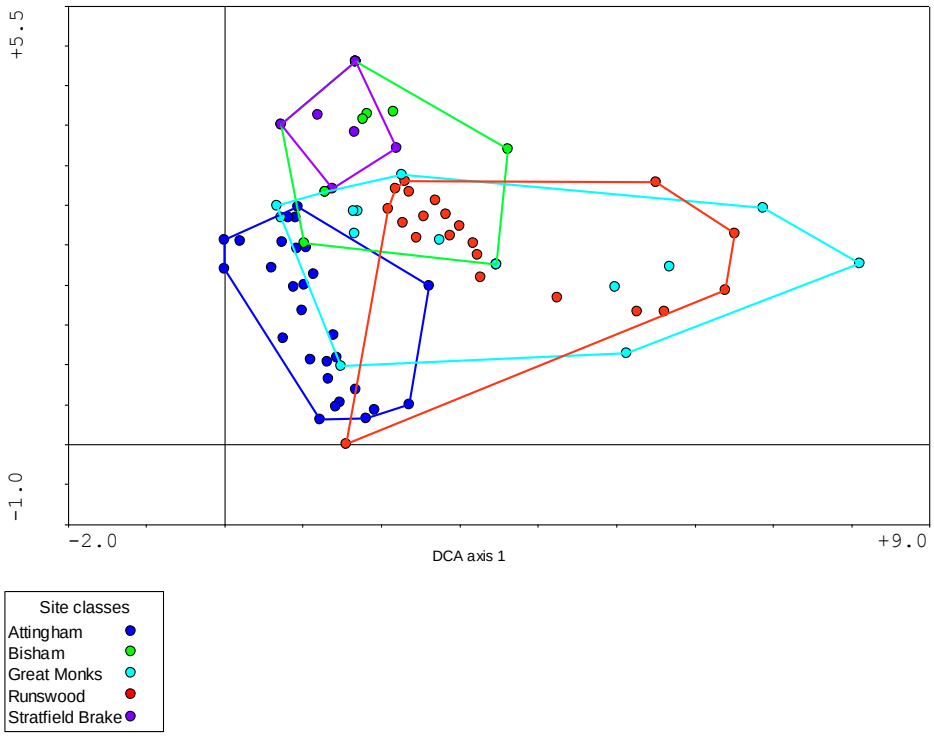
Fungi: There was variation in the incidence and occurrence of fungi, as the relationship shows some interactions it was not clarified by producing a DCA.

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

Community structure in relation to site

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

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




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.

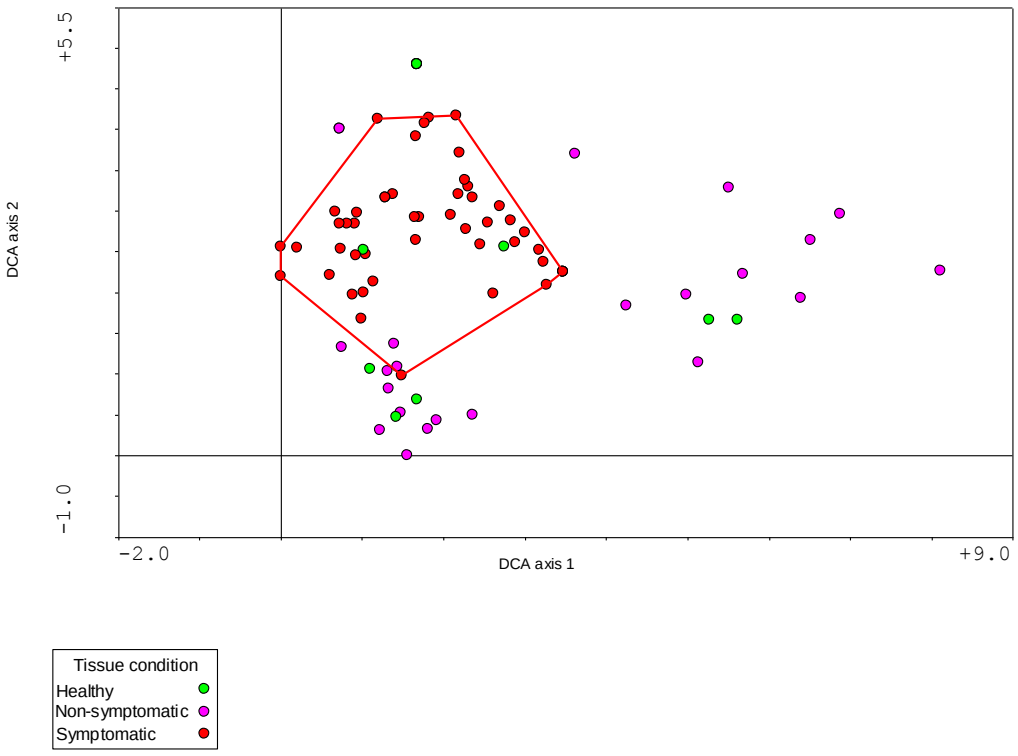
325

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

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




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

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

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

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

In summary:

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

Microbial Community Diversity (Species richness and abundance)

Bacteria: The diversity of the bacterial communities was analysed using the Shannon diversity index[b]. This identified a significant difference in species compositional diversity between tissue conditions. Diseased symptomatic tissue had higher bacterial diversity in comparison to healthy tissue ($p<0.05$). There was no significant effect of tissue type.

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

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

**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 |
|---|---|---|---|---|
| *Brenneria goodwinii* | All five sites | Healthy | Healthy | 1 |
| | | Diseased | Non-symptomatic | 4 |
| | | Diseased | Symptomatic | 22 |
| *Erwinia billingiae* | Three sites | Diseased | Symptomatic | 8 |

| | | | | |
|---|---|---|---|---|
| *Gibbsiella quercinecans* | All five sites | Healthy | Healthy | 1 |
| | | Diseased | Non-symptomatic | 1 |
| | | Diseased | Symptomatic | 21 |
| *Pseudomonas* taxon *fulva-like* | Four sites | Diseased | Non-symptomatic | 1 |
| | | Diseased | Symptomatic | 18 |
| *Rahnella victoriana* | 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*, *Pezicula* 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,

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

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

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

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

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

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

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

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

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

**Conclusions**

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

**Acknowledgements**

This study was funded by the Forestry Commission, Defra and Woodland Heritage. The authors would like to the landowners and land managers for their help and generosity in donating trees for research.

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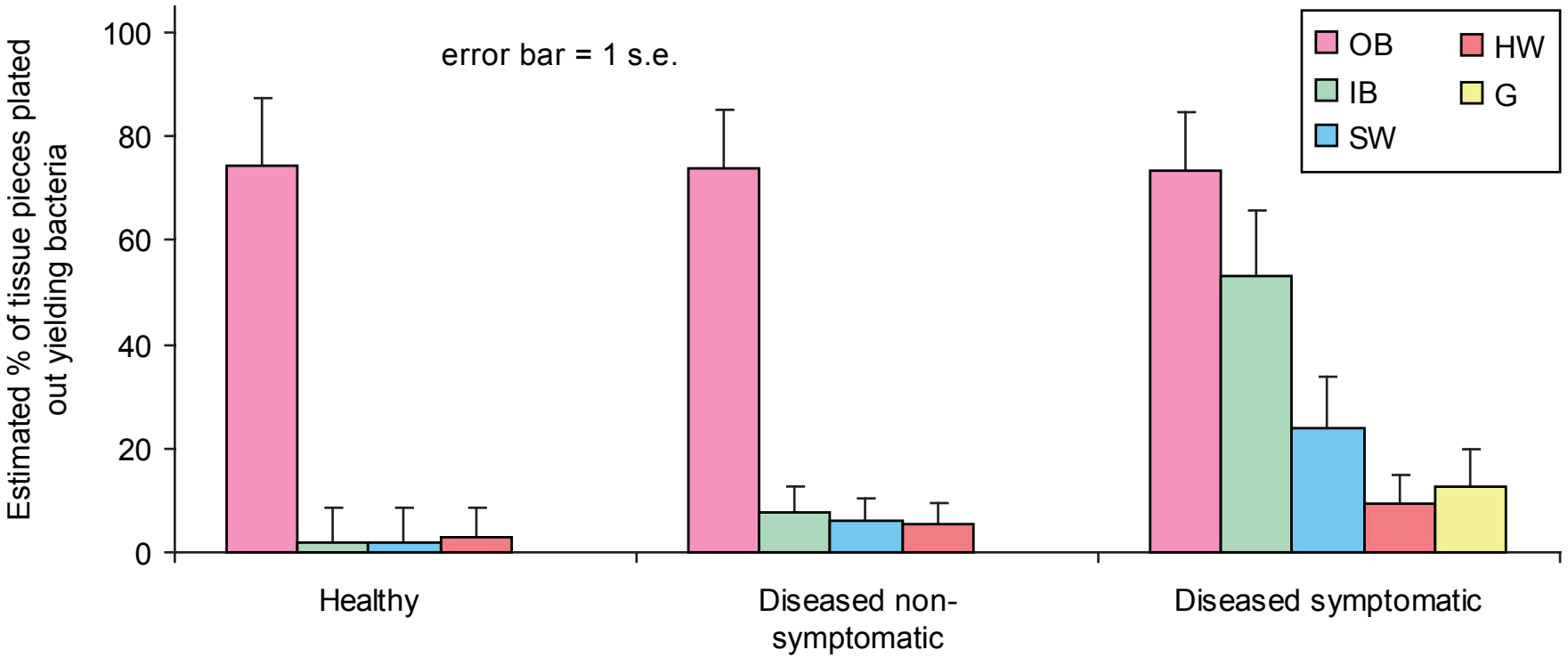
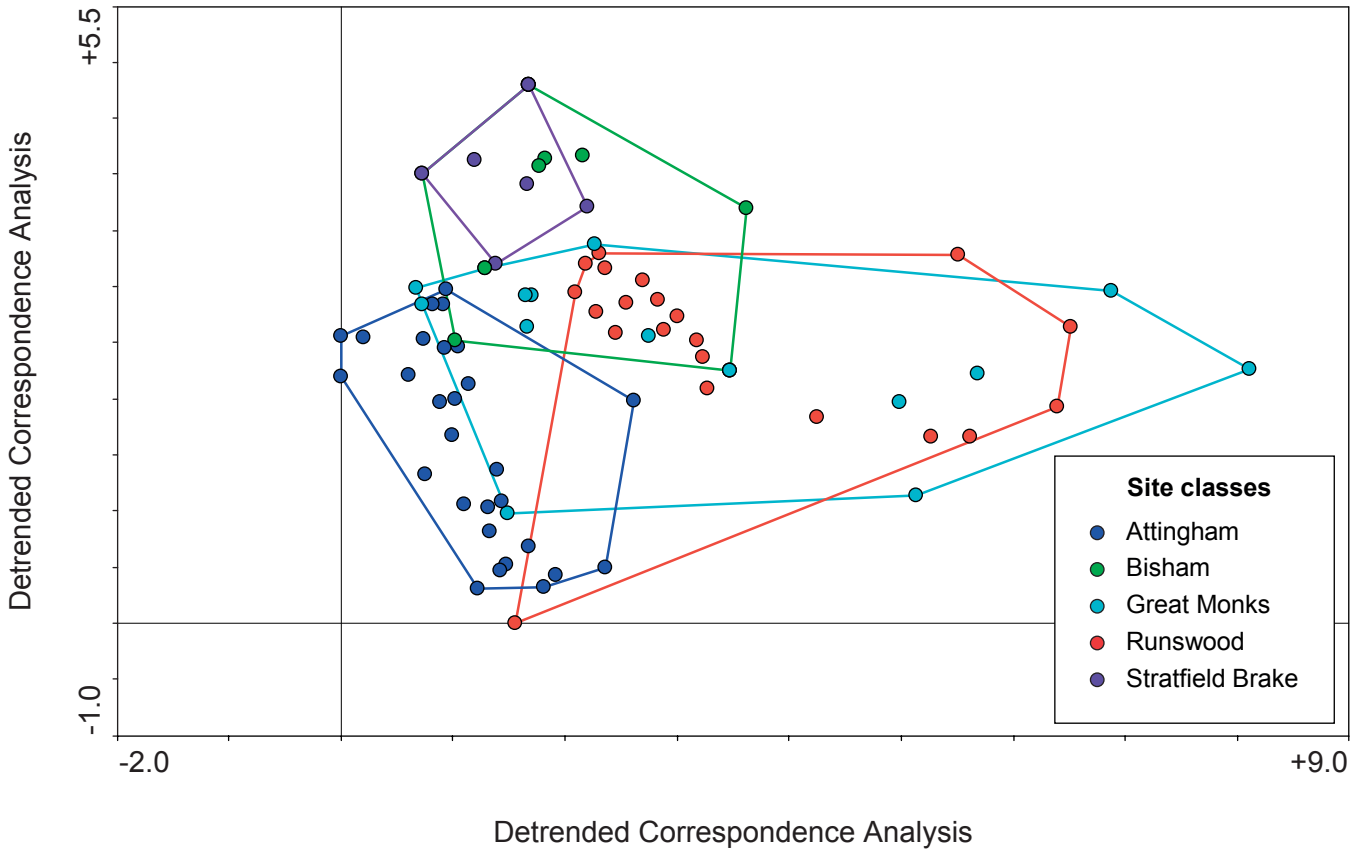
Attingham
Runs Wood
Great Monks
Stratfield Brake
Bisham Woods




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


+5.5
-1.0
Detrended Correspondence Analysis
-2.0
+9.0
Detrended Correspondence Analysis
Site classes
Attingham
Bisham
Great Monks
Runswood
Stratfield Brake

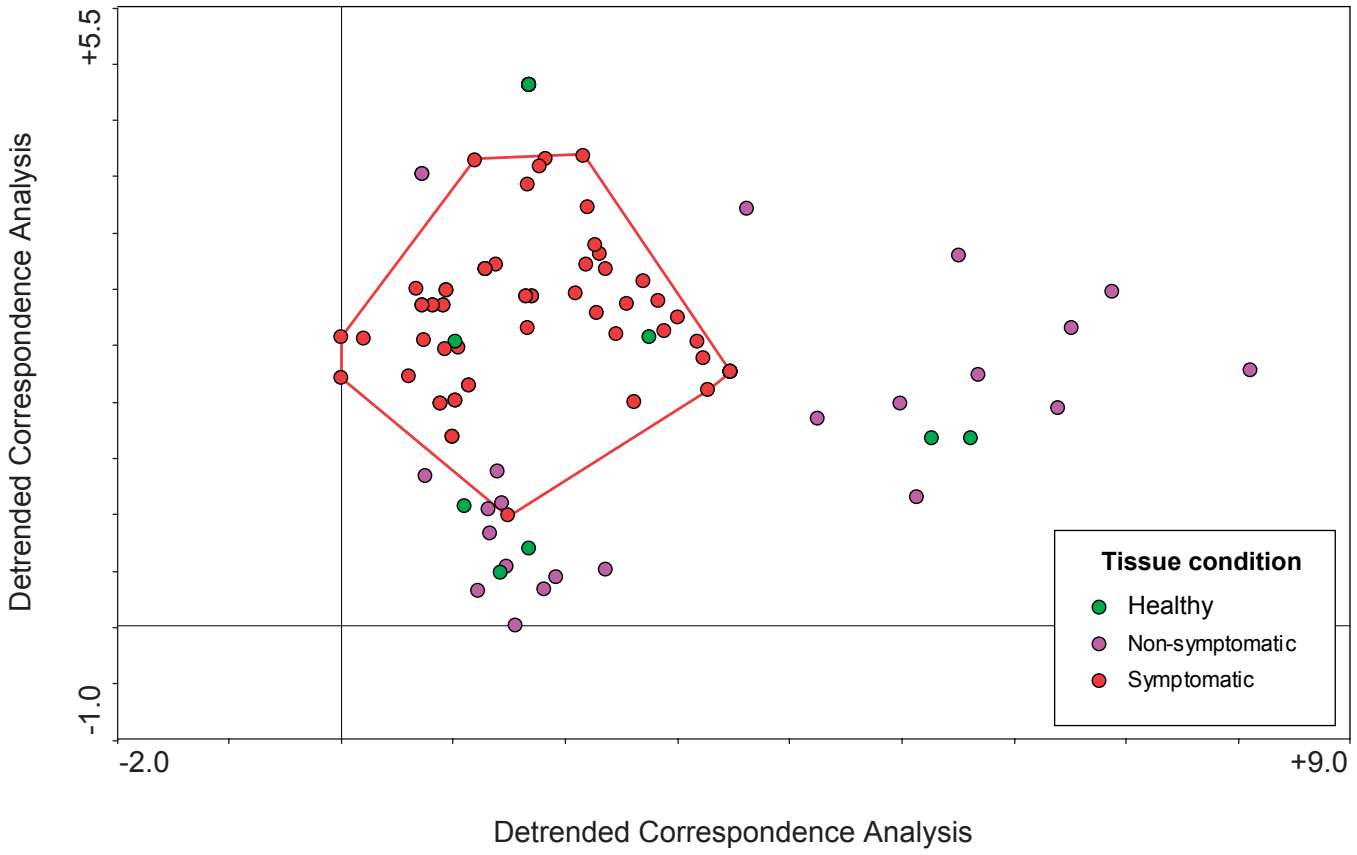

Detrended Correspondence Analysis
+5.5
-1.0
-2.0
+9.0
Detrended Correspondence Analysis
Tissue condition
Healthy
Non-symptomatic
Symptomatic

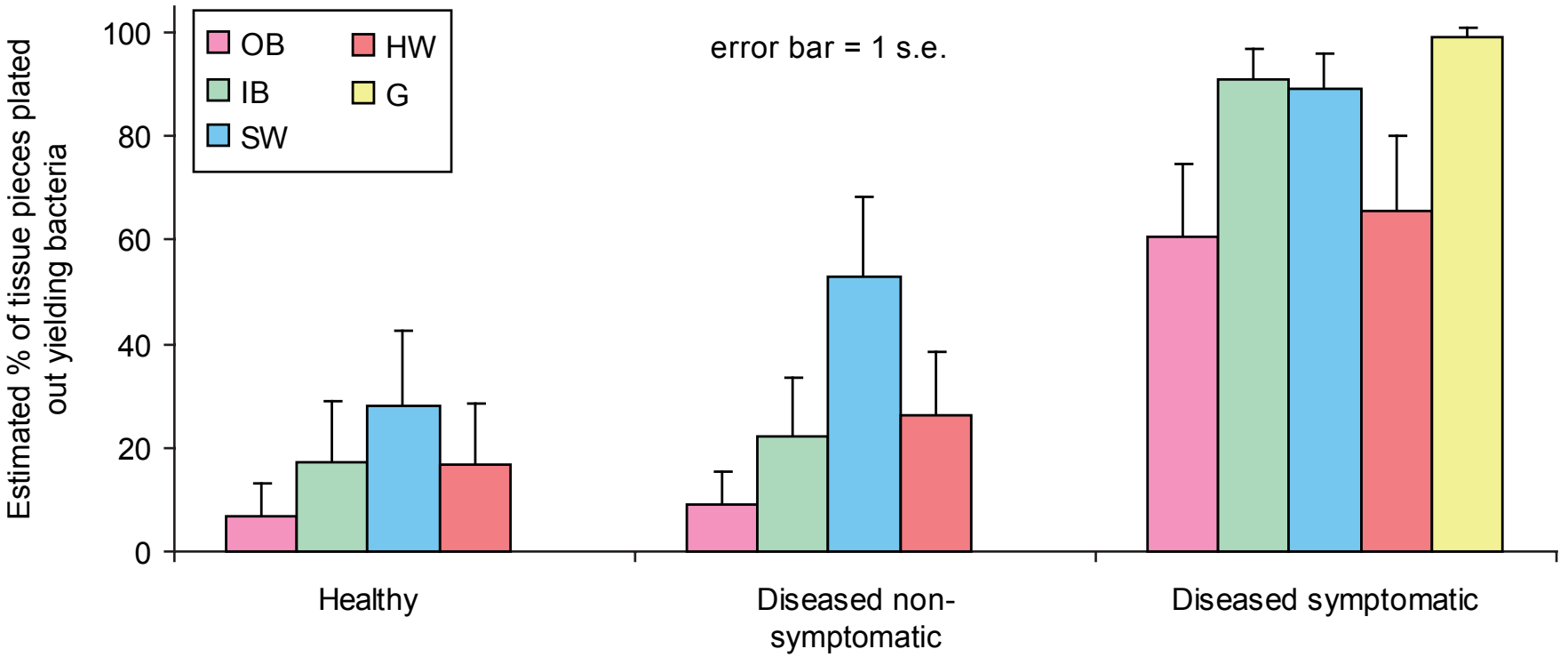
Table 1. Sampling combination[a] totals for bacteria and fungi at the site level, tissue condition level and tissue type level.

[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 |
|---|---|---|---|---|
| *Brenneria goodwinii* | All five sites | Healthy | Healthy | 1 |
| | | Diseased | Non-symptomatic | 4 |
| | | Diseased | Symptomatic | 22 |
| *Erwinia billingiae* | Three sites | Diseased | Symptomatic | 8 |
| *Gibbsiella quercinecans* | All five sites | Healthy | Healthy | 1 |
| | | Diseased | Non-symptomatic | 1 |
| | | Diseased | Symptomatic | 21 |
| *Pseudomonas* taxon *fulva-like* | Four sites | Diseased | Non-symptomatic | 1 |
| | | Diseased | Symptomatic | 18 |
| *Rahnella victoriana* | 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 *Agrilus biguttatus* in diseased trees