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Survival of *Brenneria goodwinii* and *Gibbsiella quercinecans*, Associated with Acute Oak Decline, in Rainwater and Forest Soil

Bethany J. Pettifor[1], James Doonan[1], Sandra Denman[2] & James E. McDonald[1]*

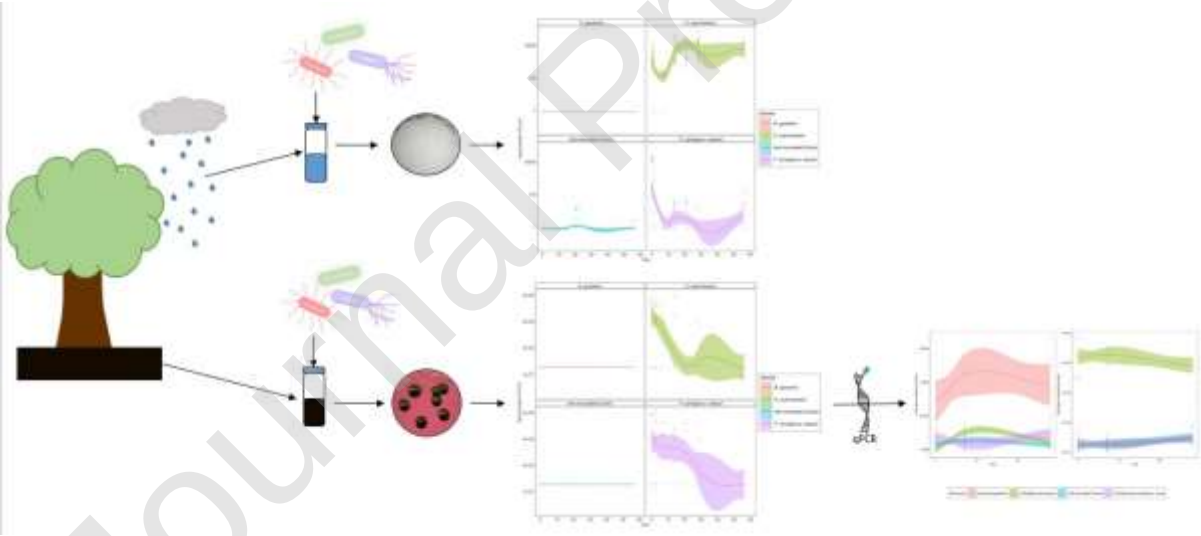
[1]School of Natural Sciences, Bangor University, Deiniol Road, Bangor, Gwynedd, LL57 2UW, UK.

[2]Forest Research, Centre for Forestry and Climate Change, Alice Holt Lodge, Farnham, Surrey GU10 4LH, UK.

*Corresponding author

E-mail address: j.mcdonald@bangor.ac.uk (James E. McDonald)

Graphical abstract



Highlights

- *Brenneria goodwinii* and *Gibbsiella quercinecans* are causal agents of AOD lesions
- The ecology of *B. goodwinii* and *G. quercinecans* in forest ecosystems is unknown
- *Brenneria goodwinii* decays rapidly in soil and rainwater
- *Gibbsiella quercinecans* can persist in soil and rainwater

Abstract

Acute oak decline (AOD) affects native UK oak species causing rapid decline and mortality in as little as five years. A major symptom of AOD is black weeping stem lesions associated with bacterial phytopathogens, *Brenneria goodwinii* and *Gibbsiella quercinecans*. However, there is limited knowledge on the ecological and environmental reservoirs of these phytopathogens. Rainwater and soils are common reservoirs of plant pathogens in a forest environment; therefore, the aim of this study was to investigate the survival of *B. goodwinii* and *G. quercinecans in vitro* when inoculated into rainwater and forest soil using a combination of agar-based colony counts and *gyrB* gene-targeted quantitative PCR (qPCR). *Brenneria goodwinii* lost viability on inoculation into soil and rainwater, but was detectable at low abundance in soil for 28 days using qPCR, suggesting a limited ability to persist outside of the host, potentially in a viable but non-culturable (VBNC) state. Conversely, *Gibbsiella quercinecans*, was re-isolated from rainwater for the entire duration of the experiment (84 days) and was re-isolated from forest soil after 28 days, with qPCR analysis corroborating these trends. These data demonstrate that *B. goodwinii* is unable to survive in forest soils and rainwater, suggesting that it may be an endosymbiont of oak trees, whereas *G. quercinecans* remains viable in soil and rainwater biomes, suggesting a broad ecological distribution. These data advance understanding of the potential epidemiology of AOD-associated bacteria and their ecological reservoirs, thus increasing the overall knowledge of the pathology of AOD, which assists the development of future management strategies.

Keywords

Environmental reservoirs, Survivability, Culture-dependant, qPCR, Acute Oak Decline, Forest pathology

Introduction

Trees play a vital role in the functioning of the Earth, delivering key ecosystem services, supporting biodiversity, and providing habitats for a huge range of plant and animal species [1,3,17,18]. Tree diseases, including diebacks, blights and bleeding cankers, have become more frequent in a range of economically important tree species [21], leading to the prioritised need to understand these diseases and how they affect host species individually, and forest ecosystems as a whole. Acute oak decline (AOD) is a decline disease affecting the UK's native oak species [12]. In recent years, AOD has spread

rapidly across the UK, from the south-east, to the midlands of England, and as far west as south Wales [8]. As part of the oak decline complex, influenced by both biotic and abiotic factors, AOD causes the rapid decline of tree health, with mortality recorded in as little as five years [11,12]. Affecting native UK oak, *Quercus robur* and *Q. petraea,* AOD is defined by the presence of four major symptoms; cracks on the outer bark plates, weeping patches on the stem, necrotic lesions in the inner bark, which are usually in close proximity to larval galleries of the bark-boring beetle, *Agrilus biguttatus* [12].

Formation of necrotic lesions is caused by a bacterial pathobiome, including *Brenneria goodwinii* and *Gibbsiella quercinecans* [8], two bacterial species that were described after being initially isolated from lesions of AOD symptomatic trees [4,5,11,32]. Isolation studies found *B. goodwinii* and *G. quercinecans* in AOD-symptomatic oak trees in various stages of decline, demonstrating that both species are clearly crucial in the development of AOD [13,16]. A contemporary adaptation of Koch's Postulates using individual and combinations of bacterial inocula, in addition to beetle larvae, proved the polymicrobial cause of the lesions [13] and analysis of the AOD lesion metagenome and metatranscriptome confirmed *B. goodwinii* as the dominant and most active organism in AOD lesions while *G. quercinecans* was present with low relative abundance in the AOD-asymptomatic metagenome but more abundant in AOD-symptomatic lesion metagenomes [13].

Although there is a strong correlation between the presence of *A. biguttatus* and AOD [9], empirical evidence of its role as a vector for necrotrophic bacteria is lacking [15]. It is suggested that beetle larvae transport AOD-associated bacteria throughout the inner bark when creating their larval galleries, as these are often located close to necrotic lesions and the bacteria have been isolated along the length of the galleries [11]. Studies have investigated the spread and distribution of *A. biguttatus* [9], however there have been no published studies exploring presence and distribution of AOD associated bacteria in the forest ecosystem, and the possible methods of entry into the host. Research on the role of bacteria in AOD has predominantly focussed on bacterial activity in necrotic lesions [8], and consequently, the ecology, transmission and survival of AOD bacteria beyond the tree host is a key knowledge gap.

G. quercinecans, associated with AOD lesions, and related species (including *G. acetica* [19] and *G. papilionis* [22]) are thought to be forest generalists, and have been isolated from various tree species (oak [6], pear and apple [19]), as well as the gut of wood boring insects [9], and the digestive system of a butterfly [22]. In contrast, *B. goodwinii* is possibly a tree endophyte, as other species in the genus *Brenneria* are host-species specific pathogens, usually with no known environmental reservoir. Members of the genus *Brenneria*, such as *B. salicis,* which causes watermark disease in willow trees, and *B. nigrifluens* which affects walnut trees, are also present as endophytes in the vascular tissue of

the host, before becoming virulent pathogens [24,26]. *B. goodwinii* may therefore be an oak endophyte, remaining dormant in the vascular tissue and becoming pathogenic in response to host and environmental factors. In addition to the virulence of latent endophytes, it is well established that rainwater and forest soils can act as environmental reservoirs for phytopathogens, such as the bacterium *Pseudomonas syringae* [10].

Here, we applied a combination of culture-counts and qPCR detection, to determine the viability of *B. goodwinii* and *G. quercinecans* in rainwater and forest soil, to address our hypotheses that (i) *B. goodwinii* is a specific tree endosymbiont that will not remain viable outside the oak host, and (ii) *G. quercinecans* has a broad distribution in forest ecosystems. This research addresses a key knowledge gap regarding the environmental reservoirs of phytopathogenic bacteria associated with AOD.

Materials and Methods

Bacterial Strains, Growth Conditions and Confirmation of Identity

The type strains of *B. goodwinii* (FRB141), *G. quercinecans* (FRB97) and *P. syringae* pv. *aesculi* (FRB130), were obtained from Forest Research and initially grown from glycerol stocks. Strains were inoculated onto standard nutrient agar (for *P. syringae* pv. *aesculi* 5% sucrose was added [20]) and were incubated for 48-hours at 28°C.

Prior to the experiment, the identity of the type strains was confirmed using PCR and sequencing of the bacterial DNA gyrase subunit B (*gyrB*) gene. DNA was extracted from bacterial cells using the boil prep method; a single colony was picked from an agar plate containing a pure culture of the isolate using a sterile inoculation loop and suspended in 20 µl of PCR-grade water. Subsequently, the cell suspension was heated to 95°C for two and a half minutes, vortexed vigorously for 10 seconds, and heated again at 95°C for a further two and a half minutes. After being briefly vortexed, 1 µl of extracted DNA was used as the template for a PCR reaction using Bioline MyTaq™ Red Mix. Each 50 µl reaction consisted of 25 µl of 2x MyTaq™ Red Mix, 1 µl of 10 pmol each of *gyrB* gene specific oligonucleotide primers [5,16] gyrB07F (5' – CMCCYTCCACCARGTAMAGT – 3') and gyrB02R (5' – CMCCYTCCACCARGTAMAGT – 3'), 22 µl of nuclease-free H2O, and 1 µl of the DNA template (from the boil prep extraction method described above). PCR reaction conditions were: initial denaturation at 95°C for 1 minute, followed by 35 cycles of; denaturation at 95°C for 15 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 10 seconds, producing a PCR product approximately 750 base-pairs in length. Ten microliters of the PCR product was isolated using 1% agarose gel electrophoresis at 100 V for 50 minutes, and 1 µl of the PCR product was quantified using the Qubit™ dsDNA HS Assay

Kit. The unpurified PCR product was then sent, for sequencing to Macrogen Europe, along with the accompanying gyrB07F sequencing primer.

The identity of each strain was confirmed by searching the *gyrB* sequence of each strain against the NCBI BLAST database using the "Nucleotide BLAST" feature [33], for *B. goodwinii, G. quercinecans* and *P. syringae* pv. *aesculi,* using >97% sequence similarity as a cut-off for species delineation.

After the identity of strains was confirmed, each strain was inoculated onto fresh nutrient agar approximately once per week for the duration of the experiment. Fresh glycerol stocks of each strain were made regularly. Stock plate cultures were wrapped in parafilm after an initial incubation at 28°C for 24-48 hours, and stored at 4°C.

Measuring Survival of *B. goodwinii* and *G. quercinecans* in Rainwater

Rainwater Collection and Microcosm Processing

Due to the failure to develop a selective culture method to re-isolate *B. goodwinii* and *G. quercinecans* from rainwater, two independent experiments were conducted using sterile rainwater. These experiments involved the following. In the first instance, rainwater was collected over a two-week period in a large bucket lined with two large autoclave bags. The bucket was situated on the roof of the Memorial Building, Bangor University, and the surrounding area was cleared to reduce the risk of ground splash contamination. One litre of rainwater was collected and frozen at -20°C prior to the start of the experiment. The frozen rainwater was thawed for six hours and then autoclaved twice, to remove endogenous rainwater microorganisms, then 30 ml aliquots of rainwater were transferred into twelve sterile 50 ml falcon tubes to generate rainwater microcosms for the experiment. In the second experiment, rainwater was collected as before, and again, autoclaved (immediately after collection, and then twice further on consecutive days). Once again, 30 ml of the sterilised rainwater was measured into twelve sterile 50 ml falcon tubes and these were used as the rainwater microcosms for the repeat experiment.

Rainwater Microcosm Inoculation Procedure

Starter cultures were produced (three biological replicates per bacterial species), by inoculating 100 µl of an overnight culture into the corresponding 10 ml broth (standard nutrient broth for *B. goodwinii* and *G. quercinecans*, and nutrient broth with 5% sucrose for *P. syringae* pv. *aesculi*). The nine broth cultures were incubated at 28°C, shaking at 150 RPM until the OD_{600} value reached 0.5. Once the desired OD_{600} value was reached, cultures were immediately stored on ice at 4°C, until all broths had reached the desired OD_{600} value.

Previous growth curve experiments, performed in triplicate, found that at an OD_{600} of 0.5; *B. goodwinii* cultures contained approximately 1.13 x 10^{14} CFU/ml, *G. quercinecans* cultures contained approximately 1.7 x 10^{8} CFU/ml, and *P. syringae* pv. *aesculi* cultures contained approximately 2.1 x 10^{7} CFU/ml. The CFU count of *B. goodwinii* at an optical density of 0.5 at 600 nm (1.13 x 10^{14} CFU/ml) was much greater than the other two species, but this observation was confirmed via three replicate growth curves with serial dilution and plate counts, and an OD_{600} of 0.5 was achieved after 12.5 hours of growth when cells were in mid-exponential phase. Subsequently, bacterial inocula for each of the three species were standardised to 1 x 10^{6} CFU/ml in each 30 ml microcosm.

Once all starter cultures had reached an OD_{600} value of 0.5, each of the nine 10 ml cultures were centrifuged at 3000 x *g* for five minutes, the supernatant was removed, and the pellet of cells re-suspended in 10 ml Phosphate Buffered Saline (PBS) solution. Prior to this step, *B. goodwinii* cultures were diluted to 10^{-6} CFU/ml due to their substantially greater cell count, in order to have a more manageable cell number. After re-suspensions in 10 ml PBS solution, 265 µl of the *B. goodwinii* suspensions was removed and transferred to a sterile Eppendorf tube, which was centrifuged at 3000 x *g* for five minutes and the cell pellets re-suspended in 200 µl of PBS solution, before being added to the corresponding microcosms. This was repeated with 176 µl of the *G. quercinecans* culture, and 1430 µl of the *P. syringae* pv. *aesculi* culture. This was to ensure that approximately 1 x 10^{6} cells were added to each microcosm. Two hundred microliters of PBS solution was added to each of the non-inoculated control microcosms to adjust for added liquid volume and salts. After this initial inoculation, all twelve rainwater microcosms were vortexed vigorously for thirty seconds.

The second experiment followed the same inoculation procedure as above, however the *B. goodwinii* colony was diluted to 10^{-3} CFU/ml, rather than 10^{-6} CFU/ml prior to inoculation. The reduced dilution was a result of the estimated CFU/ml of the initial experiment being too low after the 48-hour incubation period.

Rainwater Sampling Procedure

The first sampling session (T_0 in both the initial experiment and the second experiment) took place immediately after the microcosms were inoculated. This and all other sampling sessions were carried out in a pre-sterilised (thorough wiping with 1% Distel, and a 30 minute UV treatment) biosafety cabinet to minimise the chances of contamination, and were comprised of the following:

Two hundred millilitres of standard nutrient agar, and 200 ml of nutrient agar with 5% sucrose, were produced and poured into sterile square bioassay trays (sterilised through soaking in 5% bleach for a

minimum of 30 minutes and then UV treated before being wiped with 1% Distel prior to plating). These plates were then left to set for a minimum of three hours in a biosafety cabinet.

All twelve microcosms were vortexed briefly at a medium to high speed, and from each of the twelve microcosms, three separate samples (technical replicates A, B and C) were taken. This involved taking 100 µl from each microcosm three times, and then using these undiluted samples to produce a serial dilution to 10^{-8} CFU/ml. The serial dilution was performed in a micro-well plate where each well contained 180 µl of PBS solution and 20 µl of the neat sample was added. This was pipette mixed approximately five times to ensure thorough mixing before moving to the next row and creating the next dilution factor.

Ten microliters of each dilution was inoculated onto the bioassay trays. *B. goodwinii* and *G. quercinecans* were inoculated onto the nutrient agar plate, and *P. syringae* pv. *aesculi* and the non-inoculated control were plated onto the nutrient agar with 5% sucrose. Both plates were incubated at 28°C for 48 hours in a static incubator, and the number of visible colonies recorded.

The colony forming unit (CFU) counts of *B. goodwinii, G. quercinecans* and *P. syringae* pv. *aesculi* were carried out on day T_0, T_1, T_7, T_{14}, T_{21}, T_{28}, T_{56}, and T_{84}. The number of colony forming units per ml at each time point was calculated by multiplying by the dilution factor where countable colony numbers were present.

Survival Rates of *B. goodwinii* and *G. quercinecans* in Forest Soil

Forest Soil Collection and Microcosm Processing

Forest soil was collected from a mature oak tree at Treborth Botanic Gardens, Bangor University. The soil was collected from the base of the tree between two buttress roots. After brushing away the topsoil and any forest debris (such as twigs and leaf litter), soil was taken from around four to six inches below the surface, with as little disturbance as possible to the immediate area. The soil was collected in a clean plastic bag and a shovel disinfected with 1% Distel. The soil was immediately taken to the lab for processing.

Further processing of the soil was carried out in sterile conditions. Larger aggregates of soil were broken down by hand and then sieved using a sterile (autoclaved and UV treated) 2 mm sieve to give even sized particles. Fifty grams of sieved soil was weighed into a sterile (UV treated), re-sealable plastic bag. This was repeated twelve times giving twelve soil microcosms (three replicates for *B. goodwinii* inoculation, three replicates for *G. quercinecans* inoculation, three replicates for *P. syringae* pv. *aesculi* inoculation, and three replicates for the non-inoculated control).

Forest Soil Microcosm Inoculation Procedure

Ten millilitre starter cultures were produced for *B. goodwinii, G. quercinecans* and *P. syringae* pv. *aesculi* (in triplicate), by inoculating 1% (100 µl) of an overnight broth culture (produced from stock agar plates, incubated at approximately 25°C, shaking at 150 RPM) into 10 ml of nutrient broth. The cultures were then incubated at 28°C, shaking at 150 RPM, until reaching an OD_{600} value of 0.5. The cultures were then centrifuged at 3000 *x g* for five minutes to pellet the cells. The supernatant was removed and discarded, and the cells re-suspended in 500 µl of PBS solution, and vortexed briefly. The 500 µl cell suspension was inoculated into the soil microcosm bags, and 500 µl of PBS solution was added to the non-inoculated control microcosms to correct for moisture across the 12 microcosms. Each microcosm bag was shaken vigorously for 30 seconds to ensure even and thorough mixing. The microcosms were stored at 10°C throughout the experiment, as this is the average annual forest temperature in the UK.

Forest Soil Microcosm Sampling Procedure

The first sampling event took place under sterile conditions within one hour of initial inoculation as follows:

Four petri dishes (150 mm x 15 mm) were filled with 100 ml of autoclaved Eosin Methylene Blue (EMB) agar. These and all other equipment and consumables were UV treated for 20 minutes before starting the sampling process. Each microcosm was shaken vigorously for 30 seconds to ensure even and thorough homogenisation of the soil and bacteria.

From each microcosm, 0.5 g of soil was weighed into a sterile Eppendorf tube four times, one of which was frozen at -20°C, for qPCR analysis. The three remaining soil samples from each microcosm (technical replicates A, B and C), were used for the next stage of the process. One millilitre of PBS solution was added to the three Eppendorf tubes, and the contents vortexed at medium to high speed for 30 seconds. These undiluted samples were then used to create a serial dilution to 10^{-8} CFU/ml. Twenty microliters of dilutions 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} CFU/ml were inoculated onto EMB agar plates (1 plate per condition; *B. goodwinii, G. quercinecans, P. syringae* pv. *aesculi* and non-inoculated control). The four plates were incubated at 28°C for 48 hours and after this incubation period, the number of visible colonies was counted and recorded. Colony forming units (CFU) were determined after T_0, T_1, T_3, T_7, T_{14}, T_{21}, T_{28} and T_{56} days. The CFU/ml at each time point was calculated by multiplying by the dilution factor. EMB agar inhibits Gram-positive bacteria and fungi allowing detection of viable *B. goodwinii* and *G. quercinecans* cells in soil samples, which produce an easily identifiable green iridescent sheen.

qPCR Analysis of Soil Microcosms

Relative abundance Quantitative PCR (qPCR) assays were used to support the culture-based methods of measuring bacterial decay rates. Analysis of the bacterial gyrase B (*gyrB*) gene, using the Bioline SensiFAST™ SYBR Lo-ROX kit [2], and specific *Brenneria goodwinii* and *Gibbsiella quercinecans* primers (Table 1), were used to determine *B. goodwinii* and *G. quercinecans* gene copies in the microcosms.

Firstly, DNA was extracted from each microcosm sample (three biological replicates of *B. goodwinii*, *G. quercinecans, P. syringae* pv. *aesculi* and the non-inoculated control) using the MoBio PowerSoil® DNA Isolation Kit, following the manufacturers protocol [27].

Secondly, qPCR standards for relative abundance quantification of *gyrB* gene copy number for *B. goodwinii* and *G. quercinecans* were produced. Standards were created by amplifying the bacterial DNA gyrase subunit B (*gyrB*) gene using qPCR specific primer sets. DNA was extracted from cells grown from glycerol stocks, using the boil prep method, (described above). After being briefly vortexed, 1 µl of extracted DNA was used as the template for the PCR reaction using Bioline MyTaq™ Red Mix. Each 50 µl reaction consisted of 25 µl of 2x MyTaq™ Red Mix, 1 µl of 10 pmol each of *gyrB* gene specific oligonucleotide primers for generation of qPCR standards (Table 1), 22 µl of nuclease-free H_2O, and 1 µl of the DNA template. PCR reaction conditions were: initial denaturation at 95°C for 1 minute, followed by 35 cycles of; denaturation at 95°C for 15 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 10 seconds, producing a PCR product which was approximately 750 base-pairs in length. The PCR product was isolated using a 1% agarose gel electrophoresis at 100 V for 50 minutes, and amplicons were gel excised and further processed using the Qiagen QIAEX® II Gel Extraction kit (following the manufacturer's protocol [29]) the resulting DNA was then quantified using the Qubit™ dsDNA HS Assay. The standards were calculated using established equations from [30], where the length of the resulting *B. goodwinii* amplicon was 959 bp, and the *G. quercinecans* amplicon was 1012 bp.

qPCR analysis was performed on each individual biological replicate of each condition (*B. goodwinii*, *G. quercinecans, P. syringae* pv. *aesculi*, and non-inoculated control) from the experimental DNA extractions, from time points T_0, T_7, and T_{28}. This was to detect for accurate gene copy numbers as well as to detect any possible contamination between the conditions. Each qPCR reaction contained; 10 µl of 2 x SensiFAST SYBR Lo-ROX Mix, 0.8 µl of the forward qPCR primer (10 µM), 0.8 µl of the reverse qPCR primer (10 µM), 7.4 µl of nuclease free H_2O, and 1 µl of DNA template from the extractions above.

Results

Confirming Strain Identity Through Sequencing of the DNA Gyrase B Gene

Positive identification of *B. goodwinii, G. quercinecans* and *P. syringae* pv. *aesculi* was confirmed through *gyrB* sequencing (Table 2).

Survival of *Brenneria goodwinii* and *Gibbsiella quercinecans* in rainwater

The sterile rainwater survivability experiment (Figure 1) demonstrated that *B. goodwinii,* after being undetectable from T_0, had lost viability at the point of inoculation. This occurred in both experimental repeats. *G. quercinecans* on the other hand produced countable colonies throughout the whole experimental period of 84 days, ranging from 227 CFU/ml (on day T_7, at the lowest point of the apparent acclimatisation period) to 14818 CFU/ml (on day T_{28}, the peak before the plateau and final decline occurred indicated on Figure 1). This occurred in both repeats of the experiment. The presence of countable colonies that were not identified as any of the species used to inoculate the microcosms in the NIC condition indicated that there had been a small scale contamination event, leading to low-level microbial growth in the biological replicates of this condition.

There is a clear initial decline in the number of CFU's/ml in the *G. quercinecans* and *P. syringae* pv. *aesculi* (positive control) microcosms, followed by an increase. The *G. quercinecans* microcosms plateau before beginning a gradual decrease, with an overall increase in CFU/ml of 114% between day T_0 and T_{84}. The *P. syringae* pv. *aesculi* condition also suffers a secondary decrease before beginning a steady increase in CFUs towards the end of the experimental period, with a final overall decline of 98% between day T_0 and T_{84}.

Survival of *Brenneria goodwinii* and *Gibbsiella quercinecans* in forest soil

The soil survival experiment (Figure 2) demonstrated that once again, *B. goodwinii* lost viability immediately after resuspension of a growing culture in PBS, causing cells to be undetectable from T_0. *G. quercinecans,* on the other hand had a small decline in CFU/ml at the start of the experiment (from 2.1×10^7 CFU/ml on day T_0 to 1.1×10^6 CFU/ml on day T_1), however (with the exception of T_{21}) there were countable colonies for 28 days (with approximately 22,222 CFU/ml at day T_{28}). At day T_{56}, there were no countable colonies of *G. quercinecans* demonstrating a 100% decline over the 56-day experimental period. *P. syringae* pv. *aesculi* (the positive control in the experiment) was countable up to T_{28}, with approximately 3.5×10^6 CFU/ml at this time point, but similar to *G. quercinecans, P. syringae* pv. *aesculi* was no longer detectable at T_{56}, signifying a 100% decline. As sampling was only

carried out at T_{28} and then again at T_{56}, it is unknown when *G. quercinecans* and *P. syringae* pv. *aesculi* declined to a point where they were no longer countable.

qPCR Detection of *B. goodwinii* and *G. quercinecans* in Soil Microcosms

Results from the qPCR analysis using *B. goodwinii* specific primers show that there are detectable levels of *B. goodwinii* in the *B. goodwinii* microcosms throughout the 28 day period (Figure 3). A rapid increase in detectable levels of *B. goodwinii* over the first 7 days of the experimental period peak (from approximately 12,373 *gyrB* gene copies/µl to approximately 16,189 *gyrB* gene copies/µl) and lead to a steady gradual decline, which still had detectable levels at day T_{28} (14,824 *gyrB* gene copies/µl). Although gene ct values (*gyrB* gene copy count) values for *B. goodwinii* were obtained in the other three microcosm conditions (*G. quercinecans* microcosms, *P. syringae* pv. *aesculi* microcosms, and non-inoculated control (NIC) microcosms), it is assumed that this is due to the *B. goodwinii* specific primer partially matching with another species present in the soil. The qPCR primers used for *B. goodwinii* in this experiment were designed for use on pure cultures and did exhibit low-level cross reactivity with environmental samples. However, the level of non-specific amplification was consistent across all samples and timepoints in microcosms where *B. goodwinii* was not added. To address this, and to confirm that the detection of *B. goodwinii* gyrB gene copies in microcosms where it was inoculated was statistically significantly different to treatments without *B. goodwinii* addition that expressed low-level cross-reactivity, a Welch's two sample t-test was conducted (*P<.001*). The significantly higher detection of *gyrB* gene copies in *B. goodwinii* microcosms compared to the other conditions (which all had very similar numbers of *gyrB* gene copy detection throughout the experimental period, between 5000 and 7000 *gyrB* gene copies/µl), in combination with statistical support for the significant difference between the low-level cross-reactivity and genuine detection of *B. goodwinii,* therefore validates the qPCR data.

Results from the qPCR analysis, show that there are detectable levels of *G. quercinecans* in the microcosms throughout the 28 day period (Figure 3). Following a similar trend to the culture based study, the initially high levels of *G. quercinecans* (approximately 33,415 *gyrB* gene copies/µl at T_0 where there were approximately 2.1 x10^7 CFU/ml) steadily declines over the course of the experiment (to 22,790 *gyrB* gene copies/µl at day T_7 where there were 1.4 x 10^7 CFU/ml, and just 9,445 *gyrB* gene copies/µl at day T_{56} where there were 1.1 x 10^7 CFU/ml). The other microcosm conditions (*B. goodwinii* microcosms, *P. syringae* pv. *aesculi* microcosms, and the non-inoculated control (NIC) microcosms), have negligible amounts of detection of *G. quercinecans* (all less than 100 *gyrB* gene copies/µl at any time point), showing that the qPCR primers are *G. quercinecans* specific.

Discussion

The main aim of this study was to determine whether rainwater or forest soil provide appropriate conditions to act as environmental reservoirs of the AOD associated bacteria, *B. goodwinii* and *G. quercinecans*.

Brenneria goodwinii

The results of the culture-based survival experiments, both in rainwater and forest soil, indicate that *B. goodwinii* cannot survive in rainwater or forest soil. *B. goodwinii* cells lost culturability at the point of inoculation in two independent rainwater experiments and a soil microcosm experiment. Conversely, *G. quercinecans* was tested in parallel with the same soil and rainwater samples in each of these experiments, and remained viable throughout. This loss of culturability in *B. goodwinii* is therefore not a result of the experimental design, and is a genuine phenomenon, as this result was obtained in three independent experiments, across two different environmental matrices, each with nine replicate measurements (3 biological replicates, with three technical replicates of each) at each time point. However, qPCR analysis of the soil microcosms, provides evidence that, *B. goodwinii* DNA was detectable, albeit at low relative abundance for up to 28 days in forest soil. This suggests that the *B. goodwinii* cells may have entered a viable but non-culturable (VBNC) state, as there was a rise in quantifiable *B. goodwinii* DNA between T_0 and T_7, and a reduced, but relatively consistent level of detection between T_7 and T_{28}. This change could also be explained by the degradation of cells leading to nonviable DNA being present in the microcosms at a low level until T_{28}.

The *Brenneria* genus are plant pathogens, causing necrogenic infections of various tree species [7]. Some species in the genus *Brenneria* are known to have this endophytic lifestyle, such as *Brenneria salicis*, the cause of Willow watermark disease, which is spread through leaf to leaf contact [24]. *B. salicis* resides in willow tree tissue in a non-pathogenic form, with undescribed factors and stressors tipping the balance between endophyte and pathogen [23]. Previous studies have confirmed that the development of AOD has a polymicrobial cause, and multi-omic data has confirmed that *B. goodwinii* is a key bacterial pathogen in this process [8,14]. The results of this study address the first experimental hypothesis, that *B. goodwinii* is a specific tree endosymbiont and will therefore decay quickly outside of the oak host in rainwater and forest soil. If the bacteria does not have the ability to survive in rainwater or forest soil (the two most common environmental reservoirs in a forest habitat), then is it possible that this phytopathogenic species is a specific tree endophyte, residing in host tissues until predisposing factors and inciting factors [25] cause sufficient weakening of the host that *B. goodwinii* becomes virulent and attacks host tissues.

Gibbsiella quercinecans

The results of the rainwater survival experiment indicate that there was an initial acclimatisation period, where a preliminary reduction was followed by rapid multiplication of *G. quercinecans.* This strain has the ability to survive in rainwater for up to 84 days (approximately 3 months). This would provide sufficient time for the bacterium to transfer from one host to the next, suggesting that bodies of rainwater could provide adequate conditions to act as an environmental reservoir for *G. quercinecans.* The results of the culture-based soil survival experiment indicate that after an initial decrease in CFU/ml, *G. quercinecans* slightly recovered around T_{28}, only to decline fully between T_{28} and T_{56}. The qPCR analysis of the soil microcosms for detection of *G. quercinecans* were as expected from the initial culture-based results, following a similar reduction. These results suggest that *G. quercinecans* has the ability to survive in rainwater and to a lesser extent forest soil (although conditions in a sealed forest soil microcosm would no longer be considered natural after 28 days, due to the lack of nutrient and carbon cycling).

In addition to the type species of the genus *Gibbsiella*, *G. quercinecans*, other species have also been isolated from a wide range of environments, including diseased apple and pear trees, and decaying wood from a compost pile [19], the intestinal tract of a butterfly in Korea [22] and the oral cavities of a bear, where acorns provide a high percentage of the autumnal diet of the bear [31]. Molecular genetic analysis of various *Gibbsiella* species have also indicated divergence in a number of genes present across the genus. This has been linked to the requirement for these saprophytes to adapt to a range of conditions present in these differing environments, as well as the numerous microbial competitors present in these environments [19].

The results of this study, address the question posed by the second experimental hypothesis, that *G. quercinecans* has a broad distribution in forest ecosystems and will therefore survive outside of the oak host in rainwater and forest soil. As with other species in the genus *Gibbsiella*, the plausibility that it will similarly be a saprophyte with a broad distribution in a forest ecosystem (including rainwater and forest soil), is quite high.

Future Research Priorities

Here we demonstrate that *B. goodwinii* is unable to survive in forest soils and rainwater, suggesting that it may be an endosymbiont of oak trees, whereas *G. quercinecans* remains viable in soil and rainwater biomes, suggesting a broad ecological distribution. However, these data are based on standardised laboratory microcosm experiments which are not necessarily fully representative of natural forest rainwater and soil biomes (i.e. rainwater microcosms were sterile with no autochthonous microbiota, limited nutritional biomass, and a lack of edaphic factors). To investigate the natural ecological distribution of these species in global forest biomes, further analyses could be

performed by mining public nucleotide sequence repositories for sequences originating from these species. Results from the soil survival experiment showed variation between *B. goodwinii* CFU/ml counts (indicating almost immediate depletion of culturability) and qPCR values (indicating a relatively constant level of cells, as determined by *gyrB* gene copies). This study therefore provides evidence that *B. goodwinii* could potentially enter a viable but non-culturable (VBNC) state, although this has not been proven empirically and should be the focus of further work on this species. In addition, experiments to assess the length of time that dead *B. goodwinii* cells and/or extracellular DNA persists in soils would be beneficial [28]. Another environmental reservoir for phytopathogens is their association with insect vectors. For AOD, the larval galleries of *A. bigutattus* are associated with almost 100% of AOD lesions in the UK [12], and evidence suggests that *A. bigutattus* larvae are able to 'drag' the bacteria a short distance within the inner tissues of trees and could facilitate the 'within tree spread' of bacteria [13]. However, there is currently no empirical evidence regarding the colonisation of larvae by AOD associated bacteria, and this is an unresolved research question.

Conclusions

The aim of this study was to investigate whether rainwater and forest soil could represent environmental reservoirs of the AOD-associated bacteria, *B. goodwinii* and *G. quercinecans*, outside of the AOD lesion environment. Using culture-based experiments, *B. goodwinii* was unable to survive in rainwater (without natural microbial competitors) or forest soil (with microbial competitiors), therefore supporting the hypothesis that *B. goodwinii* is an oak specific endosymbiont and will decay quickly outside of the host in rainwater and forest soil. However, qPCR analysis of *B. goodwinii* gyrB gene copies in soil microcosms confirmed detection for 28 days, suggesting that it may have entered a viable but non-culturable (VBNC) state. For *G. quercinecans,* culture-based experiments show that the bacterium could survive in rainwater (without natural microbial competitors) for up to 84 days, and forest soil for 28 days (with qPCR data to corroborate this). This supports the hypothesis that *G. quercinecans* has the potential for a broad distribution in forest ecosystems and will therefore survive outside of the oak host. This study strengthens the ecological understanding of *B. goodwinii* and *G. quercinecans,* which is crucial to understanding the spread of Acute Oak Decline.

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Figure 1. Colony Forming Units (CFU) per ml, of bacteria over time in a rainwater environment, showing that G. quercinecans, after an initial decline, recovered and maintained relatively high numbers of CFU/ml over the whole experimental period of 84 days.

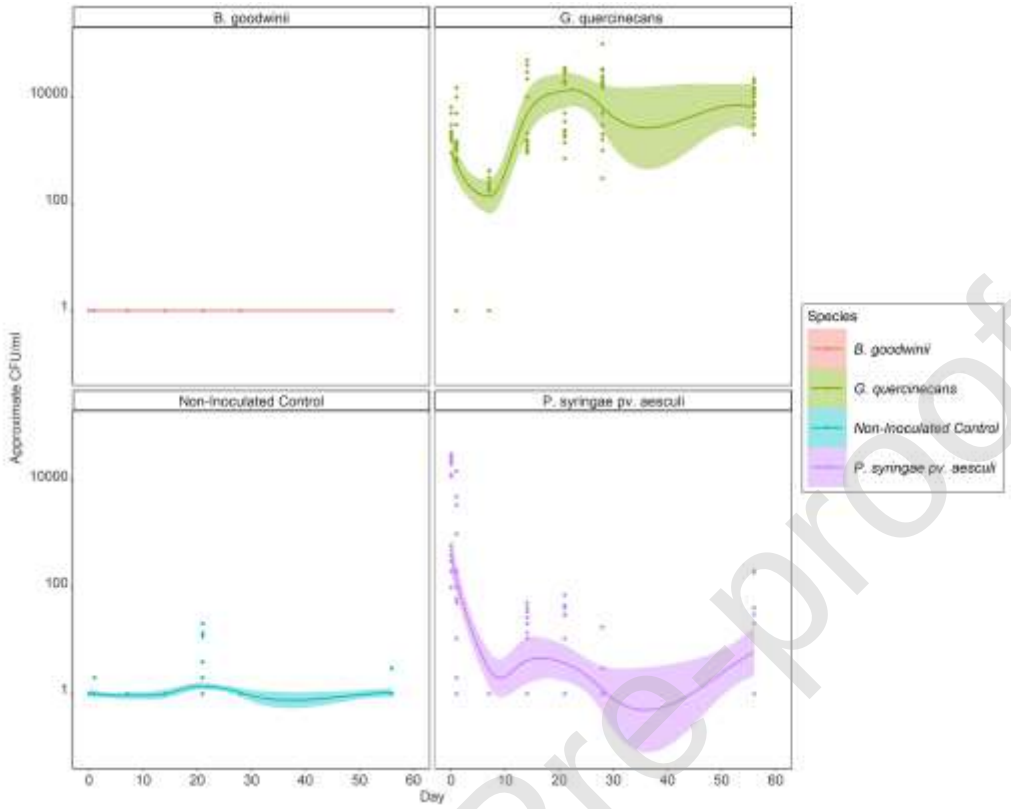

Figure 2. Colony Forming Units (CFU) per ml, of bacteria over time in a forest soil environment, showing that CFU/ml of *G. quercinecans* and *P. syringae* pv. *aesculi* fluctuated throughout the experiment, however did show an overall decline throughout the 56 day experimental period.

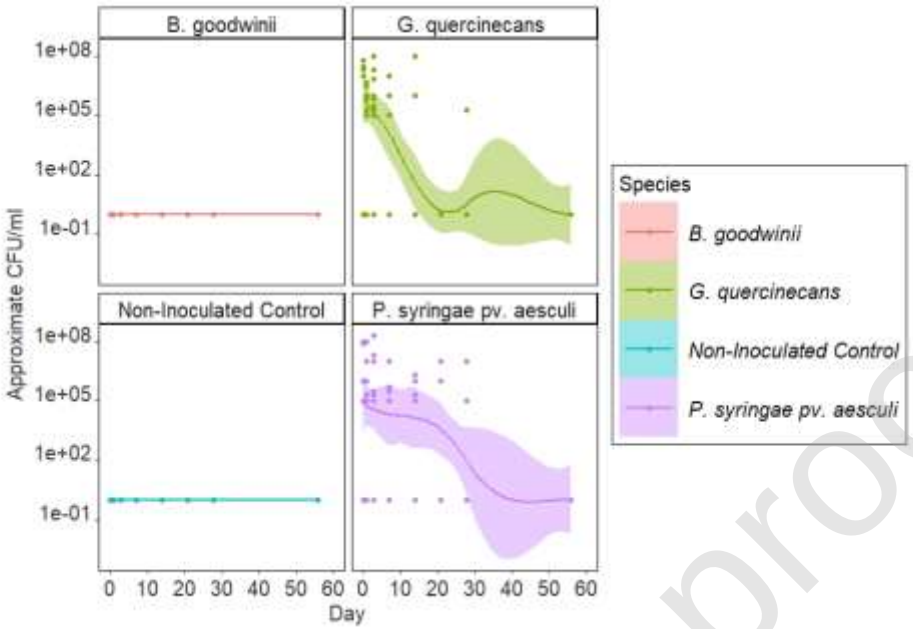

Figure 3. qPCR analysis of the each microcosm, (Left) Using *B. goodwinii* DNA specific primers . A significantly higher number of *B. goodwinii* gyrB gene copies is present in the *B. goodwinii* microcosms (as expected). Although there is a reasonable level of detection of the gene in the other conditions (which was unexpected), it is seemingly consistent across all three conditions, leading to the suggestion that this is background contamination common to all microcosms, due to a partial match of the *B. goodwinii* primers with other environmental bacteria. (Right) Using *G. quercinecans* DNA specific primers. A significantly higher number of *G. quercinecans* gyrB gene copies is present in the *G. quercinecans* microcosms, with extremely low levels of detection in the other conditions. Limit of Quantification: 250, Slope: -3.313, Y-Inter: 45.574, R^2: 0.999, Amplification Efficiency: 100%, Error: 0.024.

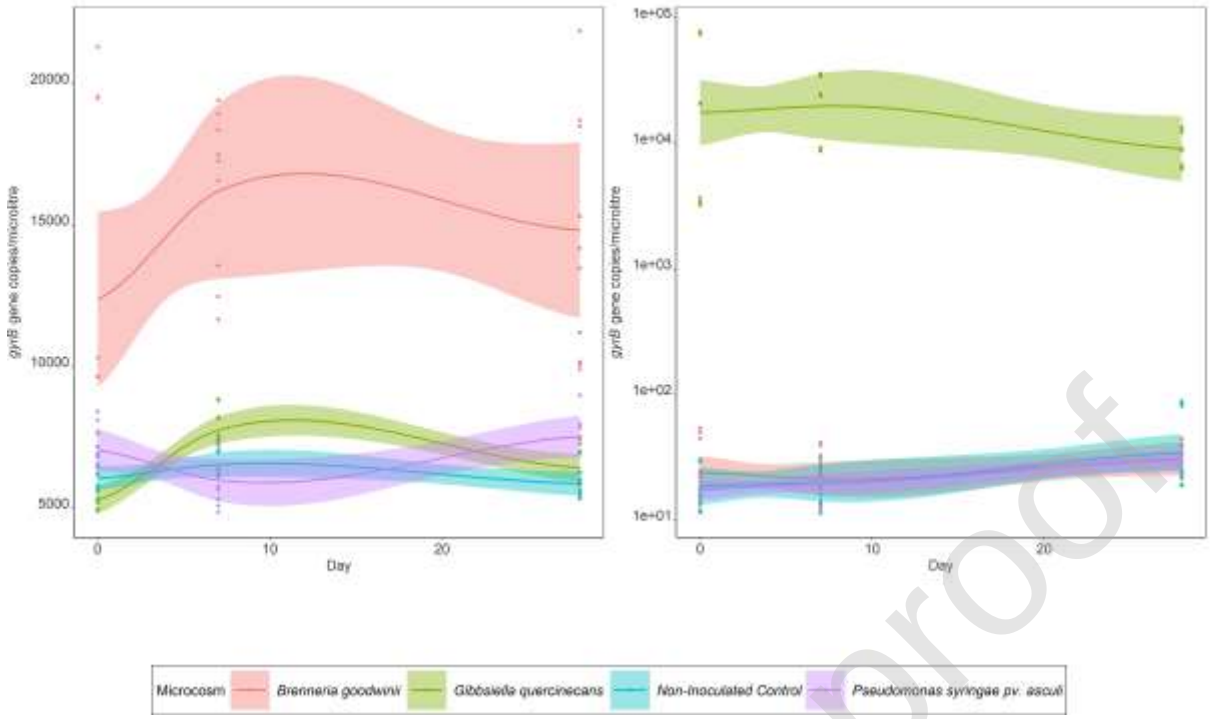

Table 1. Primer names and sequences used for the qPCR analysis of *Brenneria goodwinii* and *Gibbsiella quercinecans*. All primers designed to target the bacterial DNA gyrase B gene (*gyrB*).

Reaction	Primer Name	Sequence (5' – 3')	Product Length	Reference
Generation of qPCR Standards	BG_gyrB_F	TCGCGAAGGTAAGGTTCATC	959bp	This study
	BG_gyrB_R	CACTTCCTGGGAAGAGAGCA		This study
	GQ_gyrB_F	CATGACCCGTACCCTAAACG	1012bp	This study
	GQ_gyrB_R	CTGGTGAAACTGCTGAACGA		This study
qPCR	BG_gyrB_qPCR_F	CTGGCCGAGCCTGGAAAC	88bp	This study
	BG_gyrB_qPCR_R	AGTTCAGGAAGGAGAGTTCGC		This study
	GQ_gyrB_qPCR_F	GCGGTTGAACAACAGATG	94bp	This study
	GQ_gyrB_qPCR_R	GCCGCATCAATGATTTTG		This study

Table 2. Bacterial *gyrB* gene BLASTn results. DNA *gyrB* sequences of *B. goodwinii*, *G. quercinecans* and *P. syringae* pv. *aesculi*, were queried against the NCBI database. The closest identified alignment, similarity % and GenBank accession number are displayed below.

Species and Strain ID	**NCBI BLAST Result**	**Similarity**	**GenBank Accession Number**
Brenneria goodwinii (FRB141)	*Brenneria goodwinii* strain FRB141, complete genome	99%	CP014137
Gibbsiella quercinecans (FRB97)	*Gibbsiella quercinecans* strain FRB97, complete genome	97%	CP014136
Pseudomonas syringae pv. *aesculi* (FRB130)	*Pseudomonas syringae* SUPP817, *gyrB* gene for DNA gyrase subunit B	98%	LC364076.1