Harper Adams University

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POTENTIAL TRANSMISSION PATHWAYS OF TWO BACTERIA ASSOCIATED WITH ACUTE OAK DECLINE; *BRENNERIA GOODWINII AND GIBBSIELLA QUERCINECANS*

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Abstract

Acute Oak Decline (AOD) is a tree health condition affecting two oak species in Britain *Quercus robur* and *Q.petraea*. Symptomatic trees develop vertical fissures with associated stem bleeding due to tissue necrosis. Two newly identified bacteria species *Brenneria goodwinii* and *Gibbsiella quercinecans* have been isolated from this exudate and are now thought to contribute to the inducement of stem bleeding. Genetic analysis has indicated both bacteria have the capacity to be pathogenic and are able break down plant tissue.

How these bacteria colonise oak and any potential movement pathways allowing the spread of the bacteria within and between trees remains unknown. The association of *Brenneria goodwinii* and *Gibbsiella quercinecans* with the oak phyllosphere was first considered. Two primary pathways were then identified and explored. These were movement via insect vectors and airborne movement via rain water. A number of different techniques were used to establish whether any of these pathways had potential as colonisation routes. Stem lesions focus the collection on wood boring beetles as potential insect vectors and this will include *A.biguttatus* due to its associated distribution with symptomatic sites although as discussed there is limited evidence to support this. The presence of stem lesions and tissue necrosis symptomatic of Acute Oak Decline has led to suggestions a xylophagous (wood boring) insect may be involved. Other wood boring insects such as those with xylophagous and saproxylic lifestyles also utilise oak as a host and have potential to act as passive or active vectors as well. The second mechanism to be investigated will be the potential for airborne movement via rain to move bacteria within and between oak hosts.

Results indicate that *Brenneria goodwinii* and *Gibbsiella quercinecans* are associated with live oak leaves, leaf litter, acorns and catkins. Investigations suggest that both are part of the oak bacterial community and hint at differences in lifestyles between *Brenneria goodwinii* and *Gibbsiella quercinecans*. In addition the results suggest that both bacteria potentially have multiple means of encountering new hosts.

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List of Abbreviations

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AOD	Acute Oak Decline
CFU	Colony-Forming Unit
DNA	Deoxyribonucleic Acid
MarR	Multiple antibiotic resistance Regulator
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RpoS	RNA polymerase, sigma S
T2SS	Type 2 Secretion System
T3SS	Type 3 Secretion System
T4SS	Type 4 Secretion System
T6SS	Type 6 Secretion System
TCA	Tricarboxylic Acid Cycle
VFDB	Virulence Factor Database
VOC	Volatile Organic Compound

Chapter 1

Literature Review

1.1 Acute Oak Decline

The appearance of Acute Oak Decline (AOD) in the UK is first thought to have occurred during the 1980s (Denman *et al.,* 2010). At that time it was considered a new condition impacting the health of two oak species in Britain (*Quercus petraea* and *Q.robur*). In particular, it was noted that trees over 50 years old were most likely to be affected (Denman *et al.,* 2010).

Acute Oak Decline is described as a decline disease (Denman *et al.,* 2014) or a decline syndrome (Sapp *et al.,* 2016). This concept was established by Manion (1991) who developed a decline disease spiral model to explain the progression of declines. Within the model the interaction of abiotic and biotic elements together result in a gradual decline in tree health. Elements can be categorised into three groups (Sinclair, 1965); 'predisposing' - affecting a trees ability to withstand adverse events; 'inciting' - causing initial decline symptoms and 'contributing' - additional factors furthering decline (Table 1) (Sinclair, 1965). Trees already weakened by predisposing and inciting factors are more exposed to being affected by additional contributing factors (Manion, 1991).

Table 1. Categories and examples of abiotic and biotic elements influencing decline diseases

	Predisposing	**Inciting**	**Contributing**
Mode of action			
	Long term slow changing	Short term	Long and short term
	Site factors e.g.soil moisture[1, 2]	Defoliation[1]	Root decay fungi[1]
	Greater age[2]	Frost[2]	Canker fungi[1]
	Climate[2]	Drought[1]	Wood boring insects[1]
	Genetic makeup[2]	Injury[2]	Insect activity[1]
			Viruses[1]

[1]Sinclair (1965); [2]Manion (1991)

The decline in the health of oak due to various abiotic and biotic factors has been reported across Europe throughout the last century (Sallé *et al.,* 2014; Thomas *et al.,* 2002; Führer, 1998). Although oak declines have occurred previously in the UK these caused deteriorations in oak health by impacting foliage, via defoliation and mildew, rather than bark (Denman *et al.,* 2010). Despite the differing visual symptoms these declines are now thought to have also been caused by AOD (Denman *et al.,* 2010). This could indicate that trees affected by AOD show a variety of symptom expression or that impacts on foliage are a stage of disease infection. Defoliation could alternatively be a predisposing and mildew a contributing factor (Table 1).

Due to often multiple and interchangeable influential factors decline diseases make management strategies difficult to establish. Deciding at what point during the disease development to intervene can prove problematic when declines can also be considered part of natural succession at affected sites (Manion, 1991). Management options can also become complicated without a clear idea of disease progression. Research to date has primarily focussed on the epidemiology of AOD in the UK and potential inciting and influential factors which are discussed in the following sections.

1.1.1 Epidemiology of Acute Oak Decline

The symptoms of AOD have been specified as stem bleeds from lesions and underlying necrotic bark tissue (Denman *et al.,* 2014) (Figure 1, Figure 2). Exudate from these bleeds appears to be seasonal; usually occurring March to June and reappearing October to November (Denman *et al.,* 2014). During July to September and December to February) the exudate dries up. A further symptom of exit holes and larval galleries of *Agrilus biguttatus* (Oak jewel beetle) is often included (e.g. Broberg *et al.,* 2018) but this should be discounted, as a symptom is defined as the visible effect of a disease. Galleries and exit holes are more appropriately reported as signs (Denman *et al.,* 2014) or as associated with lesions (Denman *et al*., 2018). There is, however a lack of evidence that galleries and exit holes found are those of *A.biguttatus* and that they are linked with AOD. The identification of a *Q.robur* tree in Spain with AOD symptoms reported the absence of larval galleries (González & Ciordia, 2019) and trees have been observed with exit holes and no visible bleeds (personal observation).

Figure 1. Tree with symptomatic AOD lesions

Figure 2. Cut log section with AOD symptomatic bleeding lesions

Understanding the epidemiology and disease progression for AOD has proved difficult since there appears to be no consistent pattern of infection or symptom development. Mortality of trees showing symptoms was initially reported as occurring within four to five years of symptoms developing (Denman *et al.,* 2010). An epidemiological study using eight monitored woodland plots has, however, indicated that individual trees can enter remission following the previous years infection and that relapses in infection are also possible (Brown *et al.,* 2016).

In disease epidemiology, the epidemiological triangle (Figure 3) is a methodology which suggests that in order for disease to occur the presence of a causal pathogen alone is insufficient and factors related to the host and environment have to be present for it to establish (Page *et al.,* 1995). The use of the epidemiological triangle in relation to AOD could therefore be appropriate with indications that characteristics of individual trees and the environment are important in determining susceptibility to AOD.

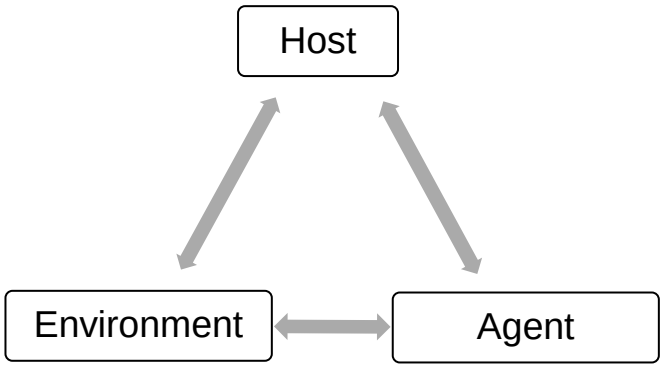


Figure 3. Example of an epidemiological triangle

Site conditions were found to be important when considering the rate of AOD infection and a linear progression of increasing disease development has not been demonstrated (Brown 2014). Local environmental factors may therefore be influencing infection (Brown, 2014). Over four study years the overall number of symptomatic trees remained stable at four sites potentially indicating AOD had reached a maximum level of potential hosts (Brown 2014). Full host resistance to a pathogen (Hughes, 1996) offers an explanation as to why not all trees are affected. Partial (Hughes, 1996) or quantitative host resistance, whereby the disease is reduced but not absent completely, is also possible (Poland, 2008).

In addition to the basic triangle other factors such as vectors, time and external reservoirs can be added in (Page *et al.,* 1995) and may be relevant to AOD. At seven of the eight woodland plots monitored by Brown, 2014 there was evidence of trees with symptoms 'clustering'; suggested as indicating the cause and movement of the condition was more likely to be due to a local biotic agent than a wider environmental cause such as movement via rain (Brown *et al.,* 2016). This supports the hypothesis that a pathogen could be influencing the pattern of infection shown (Brown *et al.,* 2016). The level of clustering of affected trees was however variable between sites and over different scales and distances (Brown, 2014) and has additionally become less clear with increasing time (Brown, 2018 conference presentation pers. comm.). Since plant pathogens can be dispersed by rain, wind, infected tissues, seeds or vectors (Esker *et al.,* 2007) the variation in distances between symptomatic trees may therefore indicate multiple dispersal mechanisms (Brown, 2014) or multiple reservoirs.

A modelling study has been carried out to investigate potential predisposing abiotic factors including soil type, climate and pollutant deposition all of which may influence the presence of AOD at a site (Brown *et al.,* 2018). Initial conclusions indicated that soil water level was a key influential factor with Acute Oak Decline more likely to be present where recorded rainfall was lower and on sites with seasonally waterlogged soil (Brown *et al.,* 2018).

Both of these may be related to soil type as most AOD was on sites with soils rich in clay (Brown *et al.,* 2018). Affected sites had a higher number of days above 11.5°C considered to indicate a longer growing season (Brown *et al.,* 2018). Total deposition of nitrogen was higher and sulphur lower at sites with AOD than those without (Brown *et al.,* 2018). This was suggested as being linked to sulphurs' antibacterial properties and a potential decline in tree health status as a result of decreased levels (Brown *et al.,* 2018). A higher level of soil nitrogen was also thought to represent lower tree health at a site (Brown *et al.,* 2018).

1.1.2 Bacterial component of Acute Oak Decline

Work on the epidemiology of Acute Oak Decline suggested the involvement of a bacterial pathogen or pathogens. Two newly identified species; *Gibbsiella quercinecans* [Family: *Enterobacteriaceae*] and *Brenneria goodwinii* [Family: *Pectobacteriaceae*] had been consistently detected together from necrotic tissue and stem bleeds and were considered likely to be involved (Brady *et al.,* 2010; Denman *et al.,* 2012; Sapp *et al.,* 2016).

Brenneria goodwinii

The genus *Brenneria* (Hauben *et al*., 1998 emend. Brady *et al*., 2012) forms a distinct phylogenetic group of gram negative bacteria 0.861-1.3 μm with flagella on their surface (peritichous flagella). Strains of *Brenneria* can cause disease on trees (Hauben *et al*., 1998). Examples of those causing bark cankers include *B.alni,* on *Alnus* (alder), *B.nigrifluens* and *B.rubrifaciens* on *Juglans* (walnut) (Hauben *et al*., 1998) and *B.populi* on *Populus* x *euramericana* (poplar).

Species of *Brenneria* also affect other tissues e.g. *B.salicis*, affecting the xylem and cause of watermark disease of *Salix* (willow) and *B.paradisiaca*, causing rotting in the roots of *Musa paradisiaca* (banana) (Hauben *et al*., 1998). One *Brenneria* species able to colonise oak is *Brenneria quercina*[1] (Brady *et al.,* 2012). This bacteria causes bark cankers and 'drippy nut disease', where acorns ooze sap and rot, (Biosca *et al*., 2003). When injected into the stems of *Nicotiana sylvestris* (tobacco) seedlings, two out of eight *B.goodwinii* isolates also initiated a hypersensitive response (Denman *et al*., 2012).

Phylogenetic analysis has revealed that *B.goodwinii* is a facultative anaerobe (Denman *et al.,* 2012) located alongside other *Enterobacteriaceae* 'soft-rot' necrotic bacterial pathogens (Doonan *et al*., 2019). It has been shown to be more associated with necrotrophs, which kill their host and utilises the dead matter, than hemibiotrophs, which can shift from living to dead tissues (Doonan *et al*., 2019).

[1]Formally *Erwinia quercina*; reclassified into a new genus with a single species, *Lonsdalea* gen. nov., as *Lonsdalea quercina* comb. nov. (Brady *et al.,* 2012).

A phylogenetic and cluster-based sequence analysis of 44 *B.goodwinii* strains from seven different locations revealed that the UK population is highly variable (Kaczmarek *et al*., 2017). The study showed no pattern to the distribution of the 22 sequence types identified and suggested there is an endemic form of *B.goodwinii* in the UK with sequence types becoming dominant in their separate locations and even on separate trees.

Gibbsiella quercinecans

The genus *Gibbsiella* (Brady *et al*., 2010, gen nov.) are gram negative, facultatively anaerobic bacteria 0.9 × 1-1.5 μm with fine fimbriae distributed over their surface (Brady *et al.,* 2010). *G.quercinecans* has been identified in Spain on both *Q.ilex* (holm oak), *Q.pyrenaica* (pyrenean oak) (Brady *et al.,* 2010) and *Q.robur* (González & Ciordia, 2019). In the UK it has also been isolated from *Aesculus hippocastanum* (horse chestnut), apple and pear (Denman *et al*., 2016).

Several other bacterial genera containing plant pathogens have been isolated from lesions in lower concentrations (Brady *et al.,* 2017). These include the cause of Poplar canker *Lonsdalea quercina* and several *Erwinia* sp., many of which cause soft rots (Brady *et al.,* 2017). Other species of *Brenneria* have also been identified along with *Rahnella victoriana,* another potential bacterial component of AOD (Brady *et al.,* 2017). As detection is a reflection of the bacteria present and capable of being detected (Gonzalez *et al*., 2012) rather than pathenogenicity the role of other bacteria in tissue necrosis may have been overlooked. There are additionally biases to the detection of bacteria via PCR amplification which may over or underestimate the proportion of each taxa within the community (Gonzalez *et al*., 2012). Nevertheless *G.quercinecans* and *B.goodwinii* are now considered the microbial cause of tissue death and stem bleeding (Brady *et al.,* 2010; Brady *et al.,* 2017) with *B.goodwinii* thought to be more dominant (Broberg *et al.,* 2018; Doonan *et al*., 2019).

Genomic analysis has shown that *B.goodwinii* and *G.quercinecans* have the capacity to become pathogenic and furthermore that *G.quercinecans* has the genes necessary to degrade oak tissue (Doonan *et al*., 2019). In order to investigate the pathogenicity potential of *B.goodwinii* and *G.quercinecans* small holes were bored into sections of cut logs and then the bacteria either applied singly, together or in combination with *A.biguttatus* eggs (Denman *et al*., 2018). As tissue necrosis then followed the conclusion was that both *B.goodwinii* and *G.quercinecans* induce necrosis with and without the presence of *A.biguttatus* (Denman *et al*., 2018). Since wound controls also produced lesions (Denman *et al*., 2018), *Brenneria goodwinii* and *G.quercinecans* do not simply induce necrosis, rather they cause lesion formation *after* a wound has been created. The results therefore indicate that lesions can be formed as a result of bacteria entering a pre existing opening or wound.

Bacterial inoculations were also carried out on non-wounded tissue although their application was only in combination and with *A.biguttatus* eggs. With no test of the bacteria individually with and without eggs and results not presented from this trial, there is no experimental comparison with wounded logs. This would have provided an evaluation of bacterial actions with and without a wound. It further remains unknown at what population level bacteria induce a lesion response. Without this knowledge the applied inoculums may not have been realistically related to that found in nature. The *Gibbsiella* genus for example, is considered common in the environment and not a causative agent of oak decline (Geider *et al*., 2015).

By using cut logs with a low moisture content, the actions of the bacteria are on compromised tissues. Larger lesions produced by introducing of eggs with bacteria could simply indicate the effect of multiple stress factors. The eggs themselves may also incite a plant defence response (Reymond, 2013). Eggs were also introduced onto a wound surface which does not replicate nature where eggs are laid on external bark. As previously mentioned, evidence of the involvement of *A.biguttatus* in AOD or vectoring the bacteria is non-existent. Isolation of bacteria along larval tunnels could mean they have entered via a wound created by an exit hole, are utilising larval frass as a resource or taking advantage of already degraded tissues. A comparison of another wood boring insect or a mechanically created tunnel would have provided evidence of how the bacteria may colonise a tree.

Both *B.goodwinii* and *G.quercinecans* have been proposed by Doonan *et al*. (2019) as acting via necrogenic enzymes or secretion of effector proteins. Both of these mechanisms of action involve the bacteria secreting enzymes to cause tissue decay (McDonald and Denman, 2015) or introducing proteins to assist in pathogen invasion or suppress a host immune system (Alfano and Collmer, 2004). The bleeding lesions and tissue necrosis that resulted from bacterial inoculations onto cut oak logs is therefore not surprising and may indicate that a hypersensitive response has been stimulated within the tree. This defence response causes tissue necrosis in reaction to the presence of a pathogen; a mechanism of plant defences which is well established (Hughes, 1996).

The development of molecular techniques has provided the ability to identify and separate bacterial species (Johnson, 2000). It is therefore possible that the symptoms now ascribed to AOD have been present for longer and attributed to other causes and it is the use of molecular identification that has resulted in the categorisation of a new condition. How *G.quercinecans* and *B.goodwinii* colonise oak tissues and from where they originate remains unknown. They may form part of an oak bacterial community or be present within a woodland and colonising from other sources or reservoirs. Whether the bacteria are transferred between individual trees and how they might transported be is also unknown.

1.2 Plants and associations with bacteria

Of all the microorganisms, bacteria are the most abundant in nature (Lindow and Brandl, 2003). Bacteria are widely present within the natural environment and are able to survive in a range of habitats, hosts and tissues. Plants in particular provide a large resource above and below ground for bacteria not all of which are pathogenic. The following sections highlight some of the associations, positive and negative, that exist between bacteria and the plant environment.

The phyllosphere is an environment colonised by a variety of microorganisms including bacteria, yeasts and fungi (Lindow and Brandl, 2003). Phyllosphere is a term first introduced in the 1950s where it was used to refer to the microbial fauna of leaves (Fokkema, 1991). It is also used to describe the above ground parts of plants (Lindow and Brandl, 2003) where the term encompasses leaves, stems and flowers. Some authors subdivide the phyllosphere into separate components using terms such as caulosphere (stems), phylloplane (leaves), anthosphere (flowers) and carposphere (fruits).

Bacteria can exist both endophytically within plant tissues, (Rosenblatt and Martinez-Romero, 2006) and/or epiphytically on external surfaces (Lindow and Brandl, 2003). Bacterial endophytes are classified as either facultative or obligate; respectively able or unable to exist without the host plant (Hardoim *et al.,* 2008). In a review of the interactions of endophytic bacteria and their hosts Rosenbleuth and Martinez-Romero (2006) discuss bacterial ability to enhance a host plants nutrient acquisition and uptake, increase plant growth, suppress pathogens and remove contaminants via phytoremediation.

Interactions between epiphytic bacteria and plants have also been widely investigated including the bacterial synthesis of growth hormones and their ability to influence the rate of plant metabolism (Lindow and Brandl, 2003). Bacteria are unable to actively move into a plant instead relying on openings such as stomata or wounds (Manion, 1991) potentially indicating why microbial species have been isolated to a greater extent from plant exterior surfaces (Lindow and Brandl, 2003).

Pathogenic bacteria in particular are able to colonise the tissues inside the leaves and avoid external limiting factors (Beattie and Lindow, 1999; Lindow and Brandl, 2003). Few estimates have been made of the number of plant pathogenic bacteria. Estimates have been put forward of at least 100 species already identified (Dickinson, 2004), with others suggesting of 7100 classified bacterial species, up to 150 cause disease by overcoming plant defences (Kannan *et al*., 2015). The most studied plant pathogens are those associated with crops (Daniels, 1998), with few associated with tree disease (Manion, 1991).

Phytopathogens fall into a range between two main types (Lucas, 1998; Glazebrook, 2005) (Table 2). Phytopathogens can also be saprotrophic – only utilising dead organic tissue (Lucas, 1998) or hemibiotrophic - shifting from biotroph to necrotroph during different life stages or situations (Glazebrook, 2005).

Table 2. Key features of the two main types of pathogen, biotroph and necrotroph

	Biotroph	**Necrotroph**
Key features		
Host	Obligate on living host tissue Not able to grow without contact with host	Kill and then utilise dead or dying host tissue Opportunistic pathogens not obligate on host Can exist as saprotrophs
Mode of action	Slow acting Invade directly into host or via natural openings Attack healthy tissue	Rapid action Invade via wounds or natural openings Attack damaged tissues
Host range	Limited range All age classes	Wide range Younger age classes Declining hosts

Pathogenic bacteria can cause the visible disease symptoms through degradation of cell walls, production of toxins or by disrupting cell division (Manion, 1991). Plant defences to resist pathogens include cell structures designed to inhibit colonisation, acquired resistance and chemical defences (Hughes, 1996). Chemical defences can be preformed inhibitors (those continually present) and induced chemical responses (activated by the presence of the pathogen) (Hughes, 1996). One induced response that could indicate a pathogen attack is the necrosis of a plants own tissues (Hughes, 1996) which can appear as stem bleeds.

1.2.1 Plants and bacterial communities – colonisation and establishment

The majority of studies on phyllosphere bacterial communities have concentrated on leaves as they represent a large available area of a plant for colonisation (Lindow and Brandl, 2003). There have been few studies on the bacterial communities of stems and fruit although some have been carried out on flowers (Aleklett *et al.,* 2014), pollen (Stefani and Giovanardi, 2011) and seeds (Rosenbleuth and Martinez-Romero, 2006; Green *et al.,* 2007).

Bacterial communities are known to vary with host species, surrounding environment and abiotic conditions (Laforest-Lapointe *et al.,* 2016). Abiotic factors limiting and influencing the colonisation of bacteria of a leaf surface are conditions such as changes in light, temperature, humidity and exposure to the elements. In an urban environment air pollution

has also been found to have an influence on the bacterial community composition (Wuyts *et al*., 2020). Other influencers are the leaf physical characteristics for example the waxy cuticle and nutritional content (Lindow and Brandl, 2003).

Scanning electron microscopy utilised to explore epiphytic bacterial communities revealed a higher density of bacteria on the underside of the leaf compared with above with concentrations along the leaf veins as well as near stomata and the bases of trichomes (Nongkhlaw and Joshi, 2014). These places of aggregation were in some cases forming biofilms, potentially as an adaptation to protect from environmental stress (Nongkhlaw and Joshi, 2014). Bacteria are also able to modify leaf surface conditions to allow a more effective colonization of plants (Beattie and Lindow, 1999). Examples include increasing nutrient concentrations on the leaf surface, producing extracellular polysaccharides and biofilms to aid surface adhesion and mitigate against desiccation (Beattie and Lindow, 1999).

All of these factors mean that selection will favour those bacteria that can overcome the prevailing environmental conditions and plant host defences (Nongkhlaw and Joshi, 2014). It has also been suggested that a colonising bacterium may have a greater chance of establishment on a leaf with fewer bacteria already present; such as after rain, or a new leaf compared with a full grown leaf (Lindemann and Upper, 1985). Both Ercolani (1978) and Thompson *et al*. (1993) found young leaves generally hosted a greater number of taxa compared to older leaves. This supports the suggestion of Lindemann and Upper (1985) that a newly colonising species may have a greater likelihood of establishment on new leaves compared with older ones. Leaves from mature oak trees were found to have a higher species richness compared with leaves from younger trees which may have been linked to canopy height and site conditions (Jakuschkin, 2015). This is contrasted by an observation that bark microcores from *Q.robur* showed a significant decline in species richness as tree age increased (Meaden *et al.,* 2016). Other influences on community composition may therefore be involved.

When studying bacterial movement the use of a control can be used to differentiate between endemic phyllosphere communities and those colonising from the environment (Osteen *et al.,* 2016). To evaluate whether there is a difference between endemic or introduced species Osteen *et al.* (2016) compared sterile plastic controls and live tomato plants. By using short sequences of 16S RNA (referred to as Operational Taxonomic Units OTUs) as a proxy for individual bacterial species Ottesen *et al.* (2016) identified the bacteria found. *Enterobacteriaceae* dominated the samples and results showed that only 10 percent of OTU's were unique to the phyllosphere. Over 90 percent found on live plants were also shared with the inanimate controls (Ottesen *et al.,* 2016).

Controls had a greater diversity of OTU's however these were present at lower abundances. The conclusion drawn from this was that a proportion of bacteria present within the air were finding a highly suitable plant host; in this case tomato leaves (Ottesen *et al.,* 2016). Few bacteria were therefore endemic to the tomato leaves and environmental factors were important in introducing bacteria to plant surfaces (Ottesen *et al.,* 2016).

When looking at establishment of bacteria via irrigation water Telias *et al. (*2011) showed that whilst the bacterial communities of two different sources of water, ground and surface, were significantly different, the communities of the irrigated tomato plants were not. Only some of the bacterial species present in the irrigation water managed to establish themselves on the leaves of the tomato plants (Telias *et al.,* 2011). This could indicate the influence of external environmental factors or alternatively the suitability of the tomato plant itself as a long term host only for certain species.

Another influence on community composition is seen where damage to *Fagus sylvatica* (beech) leaves by Lepidopteran larvae resulted in a higher species diversity of epiphytic heterotrophic bacteria compared with undamaged leaves (Müller *et al.,* 2003). There was no similar difference noted for *Q. petraea* leaves which may be due to differences in leaf structure (Müller *et al.,* 2003).

The compatibility or incompatibility of a plant and a bacteria influences the growth of the bacteria and its capacity to cause disease (Daniels, 1998). Pathogenic bacteria can have a non-pathogenic state existing on plant tissues without causing damage only transitioning to a pathogenic state in response to decreases in host defences (Lucas, 1998), environmental or nutrient changes (Toth and Birch, 2005). Humidity, for example appears to be important for certain pathogenic *Pseudomonas syringae* mutants to become established with increased growth in the resultant waterlogged apoplast observed at higher humidity (Xin *et al.,* 2016). Bacterial phytopathogens are able to multiply on the surfaces of healthy plant tissues of host and non-hosts (Leben, 1974). Phytopathogens found amongst plant microbial communities of non-hosts have been shown to cause no detrimental effects, only becoming pathogenic once transferred to a host plant (Vanette, 1982).

Bacteria with the ability to switch from an epiphytic to an endophytic lifestyle could be responsible for initiating disease (Beattie and Lindow, 1995). One example of a phytopathogenic bacteria able to colonise both the exterior leaf surface and internally is *P.syringae* pv. *syringae* (Beattie and Lindow, 1995; Meyer and Leveau, 2012). In this instance high rainfall initiates an increase in population with stomata potentially providing the opening for colonisation of internal tissues (Lucas, 1998).

Soil is another potential source of bacteria colonising plant tissues (Ryan *et al.,* 2007). The colonisation of endophytic bacteria through the rhizosphere is a known mode of entry into internal plant tissues (Compant *et al.,* 2010). Soil collected from the base of an old oak and subsequently inoculated with *B.goodwinii* and *G.quercinecans* showed that unlike *G.quercinecans*, which was detectable throughout the experiment, *B.goodwinii* had no viability although it was detectable via qPCR (Pettifor *et al*., 2020). Therefore soil as a source of these bacteria cannot be discounted. The ability of successfully colonising bacteria to move throughout the plant via intercellular spaces and xylem has also been established (Compant *et al.,* 2010). Soil was considered to contribute to the bacterial community of oak trees due to the high abundance of *Acidobacter* within younger *Q. robur* trees compared with older trees and their generally high abundance in soil (Meaden *et al.,* 2016).

1.2.2 Plants and bacterial communities – survival and persistence

Two strategies have been put forward by Beattie and Lindow (1995) that allow foliar bacteria to survive - avoidance and tolerance. Bacteria with an avoidance strategy will take advantage of spaces within leaf cells (Beattie and Lindow, 1995). Living endophytically affords protection from external conditions such as changes in temperature or moisture (Beattie and Lindow, 1995). Those with a tolerance strategy in contrast have adapted to the prevailing epiphytic conditions; producing pigments for example, to protect against U.V radiation (Beattie and Lindow, 1995).

Temporal and spatial differences in bacterial communities have been observed in different temperate forest species (Laforest-Lapointe *et al.,* 2016) and the influence of physical geographical distance has been seen for the bacterial communities of oak (Sapp *et al.,* 2016). Across five sites studied the composition of the oak bacterial communities differed by site (Sapp *et al.,* 2016). In contrast, at an individual tree level, the location of a canopy leaf sample from five different species; *Acer saccharum*, *A.rubrum*, *Betula papyrifera*, *Abies balsamea* and *Picea glauca* was not a significant factor in accounting for differences in community composition (Laforest-Lapointe *et al.,* 2016).

Individual leaves can show variation in their bacterial composition (Jakuschkin, 2015). There were observed differences between individuals of the same species and between different host species (Laforest-Lapointe *et al.,* 2016). The greater influence of a larger distance on bacterial community composition could be due to site abiotic differences determining which bacteria are present (Sapp *et al.,* 2016). Temporal differences may be due to changes occurring within the phyllosphere which alter available nutrition and/or physical condition of the colonisable surface (Lindow and Brandl, 2003).

Seasonal changes in the bacterial population of *Olea europaea* (Olive) leaves (Ercolani, 1978) and *Beta vulgaris* (sugar beet) (Thompson *et al.,* 1993) have been observed. For *B.vulgaris*, the lowest bacterial diversity recorded correlated with a warm and dry period and the highest with low temperature and high rainfall (Thompson *et al.,* 1993).

The concept of 'quorum sensing' whereby bacteria, once they reach a certain population level, communicate via 'signalling molecules' to coordinate certain actions has been applied to the interactions between bacteria and plants (Braeken *et al.,* 2008). These signalling molecules have been identified in the genes of plant pathogens including those resulting in the production of proteins and enzymes, regulation of gene expression and pathenogenicity as well as the initiation of plant defences and stress responses (Braeken *et al.,* 2008). The expression of the same molecules in different species is also suggestive of the capacity for intraspecies communication and interactions (Breaken *et al.,* 2008). Some plants are, however, able to disrupt these signals (Breaken *et al.,* 2008). So far both signalling and interference are uninvestigated for the bacteria associated with AOD.

Plant pathogens that are able to persist through gaps in host availability, such as that caused by seasonal growth changes, will have an advantage (Allen *et al.,* 2009). Persistence can therefore prevent eradication of pathogens (Martins *et al.,* 2018). Contaminated plant litter as well as other plant species can provide phytopathogens with an overwintering resource (Vanette, 1982). Others persist in soil (Brown, 1997), insects, seeds or the atmosphere (Allen *et al.,* 2009). Bacteria are also able to survive in dry diseased tissues, reduce their metabolism (hyperbiotic state) or through aggregation (Leben, 1974). Although understudied, phytopathogens may also use 'persister cells' which inactivate their metabolism to survive resistance and then reinitiate growth (Martins *et al.,* 2018).

Bacteria with very different lifestyles exhibit this strategy (Martins *et al.,* 2018). These include canker inducing bacteria e.g. *Erwinia amylovora*, responsible for stem cankers on Rosaceae and *Clavibacter michiganensis*, responsible for cankers on tomato and spread via seeds (Martins *et al.,* 2018). Other bacteria with this strategy are *Ralstonia solanacearum* a wilt inducing bacteria on a range of crops, *Xylella fastidiosa*, which causes leaf chlorosis, and is an inhabitant of Xylem and the fore gut of 'sharpshooter' insect vectors (Martins *et al.,* 2018). Opportunistic pathogens e.g. *Pseudomonas syringae* can also avail themselves of this strategy (Martins *et al.,* 2018). Long term survival will however be dependent on environmental conditions (Leben, 1974).

It is possible the bacteria found within bleeds on trees diagnosed with AOD exist epiphytically or endophytically on external oak tissues. They may, alternatively, be found within the environment and as Ottensen *et al. (*2016) suggest find oak a suitable host to

colonise in larger numbers. Entering via leaves may be one way the bacteria associated with AOD are able to colonise oak tree tissues. Alternatively, if they are already part of any aspect of the phyllosphere certain environmental conditions may induce pathenogenicity or an increase in population which overwhelms plant defences.

1.2.3 Plants and bacterial communities – dispersal and movement pathways

Persistence and dispersal are understudied aspects of bacterial life stages (Allen *et al.,* 2009). Identifying how bacteria disperse is an important aspect in understanding their interactions with plants (Frank *et al.,* 2017) with the origin of the microbiota associated with plant surfaces highlighted by Ottesen *et al.* (2016) as a research gap. Bacteria are not able to actively disperse to new hosts and rely on transmission (Frank *et al.,* 2017) and opportunistic encounters (Vanette, 1982).

Although bacteria can be dispersed solely by air, direct movement is not thought to be a common mechanism (Kannan and Bastas, 2015). Movement is more likely to be via insect vectors (Kannan and Bastas, 2015), through rain picking up airborne bacteria or rain splash directly on plant surfaces or via soil water (Frank *et al.,* 2017). Bacteria can also be moved by multiple methods, for example bacteria able to colonise new hosts independently may still form a relationship with an insect for transmission (Orlovskis *et al.,* 2015). This section focuses on the movement pathways of bacteria with an emphasis on phytopathogens.

1.2.3.1 Insect vector transmission of bacteria

The ‘microbial colonization’ of insects and whether they have indigenous bacteria is unknown for most species, as is the function of the bacteria themselves (Dillon and Dillon, 2004). The role of bacteria within an insect, can be to aid and improve digestion of food or provide nutrients or digestive enzymes (Dillon and Dillon, 2004; Douglas, 2009; Klepzig *et al.,* 2009; Engel and Moran, 2013). Bacteria can confer other benefits to insects such as resistance to pathogens (Eleftherianos *et al.,* 2013), resistance to parasites, defence against predation (Engel and Moran, 2013) and pheromone production (Dillon and Dillon, 2004).

Insect vectors can be important to the movement of bacteria and is one of the more studied movement pathways for bacteria to colonise new hosts (Kannan and Bastas, 2015). It is therefore one way in which *B.goodwinii* and *G.quercinecans* may be transferred between trees. Insect transmission of plant pathogens has evolved independently several times over and many different insect species are utilised by plant pathogens for the transmission of bacteria (Orlovskis *et al.,* 2015). For many insects the microorganisms present within their guts are also widespread within the environment (Engel and Moran, 2013).

For an insect-bacteria relationship to be successful or persistent the two must have the opportunity to come into contact (Nadarash and Stavrinides, 2011). Some bacteria can alter plant olfactory signals to insects thereby increasing the chances of successful encounter (Orlovskis *et al.,* 2015). Movement of bacteria can additionally be through incidental encounter by insects which act as passive agents of transmission or through a closer association between specific bacteria, plant host and insect (Orlovskis *et al.,* 2015).

Pathogens have adapted to use insect feeding routes to enter plant tissues (Orlovskis *et al.,* 2015). Some are dependent on insects for movement to new hosts particularly those that are phloem restricted such as *Candidatus* species (Bové and Garnier, 2002). Xylem restricted bacteria vectored by insects include *Xylella fastidiosa*, *Clavibacter xyli subsp. xyli*[1] and *Pseudomonas.syzygii*[2] (Bové and Garnier, 2002). These restricted bacteria enter via the piercing mouth parts of insects e.g. hemipterans, aphids (Orlovskis *et al.,* 2015).

Others enter plant tissues through decaying tissues caused e.g. by flies (Basset *et al.,* 2003). Bacteria can also colonise through wounds created as insects with chewing mouthparts e.g. beetles feed. Another example of a close association between insect and bacteria is that of *Erwinia carotovora*[3] and the black onion fly (*Tritox aflexa*) (Venette, 1982). The surface of the egg has bacteria on and the larva create an entry hole for colonisation while the larva use nutrients from the bacterial infection to develop (Venette, 1982).

A different association has evolved between *E.carotovara*[1] and the iris borer (*Macronoctua onusta*) whose larva use bacterially degraded tissues as food, the bacteria survive in the digestive system and are deposited on frass (Venette, 1982). A similar association has developed between *Erwinia tracheiphila*, a bacterial wilt of cucurbits, and the striped cucumber beetle (*Acalymma vittatum*) (Mitchell and Franks, 2009). Bacteria are spread through wounds created as the beetle feeds (Orlovskis *et al.,* 2015) as well as deposited in the beetle frass where it remains viable up to 48 hours (Mitchell and Franks, 2009).

In an extension of a vector relationship some phytopathogenic bacteria are capable of using insects as alternative hosts in very specific associations (Nadarash and Stavrinides, 2011). (Nadarash and Stavrinides, 2011). These bacteria are divided into 'circulative nonpropagative' or 'circulative propagative' modes of action (Nadarash and Stavrinides, 2011). Nonpropagative bacteria do not replicate within the insect and ingestion by feeding is followed by bacterial movement to the mid or hindgut (Nadarash and Stavrinides, 2011). Propagative bacteria replicate within the insect and spread through the body (Nadarash and

[1]Reclassified as *Leifsonia xyli* subsp. *xyli* (Davis *et al*. 1984) Evtushenko *et al*. 2000
[2]Reclassified as *Ralstonia syzygii* (Roberts *et al.* 1990) Vaneechoutte *et al. 2004 emend*
[3]Reclassified as *Pectobacterium carotovorum* subsp. carotovorum (Hauben *et al*., 1998)

Stavrinides, 2011). Transfer to a new host in both cases is via salivary glands during feeding (Nadarash and Stavrinides, 2011). Figure 4. taken from Orlovskis *et al*. (2015) illustrates these different strategies phytopathogens use for dissemination via insect transmission.

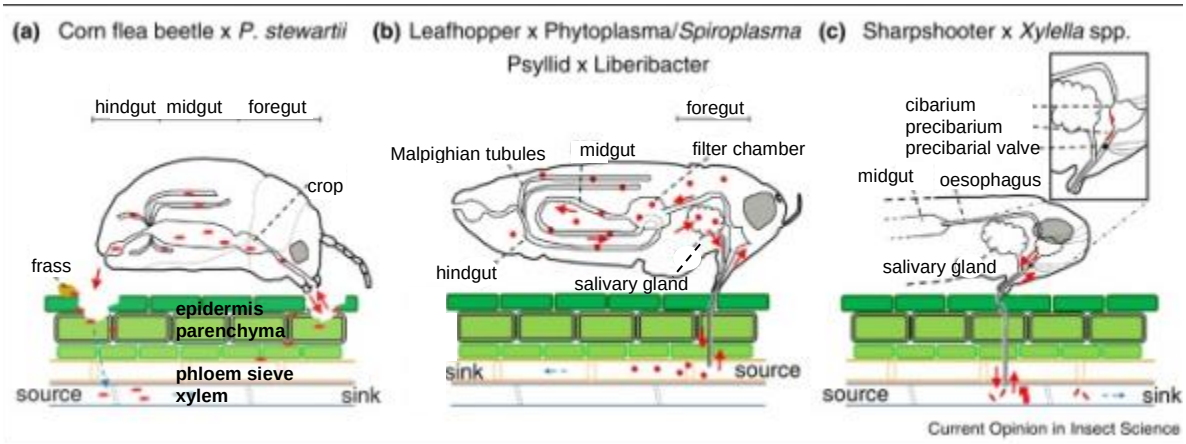


Figure 4. Three strategies phytopathogens use for dissemination via insect transmission (Orlovskis *et al.,* 2015). Whilst feeding bacteria are acquired by the insect (a, b, c). Diagrams (a) and (b) represent circulative propagation where bacteria grow within the gut or insect body. Diagram (c) represents non-circulative propagation where bacteria attach to the foregut. Bacteria transfer into plants via feeding wounds (a), in saliva through mouthparts (b, c) or infectious frass (a). Bacteria then spread through xylem (a, b) or phloem (c)

Bacterial propagation within an insect can be from days to months (Orlovskis *et al.,* 2015). Genetic adaptations for survival have been uncovered including those for adhesion to gut walls, enhancing survival and for persistence and forming biofilms (Orlovskis *et al.,* 2015). Bacteria may alter plant physiology to attract insects e.g. changes to volatile compounds, leaf yellowing or through immune response suppression (Orlovskis *et al.,* 2015). Conversely bacteria may increase insect movement to new hosts by blocking xylem or phloem, decreasing available resources (Orlovskis *et al.,* 2015).

A modelling study into insect-pathogen relationships highlighted several interactions that influence insect behaviour and therefore pathogen spread (Crowder *et al*., 2019). Overall, interactions that increased vector fitness e.g. predator reduction, increased pathogen spread, with the converse also true (Crowder *et al*., 2019). When pathogens were categorised as persistent (circulating through an insect prior to transmission) or non persistent (brief contact prior to transmission) they responded differently in the model to interactions (Crowder *et al*., 2019). Decreased insect feeding rate lowered the spread of both types, however increased vector-host encounters increased the spread of non persistent but not persistent pathogens (Crowder *et al*., 2019).

Bacteria are not necessarily harmful to the insect (Basset *et al.,* 2003). For example only three out of the 16 *E.carotovora*[1] strains tested induced an immune response in *Drospophila melanogaster* larvae after ingestion (Basset *et al.,* 2003). The presence of a single gene was identified as necessary for colonisation and spread within the body of *D.melanogaster* (Basset *et al.,* 2003). It was also suggested that the pathogenic nature of *E.carotovora* is somehow recognised by the immune system of *D.melanogaster* (Basset *et al.,* 2003).

The stem lesions and tissue necrosis of AOD has led to suggestions an insect, *A.biguttatus*, may be involved (Denman *et al.,* 2018). Xylophagous insects nearly always feed on wood as adults or larvae and many utilise oak as a host. Insects with a saproxylic lifestyle also depend on dead or decaying wood for all or part of their lifecycle. Insects with either lifestyle therefore have the potential to act as passive or active vectors.

1.2.3.2 Environmental transmission of bacteria

Wind and rain are mechanisms by which bacteria can be transferred to new hosts. Although the amount of bacteria removed will be related to the population density rain is an effective disperser of bacteria over short distances (Butterworth and McCartney, 1991). Raindrops are known to be able to disperse soil bacteria into the atmosphere where the bacteria can survive for more than an hour (Joung *et al.,* 2017). Tracking of atmospheric pathogen movement is an emerging area of research in developing plant health risk management strategies (Schmale and Ross, 2015) and new sampling methods (West and Kimber, 2015).

Rainsplash can also dislodge bacteria from the surfaces of leaves (Butterworth and McCartney, 1991). To show this experimentally three bacteria, *Pseudomonas syringae, Klebsiella planticola* and *Bacillus subtilis* were sprayed onto a leaf surface and then rainsplash was artificially simulated with sterile water (Butterworth and McCartney, 1991). A comparison of bacterial presence on leaves before and after indicated that both splash droplets collected onto agar and the water runoff from the leaf contained bacteria (Butterworth and McCartney, 1991). A greater proportion of the dislodged bacteria were found in the runoff compared to the splashes (Butterworth and McCartney, 1991). A similar experiment found that artificially generated wind and rain were able to dislodge the bacteria *Xanthomonas axonopodis* pv. citri, which causes cankers on citrus, from the canopies of 1.5 m tall grapefruit trees (*Citrus* × *paradisi*) (Bock *et al.,* 2005). These cankers appear as necrotic lesions on leaves, shoots, branches and fruit (Bock *et al.,* 2005).

The distance that bacteria can travel via wind and rain is one area of investigation for plant pathogens. Bacteria are able to be dispersed thousands of kilometres through the atmosphere (Frank *et al.,* 2017).

Rain and wind dislodged bacteria from the canopies of grapefruit saplings were found up to 12m downwind of the leaf source area in a time frame of 10 minutes (Bock *et al.,* 2005). The highest concentration of bacteria was at 1m with bacterial concentration decreasing with time and distance (Bock *et al.,* 2005). In an investigation on the deposition of bacteria dislodged from the surfaces of bean leaves researchers noted that with a windspeed of ~6 km $h^{-1}$and peak deposition occurring 6-9 hours after initial displacement the bacteria could potentially travel 40-65 km from their original source (Lindemann and Upper, 1985).

Collectors placed on the ground below the grapefruit sapling canopies contained higher bacterial concentrations compared with those placed in front of the canopy possible related to the angle of spray related to the collection funnel (Bock *et al.,* 2005). A greater amount of bacteria were also dispersed from trees with lesions less than 6 months old (Bock *et al.,* 2005). Strategies that may enhance the environmental transmission of bacteria include biofilm development (Morris *et al.,* 2008) and formation of lesions (Allen *et al.,* 2009). Although lesions are thought more likely to facilitate short term rather than long term persistence and dispersal (Leben, 1974).

The diversity of bacteria makes them a challenging area of study. Much about their lifestyle and modes of action remains understudied and this includes *Brenneria goodwinii* and *Gibbsiella quercinecans*.

1.3 Thesis objectives

The interactions of *Brenneria goodwinii* and *Gibbsiella quercinecans* with the oak phyllosphere remains an unexplored area as does their potential dispersal and colonisation pathways. The focus of this thesis will therefore be to research how *Brenneria goodwinii* and *Gibbsiella quercinecans* are associated with oak and investigate potential transmission mechanisms.

The objectives of this thesis will be to:

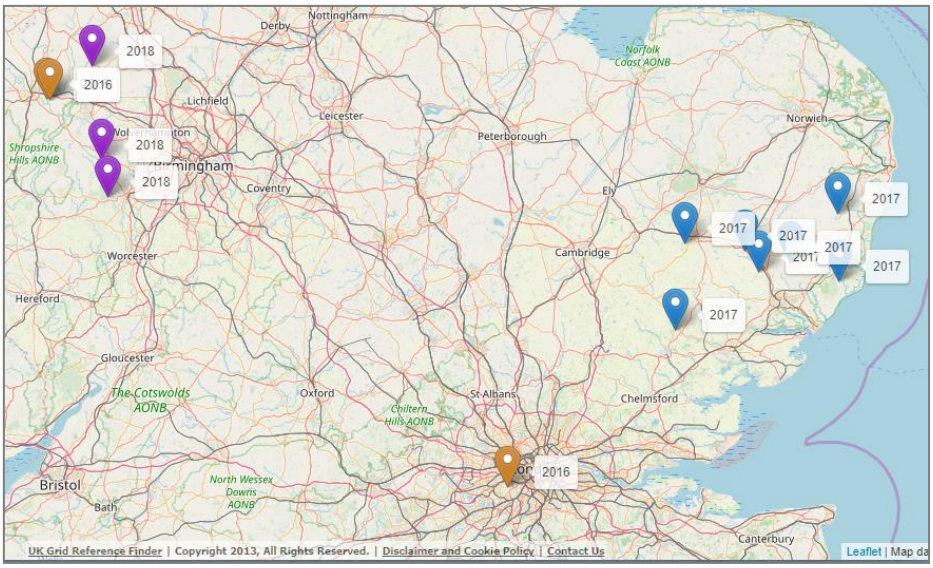
1) Identify the associations of *B.goodwinii* and *G.quercinecans* with the oak phyllosphere, focusing on live oak leaves, leaf litter, acorns and catkins.

2) Investigate two potential transmission mechanisms
    i. movement via insect vectors to focus on xylophagous coleoptera.
    ii. airborne movement via rain.

Chapter 2

Site descriptions and general methodologies

Over the three years of study insect and phyllosphere samples were collected from different woodlands, with and without AOD, in different years (Figure 5). The following chapter provides descriptions of each site and details the sampling carried out. Methodologies and sampling protocols used at all sites are outlined along with lab techniques.



- Year one: 2016 - two sites; both with AOD
- Year two: 2017 - six sites; 5 with and 2 without AOD
- Year three: 2018 - three sites; 2 with and 1 without AOD

Figure 5. Map with locations of sampling sites used over three years 2016-2018

2.1 Site descriptions

2.1.1 Initial investigation 2016

The initial investigation in 2016 involved two locations where AOD is present and confirmed by a positive detection of *G.quercinecans* or *B.goodwinii* in an active stem bleed. Insect trapping was carried out throughout July and August. Autumn leaves and acorns were collected in September. No spring leaves, catkins or litter were collected. Due to a limited number of trees with active bleeds and accessible canopies, trees were not selected randomly but chosen based on those available.

Attingham Park: **Rookery wood** (Grid Ref. SJ 54882 09933) (Figure 6) is an area of oak dominated woodland approximately 1.4 ha within the larger parkland. There is little understorey vegetation. The soilscapes classification is 18: 'Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils' (Cranfield University, 2017). The site was used for insect sampling and autumn leaves.

Attingham Park: **South East plantation** (Grid Ref. SJ 55840 09593) (Figure 7) is an area of oak woodland approximately 1.7 ha within the parkland. There is a stream on the south eastern side and little ground vegetation. The soilscapes dataset classification is 6: 'Freely draining slightly acid loamy soils' (Cranfield University, 2017). The site was used for insect sampling and autumn leaves.

Figure 6. Rookery wood

Figure 7. South East plantation

Richmond Park (Grid Ref. TQ 20256 72967)

Ham Cross plantation (Figure 8) is an area of oak dominated woodland approximately 6 ha within Richmond Park. Throughout the wood are a series of drainage ditches and a dense undergrowth of bracken. The soilscapes dataset classifies the soil as 18 (Cranfield University, 2017). This site was used for insect sampling, autumn leaves and acorns.

Figure 8. Ham Cross plantation

2.1.2 Further investigation 2017

A further investigation in 2017 involved seven sites in Suffolk. Five of these sites had AOD present at varying degrees of infection. Presence of AOD was confirmed by a positive detection of the bacteria *G.quercinecans* or *B.goodwinii* in an active stem bleed. A further two sites where AOD is absent were used as comparison sites; one for insect and the other for phyllosphere samples.

The focus of the further investigation was an expansion of insect trapping and a focus on collection of phyllosphere samples. Insect trapping took place throughout July and August. Spring leaf samples were taken in June and autumn leaf and litter samples in September. No catkin or acorn samples were collected in 2017.

Priestley wood (Grid Ref. TM 08142 52929)
Priestley wood (Figure 9) is a mainly oak woodland approximately 23.68 ha. The soilscapes dataset is 9 'Lime-rich loamy and clayey soils with impeded drainage' (Cranfield University, 2017). Considered for this study as without AOD and used as a comparison site for insect sampling.

Broaks wood (Grid Ref. TL 79052 31263)
Broaks wood is 62 ha of mixed woodland. The area used (Figure 10) was around 16 ha. The soilscapes dataset classifies the soil as 8 'Slightly acid loamy and clayey soils with impeded drainage' and 6 (Cranfield University, 2017). Considered for this study as being without AOD and used as a comparison site for phyllosphere samples of spring leaves, autumn leaves and litter.

Figure 9. Priestley wood

Figure 10. Broaks wood (Geograph.org 2016)

Site one (Grid Ref. withheld at request of site owner)

Site one (Figure 11) is an area of c. 3.0 ha oak dominated woodland within a larger parkland landscape of new plantings and mixed farmland. Considered as an early stage infection site. Several trees show visibly active bleeds. The soilscapes classification is 18 (Cranfield University, 2017). This site was used for insect sampling.

Figure 11. Site One Western side

Northfield wood (Grid Ref. TM 02822 60198)

Northfield wood (Figure 12, Figure13) is 33.26 ha of mixed woodland with areas of mainly oak. Two smaller areas c. 4.7 ha and c. 1.7 ha were used. Considered for this study as an early stage infection site. One tree in the woodland shows a visibly active bleed which tested positive for *B.goodwinii*. The soil is soilscape 8 (Cranfield University, 2017). This site was used for insect sampling.

Figure 12. Northfield wood area one

Figure 13. Northfield wood area two

Site Two (Grid Ref. withheld at request of site owner)

Site two (Figure 14) is a stand of ancient oak approximately 1.5 ha; part of a larger landscaped park of around 160 ha of mainly ancient or veteran oak trees alongside newly planted individual trees. Considered for this study as a late stage infection site. Four trees have visibly active bleeds. The soilscape is 18 (Cranfield University, 2017). This woodland was used for insect sampling.

Figure 14. Site Two

Site Three (Grid Ref. withheld at request of site owner)

Site three (Figure 15, Figure 16) is an area of c. 2.5 ha ancient and veteran oak trees within a larger parkland, over 80 ha in size dominated by ancient and veteran trees. Considered as a late stage infection site. Several trees in the area show visibly active bleeds. The soilscapes classification is 10 'Freely draining slightly acid sandy soils' (Cranfield University, 2017). This site was used for insect and phyllosphere sampling of autumn leaves and litter.

Figure 15. Site Three in spring

Figure 16. Site Three in summer

Ickworth Park (Grid Ref. TL 81500 62210)

A large parkland of c.300 ha dominated by ancient and veteran trees alongside younger plantings. A smaller 6.5 ha area (Figure 17, Figure 18) was used. Considered as a late stage infection site. The soilscape is 8 (Cranfield University, 2017). Used for insect and phyllosphere sampling of spring leaves, autumn leaves, acorns and litter.

Figure 17. Ickworth park

Figure 18. Ickworth park

2.1.3 Additional investigation 2018

In 2018 two sites with AOD and one where AOD is absent were used. The focus in 2018 was to continue the phyllosphere and litter sampling. In addition the potential for airborne movement of *G.quercinecans* and *B.goodwinii* would be investigated through the collection of rain water.

Spring leaf samples were taken in June and autumn leaf and litter samples in September. No catkins or acorns were sampled in 2018.

Rain water was collected in June and then again in September.

Harper Adams University Woodland (Grid Ref. SJ 70850 21266)
An area of mature woodland c. 10 ha dominated by oak with a dense. A smaller area about 3 ha (Figure 19) was used. No AOD is present on the site. The soilscapes dataset classifies the soil as a mix of 18 and 10 (Cranfield University, 2017).

Figure 19. Harper Adams University woodland

Dudmaston Hall (Grid Ref. SO 74145 88500)

A parkland of c.1214 ha with ancient and veteran trees and younger plantings surrounded by farmland. A smaller area was used (Figure 20, Figure 21). Considered as a late stage infection site; four trees with active bleeds. The soilscapes dataset classifies the soil as soilscape 6 (Cranfield University, 2017).

Figure 20. Dudmaston in Winter

Figure 21. Dudmaston in Spring

Uncylls, Wyre Forest (Grid Ref. SO 76139 75282)

The Wyre forest is over 2500 ha of mature mixed woodland, plantations and grassland. A smaller 2 ha area (Figure 22) was used. Considered as a late stage infection site; seven tree were recorded with active bleeds. The area is soilscape 8 (Cranfield University, 2017).

Figure 22. Uncylls woodland

2.2 General Research Methodologies

2.2.1 Biosecurity and Aseptic techniques

Throughout the field and laboratory work a number of biosecurity measures, ascetic and sterile techniques were used to minimise contamination. These are described below:

Field sampling

- Between uses insect traps were washed with detergent and then sterilised by submerging in 10 % sodium hypochlorite solution before being rinsed.
- During collections of phyllosphere samples to avoid contamination from touching the leaves the bag was placed over the leaves whilst still attached to the twigs and then the leaves removed using the bag itself.
- Any equipment used was alcohol and flame sterilised between trees.
- Sites were visited from low to high risk to prevent potential spread of AOD.
- Shoes were rinsed and sprayed with disinfectant between sites.
- Collected samples were stored separately for transportation.
- Samples were placed into clean clear sealable plastic bags and frozen at -26°C until used.

Laboratory practice

- Gloves were worn and replaced between samples from different trees.
- Sample bags were weighed before collection to avoid removing the samples when weighed after collection.
- Where required equipment and chemicals were autoclave sterilised before use.
- Any water used was distilled water which was autoclave sterilised before use.
- DNA was extracted in a separate area from Polymerase Chain Reaction (PCR) preparation.
- PCR reagents were prepared within a U.V. sterilised PCR cabinet.
- PCR reagents were kept on ice while in use.
- Sterile gloves were used for all DNA extraction and PCR work.
- Sterile filter tips were used when pipetting and filter tips were used with PCR reagents.
- Extracted DNA was kept in a different freezer to unprocessed samples and reagents.
- Preparation of agar plates was carried out in a fume cupboard.

2.2.2 Methodology used for field collection of phyllosphere and litter samples

Oak phyllosphere samples were collected across three years (Table 3). All were collected from the lower canopy since this has been shown to be suitable for quantifying differences in phyllosphere communities (Laforest-Lapointe *et al.,* 2016). Litter was collected from the base of the tree.

Table 3. Phyllosphere and litter samples collected from sites 2016-2018

Year	**2016**		**2017**		**2018**	
Sites	Symptomatic 2	Asymptomatic 0	Symptomatic 2	Asymptomatic 1	Symptomatic 2	Asymptomatic 1
Spring leaves	✓	-	✓(1 site)	✓	✓	✓
Autumn leaves	✓	-	✓	✓	✓	✓
Acorn	✓(1 site)	-	✓(1 site)	✖	✖	✖
Catkins	✖	-	✓(1 site)	✖	✖	✖
Litter	✖	-	✓	✓	✓	✓

Spring and autumn leaves: Leaves were collected in June and September respectively from the lower canopy at each of the four cardinal points and placed all together into a sample bag. A separate bag was used per tree. Long handled loppers were used where the canopy was not in arms reach to either hold down the branch or, where possible, sever a small branch, which was caught before touching the ground and leaves stripped from twigs. Approximately 20 g were collected per tree.

Acorns: Where present, 5-10 acorns were collected in September from each tree and placed into separate sample bags. Collection followed the same protocol as the leaves.

Catkins: Catkins were collected in June from each tree and placed into separate sample bags. Collection followed the same protocol as the leaves.

Litter: Samples were collected in September from directly under the canopy of the same trees used for spring and autumn leaf collection. Litter was collected into a sample bag; one per tree with a final average collection weight of 26 g per tree.

2.2.3 Methodology used for collection of xylophagous Coleoptera

There are a number of different methods of collecting Coleoptera ranging from direct acquisition from searching e.g. stumps, logpiles, standing dead wood to the use of traps in the field (Aguilar, 2010). When collecting on a large scale there are other considerations such as cost and practicality that have to be taken into account and these influence the method chosen. Trap location within a sampling area is an important consideration as is trap height and may influence success.

In 2016 two sites and in 2017 six sites were sampled for xylophagous beetles. Three different trap types were used in various combinations across different sites and years (Table 4). The combinations of trap types used were:

- Yellow blue vane traps (SpringStar, Inc) (Figure 23).
- Purple blue vane traps; created by spraying the containers of the yellow blue vane traps with PlastiKote™ Sumptuous purple (Figure 23).
- Purple panel traps (AgBio, Inc) (Figure 23); cardboard traps (20 x 25 cm) with a sticky surface. A double panel trap was also created by securing two together with staples to create a trap 40 x 50 cm.
- Yellow blue vane + purple panel; a yellow blue vane trap immediately above which was a purple panel trap (Figure 23).
- Purple blue vane + purple panel; a yellow blue vane trap immediately above which was a purple panel trap.

These traps were selected based on the use of visual cues used by *Buprestidae*, specifically *Agrilus* species. Several *Agrilus* species have been found to have a preference for purple traps namely *A.planipennis* (Francese *et al.,* 2008), *A.sulcicollis* (Haack *et al.,* 2009), *A.auroguttatus* (Coleman *et al.,* 2009), *A.laticornis* and *A.biguttatus* (Brown, 2014).

Propylene glycol acted as a preservative and all traps were emptied and preservative and purple panels replaced fortnightly. Traps were hung approximately 6 m above the ground for practicality on metal hangers in the outer canopies of trees in open situations to maximise visibility. Trees in sunny situations were selected as a positive association between the number and abundance of buprestid species caught and forest edges has been demonstrated (Wermelinger *et al.,* 2007).

Figure 23. Purple blue vane trap (L) and yellow blue vane trap with purple panel above (R)

Table 4. Trap combinations used for insect sampling in 2016 and 2017

Year	**2016**		**2017**	
Number of Sites	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic
	2	0	5	1
Trap details	**Number of traps used per site**			
Yellow blue vane	9	-	5	5
Purple blue vane	9	-	5	5
Purple panel (single)	0	-	5	5
Purple panel (double)	0	-	5	5
Yellow blue vane + purple panel	0	-	5	5
Purple blue vane + purple panel	0	-	5	5

In 2016 traps were hung singly per tree, alternated by colour and placed within oak trees approximately 20-30 m apart. In 2017 seven symptomatic trees were selected across each site used and a combination of four trap types placed in each oak tree with two trees having five per tree.

2.2.4 Methodology used for field collection of rainwater samples

In 2018 rainwater was collected to investigate the potential of rain and wind to disperse *B.goodwinii* and *G.quercinecans*. Two symptomatic and one asymptomatic site were used.

Rainwater traps were constructed from 1 L plastic bottles. The tops were cut off the bottles, upturned and secured with tape to create a funnel (Figure 24). A piece of mesh was stretched over the open end of the created funnel to exclude debris. Ground traps were created by attaching the trap to short canes secured in the ground so that trap bases were level with the soil. Canopy traps were hung with string in the lower canopy at approximately 3 m from the ground.

Figure 24. Rain trap

One trap was placed in each of three symptomatic and asymptomatic oak trees. Three additional traps were placed in open ground within the woodland. New traps were made for each sampling period and the used traps recycled. Traps were deployed in June and then again in September. Traps were emptied after one week. The volume of water collected was dependent on the weather however at least 50 ml was collected per trap per site however at one symptomatic site no sample was collected in autumn due to lack of rain.

Table 5. Details of raintraps deployed at three sites in 2018

	Symptomatic	**Asymptomatic**
Number of sites	2	1
Rain traps		
Spring canopy	✓	✓
Spring ground	✓	✓
Autumn canopy	✓	✓
Autumn ground	✓	✓

2.2.5 Methodology used for molecular analysis

There are two main techniques for studying bacterial populations; culture and culture independent methods. The first involves isolating and cultivating the bacteria on growing media. The second involves the use of molecular analysis through PCR (Polymerase Chain Reaction). Since culture dependent methods do not necessarily detect all bacterial species present (Yang *et al.,* 2011) and may be also point out that be restricted by the cultivability of bacteria (Lindow and Brandl 2003). A culture independent method was followed and molecular analysis followed a two step process of DNA extraction and real time PCR for detection of the target bacteria which are outlined in the next sections.

2.2.5.1 Methodology used for DNA extraction

DNA extraction for phyllosphere samples followed the protocol set out in the FastDNA® SPIN Kit (MP Biomedicals) with a modification at the elution stage of the DNA pellet being eluted by resuspension with DES (DNA Elution Solution - Ultra pure DNAse free water) and incubating for 30 minutes at 55°C before centrifuging to elute the DNA into a clean tube. DNA extraction for insect samples followed the protocol set out in the DNeasy Blood and Tissue kit (Quiagen).

Sample preparation - initial investigation 2016

Leaf samples - Three visibly undamaged green leaves were selected from the collected leaves and a 200±10 mg sample was used for DNA extraction.

From each individual leaf a section was cut, using a scalpel, from the tip, centre and midrib onto a ceramic tile. Using forceps the sections of each leaf were next transferred to the lysing tube. A single lysing tube was used for each leaf; resulting in three lysing tubes each containing sections from an individual leaf. The lysing tube was then weighed and a final sample weight of 200±10 mg achieved. The samples were then homogenised with lysing agent according to the FastDNA® SPIN Kit instructions and the resulting supernatant from each of the leaves pooled to give a single leaf sample per tree.

Acorns - Three visibly undamaged acorns were selected from those collected. A 200±10 mg sample was used for the DNA extraction which included sections of inner and outer tissue. All preparation followed the same protocol as the leaf samples and the supernatant from each of the acorns was pooled to give a single acorn sample per tree.

Insects - Xylophagous beetles were sorted from the collected samples. Samples were macerated prior to DNA extraction.

Sample preparation further and additional investigations 2017-2018

A different approach was used for phyllosphere samples taken in 2017 and 2018 in order to analyse a greater proportion of the sample for bacteria.

Leaf samples - The collected leaves were flash frozen before use at -80°C. Distilled water that had been autoclave sterilised was added to the bag using 1 ml for every 1 g of material. The leaf material was then macerated for 1 minute per 10g within the same sample bag. After maceration 2 ml of the resultant supernatant was pipetted off using sterile filter pipette tips into a 2 ml microcentrifuge tube. This was then frozen at -26°C.

Before use the sample was defrosted at room temperature and spun at 13,000 rpm for 1 minute. 200µl was pipetted off with sterile filter pipette tips for use in extraction.

Litter samples - Litter was processed in the same way as the leaf samples.

Acorns - Three visibly undamaged acorns were selected and placed in small bags with enough perlite added to just cover the acorns. The perlite was then dampened with tap water. The bags were placed outside until the acorns germinated.

Sections of the outer and inner tissue were then cut, using a scalpel, onto a ceramic tile. Using forceps 200±10 mg was transferred to four separate lysing tubes; two for inner and two for outer tissue.

Rain - The collected rain water was first filtered with filter paper into autoclaved sterile glass beakers. The filtrate was then transferred 50 ml at a time to 50 ml filter centrifuge tubes into which a 2 µl filter had been placed and spun at 3000 rpm for 20 minutes. The supernatent was carefully poured off and 20 ml kept for analysis.

Insects - Xylophagous beetles were sorted from the collected samples. These were separated and species were grouped up to a maximum of four individuals by date collected. Samples were macerated prior to DNA extraction.

2.2.5.2 Methodology used for Real-Time PCR (Polymerase Chain Reaction)

Initial investigation 2016

A multiplex method developed by Kaczmarak *et al*. (unpublished; pers.comm) using the gene sequences presented was used in 2016. Although not a focus for this investigation this method included a probe and primer for the detection of *Rahnella victoriana* which has been detected in exudate (Denman *et al*., 2018). A reaction mix for 100 10 µl reactions was prepared in a 2 ml reaction tube.

Each reaction contained:

5.0μl Taq DNA polymerase (Thermo Fisher)

1.3μl Molecular water

0.25 μl each forward and reverse *Rahnella* primer

[15F 5'-CACCCAGACTTACGTGCAT] [134R 5' TCAGTGTGATTGGTGAAGGT]

0.25 μl each forward and reverse *Gibbsiella* primer

[284F 5'-GGCTTTGATAGTGGTGGCC] [418R 5' CGTTCCGTTATCACCGTGG]

0.10 μl *Rahnella* probe

Rv57P [5'-Rox AGTGATTGGCGATACTGACGTGACC]

0.10 μl *Gibbsiella* probe

GQ342P [5'-Cy5 AACAGTTCCAGCGCCATTTTCTT]

0.50 μl *Brenneria* mixed probe and primer

forward primer [5'-CTGGCCGAGCCTGGAAAC], reverse primer [5'AGTTCAGGAAGGAGAGTTCGC], probe [5-FAM-CCAGAATCTCATATTCGAACTCCACCATGTT]

2.5 μl template DNA

Using a 96 well plate kept on ice, 45 μl of the mix was pipetted into the 1st and 7th column. A multichannel pipette was then used to transfer 7.5 μl to each cell. DNA was quantified for each sample using a NanoDrop™. Into each well 2.5 μl of template DNA was then added with three replicate wells used per sample. Positive controls with DNA concentration of 5ng/μl for *B.goodwinii* (Strains FRB 21, FRB 23) and *G.quercinecans* (Strains FRB 31, FRB 33, FRB 36) were used along with molecular grade water negative control. A cover was attached to the plate which was then spun in a centrifuge at 3700 rpm for one minute. A Roche LightCycler ® 480A was set up for a 10μl volume reaction (Table 6):

Table 6. Roche LightCycler ® 480A programme setup

Stage	**Cycles**	**Time (mm:ss)**	**Temperature (°C)**
Initial denaturation	10	10:00	95
qPCR	40	00:10	95
		00:30	63
		00:01	72
Cooling	1	00:01	40

In real time PCR the amount of fluorescence is read after each amplification cycle. A positive reaction results when the fluorescence intensity exceeds background levels. For a positive detection to be declared at least two control wells for the relevant bacteria and two of the sample replicates had to show a positive detection curve and detection before 35 detection (Ct) cycles.

Further and additional investigations 2017-2018

For samples taken in 2017 and 2018 two separate single assays were used for *Brenneria goodwinii* and *Gibbsiella quercinecans.* The reaction mix was prepared for 100 25µl reactions using two 2 ml reaction tubes. Primers designed by Forest Research to target bacterial DNA gyrase B gene (gyrB) were used along with probes.

G.quercinecans each reaction contained:

12.5 µl Taqman® Environmental Master Mix 2.0 (Fisher Scientific)
7.125 µl PCR grade water (Merck)
0.125 µl probe (100 pmol/µl Eurofins Genomics)
G.quercinecans: [5'-Cy5 AAAACCCATCTGATGCCAAAA 3']
0.125 µl forward primer (60 pmol/µl Eurofins Genomics)
G.quercinecans gyrB : [5' GCGGTTGAACAACAGETG 3']
0.125 µl reverse primer (60 pmol/µl Eurofins Genomics)
G.quercinecans gyrB: [5' GCCGCATCAATGATTTTG 3']
5.0 µl template DNA

B.goodwinii each reaction contained*:*

12.5 µl Taqman® Environmental Master Mix 2.0 (Fisher Scientific)
7.125 µl PCR grade water (Merck)
0.125 µl probe (100 pmol/µl Eurofins Genomics)
B.goodwinii: [5'-FAM CCAGAATCTCATATTCGAACTCCACCATGTT 3']
0.125 µl forward primer (100 pmol/µl Eurofins Genomics)
B.goodwinii gyrB: forward primer [5' CTGGCCGAGCCTGGAAAC 3']
0.125 µl reverse primer (100 pmol/µl Eurofins Genomics)
B.goodwinii gyrB: [5' AGTTCAGGAAGGAG AGTTCGC 3']
5.0 µl template DNA

DNA was quantified for each sample using a NanoDrop™. Concentrations of DNA varied so DNA with similar concentrations were selected for analysis for each plate. A 96 well plate

kept on ice was used for the subsequent PCR reaction. The plate was within a U.V sterilised cabinet. 60 μl of the mix was pipetted into the 1st, 5th and 9th column. A multichannel pipette was then used to transfer 20 μl of the mix into each well. 5 μl of template DNA was then added with three replicate wells used per sample.

Positive controls (5ng/μl -100ng/μl) for *B.goodwinii* (Strains FRB 171, FRB 186) and *G.quercinecans* (Strains FRB 97, FRB 24) were used along with a negative control of molecular grade water. A cover was attached to the plate which was then placed in a BioRad CFX96 which was set up for a 25 μl volume reaction. Analysis settings were adjusted for 37 cycles, baseline subtracted curve fit, Cq determination single threshold. Baseline threshold was altered manually where needed. This was then run as follows for *G.quercinecans* (Table 7) and for *B.goodwinii* (Table 8).

Table 7. BioRad CFX96 programme set up for *G.quercinecans*

Stage	**Cycles**	**Time (mm:ss)**	**Temperature (°C)**
Initial denaturation	1	10:00	95
qPCR	40	00:15	95
		01:00	62
		00:01	72
Cooling	1	00:30	40

Table 8. BioRad CFX96 programme set up for *B.goodwinii*

Stage	**Cycles**	**Time (mm:ss)**	**Temperature (°C)**
Initial denaturation	1	10:00	95
qPCR	40	00:15	95
		00:30	65
		00:01	72
Cooling	1	00:30	40

Chapter 3

Investigating the relationship between the oak phyllosphere (leaves, acorns, catkins) and two bacteria associated with Acute Oak Decline; *B.goodwinii* and *G.quercinecans*

3.1 Introduction

The phyllosphere, where the definition encompasses the above ground parts of plants, represents a large area of study for bacteria as covered in section 1.2 of the literature review the majority of research has concentrated on leaves as they represent a large available area of a plant for colonisation (Lindow and Brandl, 2003).

Plant pathogens, including bacteria, are not widely considered to be obligate on a single host indicating their potential to disperse and exist in multiple hosts and environments (Morris *et al.,* 2008; Frank *et al.,* 2017). Bacteria are known to be moved via wind, by water through soil, via rain splash directly on plant surfaces or soil and through rain picking up airborne bacteria (Frank *et al.,* 2017). Those with a more stable means of transmission can however show a greater level of host specificity (Engel and Moran, 2013). The presence of bacteria on external tissues such as leaves suggests potential movement pathways for the bacteria to reach new hosts.

Leaves and acorns represent external surfaces and possible routes of entry for bacteria. They are not thought able to penetrate directly through the outer cuticle of leaves, relying instead on naturally created openings such as damage or stomata (Underwood *et al.,* 2007; Frank *et al.,* 2017). Bacteria acquired via flowers are also known to be able to transfer into developing seeds (Frank *et al.,* 2017). Oak leaves represent a difficult habitat for microorganisms to colonise due to their waxy surface and presence of tannins that can interfere with establishment and growth (Müller *et al.,* 2003). This could indicate that bacteria colonizing oak leaves are able to overcome these defences in some way.

3.1.1 The oak phyllosphere and Acute Oak Decline (AOD)

The sole area of investigation to date for bacteria associated with AOD has been various bark tissues and the necrotic tissue of the lesions. Both *B.goodwinii* and *G.quercinecans* have been consistently isolated from the necrotic tissue in the UK (Brady *et al.,* 2010;

Denman *et al.,* 2012; Sapp *et al.,* 2016; Broberg *et al*., 2018; Denman *et al.,* 2018) and from a tree in Spain (González and Ciordia, 2019). *Brenneria goodwinii* was more predominant in lesions compared to *G.quercinecans* suggesting it is the most active (Broberg *et al*., 2018).

A comparison of the bacterial communities in different bark layers of AOD symptomatic and asymptomatic oak has been carried out (Sapp *et al*., 2016; Denman *et al*., 2016). Bark panels were removed from 5 asymptomatic and 10 symptomatic trees across five sites. A further set of panels were taken from non symptomatic areas of AOD symptomatic trees. Tissues were then separated into heartwood, sapwood, inner bark and outer bark in Denman *et al*. (2016) with *A.biguttatus* larval galleries additionally investigated by Sapp *et al*. (2016).

A comparison of the bark tissues found that there were differences in bacterial communities between the symptomatic tissue and both asymptomatic and non symptomatic tissues (Denman *et al*., 2016). A shift in the bacterial community with a transitional phase was concluded (Denman *et al*., 2016). There was however a lower yield of bacteria from samples of asymptomatic (18%) and non symptomatic tissues (33%) compared to symptomatic tissues (82%) (Denman *et al*., 2016) which may have influenced results. The level of similarity between the asymptomatic and non symptomatic tissues was also low (Denman *et al*., 2016) at odds with the conclusion there was a transitional community.

The associated study by Sapp *et al.* (2016) found there was no significant difference in the overall bacterial community of asymptomatic compared to symptomatic trees (Sapp *et al*., 2016) contradicting the study by Denman *et al*. (2016). For symptomatic trees, overall bacterial community did differ between symptomatic and asymptomatic tissues (Sapp *et al*., 2016). Bacteria were not however found to be consistently separated by bark tissue type across all sites (Sapp *et al.,* 2016). This indicates a potential lack of niche partitioning for resources (Sapp *et al*., 2016) although since the species identified in the different tissues were not reported this cannot be further substantiated.

An explanation put forward for the two studies dissimilar results was a dilution effect as a consequence of the Sapp *et al*. (2016) analysis of non symptomatic and symptomatic tissues as two tissue types (Denman *et al*., 2016). The results reported could be considered as being from different spatial scales. This could account for the apparent disappearance of the significant effect at the larger scale and raises the question of what scale is more appropriate. The differentiation of microbial communities at separate spatial scales has been observed in ecology (Ladau *et al*., 2019) and can be an influence on species diversity (Martiny *et al*., 2011).

One of the bacteria, *B.goodwinii,* put forward by Denman *et al*. (2018) as a causal agent of AOD, was additionally highlighted as being present in the tissues of trees without evidence of AOD as well as trees with advanced symptoms (Sapp *et al*., 2016; Broberg *et al.,* 2018). Although there was no indication of whether *G.quercinecans* was similarly present in healthy as well as symptomatic trees Geider *et al*. (2015), examined the nucleotide sequences from 16S rRNA and the housekeeping genes dnaJ, gyrB, recA and rpoB of several species of *Gibbsiella*, and concluded that they are commonly occurring, including *G.quercinecans*.

The presence of *B.goodwinii* in diseased trees is not unexpected however the detection on healthy trees could indicate an epiphytic or endophytic lifestyle and this has been alluded to by Denman *et al*., (2018). Alternatively it may indicate their presence in the wider environment or the ability of these bacteria to switch from non-pathogenic to pathogenic lifestyles (Broberg *et al.,* 2018). Both bacteria have been detected by qPCR after inoculation into soil although only *G.quercinecans* was concurrently culturable (Pettifor et al., 2020). For this reason *B.goodwinii* was proposed as an endosymbiont as it may have entered a viable but non-culturable state (Pettifor *et al*., 2020). The well-studied pathogenic bacteria *Pseudomonas syringae* pv. *syringae* for example has been shown to exist on both symptomatic, non-symptomatic and non-host plant leaves (Lindemann *et al.,* 1984). Its capacity to cause disease has also been highly correlated with the population size present on leaves (Rouse *et al.,* 1994). Although there has been no investigation into whether *B.goodwinii* and *G.quercinecans* have any association with oak leaves a similar situation may be occurring where a population threshold needs to be passed or environmental conditions met before its presence becomes pathogenic and symptoms are expressed.

In a study on the microbiome of healthy *Q.robur* leaves the largest bacterial components were from the Phylum Proteobacteria (Jakuschkin, 2015). For comparison the three most abundant phyla in bark microcores were Acidobacteria, Actinobacteria and Proteobacteria (Meaden *et al.,* 2016). A study using microcores that investigated the bacterial bark microbiome of 64 healthy *Q.robur* trees on a site without AOD, found no evidence of sequences that were identified as belonging to the *Brenneria* genus (Meaden *et al.,* 2016). The detection by Sapp *et al*. (2016) on asymptomatic trees may therefore be as a result of it already being present on the AOD site and colonising but not affecting an oak host.

One aspect that may be significant is that *B.goodwinii* has flagella (Denman *et al.,* 2012) compared to the fimbriae of *G.quercinecans* (Brady *et al.,* 2010). These structures have been found to be important in binding to host cells and formation of biofilms (Suoniemi *et al*., 1995; Haiku and Westerlund-Wikström, 2013; Rossez *et al.,* 2015) and are also important for the movement of bacteria (Haiko and Westerlund-Wikström, 2013; Rossez *et al.,* 2015).

3.1.2 Pathogenic capability of *Brenneria goodwinii* and *Gibbsiella quercinecans*

The evolution of virulence factors within phytopathogens has enabled them to manipulate the processes of a hosts cell in order to effectively colonize and survive within the host plant (Speth *et al*., 2007). Identified virulence factors include plant cell wall degrading enzymes , protein secretion systems (notably T3SS) and their associated effector proteins (Doonan, 2019). Secretion systems are vital to pathogenic bacteria as they are used to secrete proteins across phospholipid membranes into host cells to aid colonisation (Green and Mecsas, 2016). These proteins can enhance bacterial virulence through e.g. improving attachment to cells, enhancing resource availability, or direct toxicity to host cells (Green and Mecsas, 2016).

Bacterial virulence factors have been identified in samples of AOD lesion tissue including those involved in regulation, signalling, membrane transport and plant cell wall degrading enzymes (Broberg *et al*., 2018). Genomic analysis of *B.goodwinii* and *G.quercinecans* indicates they encode for several different types of secretion systems (Doonan *et al*., 2019). A T2SS and T6SS have been identified in *G.quercinecans* and a T2SS, T3SS, T4SS and T6SS in *B.goodwinii* (Doonan *et al*., 2019). All of these secretion systems are involved in protein transport into host cells with the T4SS also known to transfer DNA and the T6SS utilised to transfer proteins into bacterial cells (Green and Mecsas, 2016).

Doonan *et al*. (2019) have proposed that the bacteria they act via necrogenic enzymes or the secretion of effector proteins to alter biological action, degrade host tissue and overcome host defences. Proteins have been identified from symptomatic samples; 121 proteins identified, 23.5 % of which were bacterial and 18 associated with *B.goodwinii* and 3 with *G.quercinecans* (Broberg *et al*., 2018). Those associated with *B.goodwinii* included effector proteins involved in tissue degradation and suppression of host defences (Broberg *et al*., 2018).

The presence of a T2SS secretion system (Doonan, 2016), used in obtaining nutrients from dead cells (Kraepiel and Barny, 2015) suggests both have the capacity to utilise decayed tissue and this is supported by the evidence of their presence in necrotic bark tissue. In addition when single cultures were applied to a wound created in an oak log, tissue decay followed (Denman *et al*., 2018). Since *G.quercinecans* and *B.goodwinii* are already known to have genes necessary to degrade plant tissue (Denman *et al.,* 2018) the resulting decay is not unexpected. It is not clear though whether these bacteria require an initial wound to begin colonisation or whether they are able to degrade the outer bark layer since the results of a comparison with non-wounded logs were not presented.

Another secretion system, T3SS, known to be involved in the suppression of host defences (Kraepiel and Barny, 2015) and transition to a necrotrophic lifestyle (Lee and Rose, 2010) was absent in the genomic analysis of *G.quercinecans* (Doonan *et al*., 2019). An example of this can be found in *P. syringae* pathovars which utilise a T3SS to manipulate host defences and metabolism in order to enhance colonisation and maintain a nutrient supply (Toth *et al*, 2006).

The *B.goodwinii* genome has been shown to be more aligned to that of a necrogenic phytopathogen compared that of *G.quercinecans* which was allied to saphrophytes as well as pathogens (Doonan *et al*., 2019). The predominance of *B.goodwinii* within lesion samples (Broberg *et al*., 2018) and its greater genome encoded pathogenic potential compared to *G.quercinecans* implicates *B.goodwinii* as the major causal agent (Doonan *et al*., 2019).

A high genetic diversity has been identified in the genomic analysis of *G.quercinecans* (Doonan *et al*., 2019). A separate study into *Gibbsiella* spp. has also indicated that certain genes (recA and ppiD)[5] had a greater variability in expression than would be required for a pathogen with a specific host (Geider *et al.,* 2015). This was suggested as indicating an ability to adapt to different environments and a wide host range (Geider *et al.,* 2015) which would be an advantage for a commonly occurring generalist bacteria.

Bacteria have been shown to be able to work as pairs to initiate disease (Allen *et al*., 2009). The T6SS identified in both bacteria and the presence of the T3SS secretion system (Doonan *et al*., 2019) further hints at a potential mutualistic relationship between the two bacteria and why they are consistently isolated together. It has been suggested *G.quercinecans* is the primary coloniser creating the conditions required for *B.goodwinii* to colonise and express virulence factors (Doonan *et al*., 2019). Alternatively the presence of the T3SS secretion system in *B.goodwinii* and not in *G.quercinecans* (Doonan *et al*., 2019) could indicate host defences already need to be compromised for *G.quercinecans* to be able to effectively colonise internal tissues.

The possibility of survival on other oak tissues is another important question in understanding the epidemiology of AOD. Both *B.goodwinii* and *G.quercinecans* were separately inoculated into 15 acorns from an asymptomatic tree through a needle puncture (González and Ciordia, 2019). After 15 days a rotting was observed around the wound and inside the acorns of those applied with *B.goodwinii* and *G.quercinecans* whilst the sterile water control showed no necrosis. Both bacteria were reisolated from the inoculated acorns (González and Ciordia, 2019).

[5]The recA gene is involved in genetic recombination (Selbitschka *et al*., 1991) and the ppiD (peptidyl-prolyl cis-trans isomerase D) gene is linked to protein folding (Dartigalongue & Raina).

Although the genomes of *B.goodwinii* and *G.quercinecans* have been sequenced we lack information on much of their basic ecology. Key questions over host specificity and potential movement mechanisms remain uninvestigated. Live leaves represent a large available and uninvestigated area for *B.goodwinii* and *G.quercinecans.* Leaves could be a potential way for bacteria to enter the trees and a pathway from which bacteria colonise new hosts and leaf litter may offer an overwintering resource. Similarly acorns and catkins are both a potential dispersal mechanism and colonisation entry point.

3.1.3 Chapter Aims

The aim of this chapter is to investigate whether there is any association between the oak phyllosphere, here represented by leaves (live and litter), acorns, catkins and the bacteria *B.goodwinii* and *G.quercinecans.*

3.2 Methodology

3.2.1 Field investigation

Leaves, litter, acorns and catkins were collected over three years (Table 10) according to the protocol in section 2.2.2. Sample preparation and DNA extraction followed section 2.2.5.1. Detection of *G.quercinecans* and *B.goodwinii* was via PCR as covered in section 2.2.5.2.

Table 10. Locations and number of trees from which leaf, litter and acorn samples were taken between 2016 and 2018 at symptomatic (S) and asymptomatic (A) sites

Site	**ATT**[1]	**RIC**[2]	**ICK**[3]	**S3**[4]	**BRO**[5]	**WYR**[6]	**DUD**[7]	**HAU**[8]
Status	S	A	S	S	A	S	S	A
Year	2016	2016	2017	2017	2017	2018	2018	2018
Number of trees sampled								
Autumn leaves								
Symptomatic	5	4	6	5	N/A	5	4	N/A
Asymptomatic	7	4	9	9	9	9	9	9
Total	12	8	15	14	9	14	13	9
Spring leaves								
Symptomatic	0	0	0	0	N/A	5	5	N/A
Asymptomatic	0	0	9	0	9	9	9	9
Total	0	0	9	0	9	14	14	9
Acorns								
Symptomatic	2	2	2	0	0	-	-	-
Asymptomatic	2	3	4	0	0	-	-	-
Total	4	5	6	0	0	-	-	-
Litter								
Symptomatic	-	-	6	5	N/A	5	4	N/A
Asymptomatic	-	-	9	9	9	9	9	9
Total	-	-	15	14	9	14	13	9
Catkins								
Symptomatic	-	-	2	0	0	-	-	-
Asymptomatic	-	-	6	0	0	-	-	-
Total	-	-	8	0	0	-	-	-

[1]Attingham [2]Richmond [3]Ickworth [4]Site 3 [5] Broaks [6]Wyre [7]Dudmaston [8]Harper Adams. For site descriptions see section 2.1

3.2.2 Additional field and glasshouse investigations

In addition to the field collection of phyllosphere samples two further studies outlined below were set up.

3.2.2.1 Field investigation into the presence of *G.quercinecans* and *B.goodwinii* on leaves via culture of washings from leaves and litter

The aim of this investigation was to establish whether *G.quercinecans and B.goodwinii* were present on leaf surfaces as live bacteria by washing a collected bag of leaves and culturing a sample of the washings.

Sampling followed the same protocol detailed in section 2.2.2. In September 2018 leaf samples were taken at Uncylls woodland (Figure 22) from four symptomatic trees and four asymptomatic trees. Litter samples were collected from below the same trees and combined to create one bag of litter from symptomatic trees and one from asymptomatic trees.

Whilst on site 100 ml of distilled sterilised water was added to each of the bags and the sample macerated for 1 minute. A single use pipette was then used to pipette off 1 ml onto general nutrient agar plates (Oxoid). The plates were then returned to the lab and incubated at 37°C for 24 hours after which the surface was scraped using a single use spreader into a 2 ml microcentrifuge tubes.

DNA extraction then followed the protocol outlined in section 2.2.5.1. Detection of the presence of *G.quercinecans* and *B.goodwinii* was via PCR as covered in section 2.2.5.2.

3.2.3.2 Glasshouse investigation into the survival of *G.quercinecans* and *B.goodwinii* on leaves after inoculation

The aim of this investigation was to identify whether both bacteria singly and in combination were able to survive 24 hrs after being spread onto newly opened leaves of oak saplings.

Sixty *Quercus robur* 40-60 cm high bare root saplings (Maelor Nursery) were planted into 20 cm diameter and 60 cm depth pots filled with 65°C heat treated soil (John Innes). Trees were planted before leaves had opened and were placed into the experimental glasshouse room. Trees used for the control placed into a separate glasshouse room. Watering was carried out as necessary.

A single colony of *G.quercinecans* and *B.goodwinii* were separately added to 50 ml of nutrient broth. After 48 hrs of room temperature agitation a 10 fold serial dilution of 1 ml original starting culture was then carried out in a fume cupboard using distilled sterilised water. Three of the dilutions for each of the bacteria individually (10^{-2}, 10^{-3}, 10^{-4}) plus a combination of *G.quercinecans* and *B.goodwinii* and a control of sterilised water were then used in the investigation.

Six replicates were used for each treatment. In the experimental glasshouse room three benches were arranged. One each for the single applications and one for the combination. The treatments were then applied in a randomised block design.

For each tree 300 µl of each of the treatments was pipetted into an individual well of a 96 well PCR plate and then sealed. This was then used to inoculate three newly opened leaves per tree with a separate sterile cotton bud used for each tree.

After 24 hrs the three leaves were harvested and placed individually into clean bags. A single leaf from three different trees per treatment was then randomly selected for analysis. To the bag selected 2 ml of distilled sterile water was added and gently agitated and 1 ml pipetted and spread onto general nutrient agar (Oxoid). The plates were then incubated at 37°C for 24 hours after which the surface was scraped using a single use spreader into a 2ml microcentrifuge tubes.

DNA extraction then followed the protocol outlined in section 2.2.5.1. Detection of the presence of *G.quercinecans* and *B.goodwinii* was via PCR as covered in section 2.2.5.2.

3.3 Results

3.3.1 Field investigation

3.3.1.1 Spring and autumn Leaves

In this section results are presented for spring and autumn leaves tested in 2016, 2017 and 2018. For site identification refer to Table 10.

Table 11 indicates the percentage of positive detections per leaf sample type based on tree health status, independent of site health status.

Figures 25-28 show the overall number of trees per site, sample period and year which were positive or negative for *B.goodwinii* or *G.quercinecans*. Results are presented separately for asymptomatic and symptomatic trees and separately for each bacteria.

Tables 12-15 show the detail behind the graphs with the results per site and year of the trees which had a positive detection of *B.goodwinii* or *G.quercinecans* in spring and/or autumn leaf samples. Results are presented separately for asymptomatic and symptomatic trees and separately for each bacteria.

Table 11. The percentage of positive samples for *G.quercinecans* and *B.goodwinii* in each of the different sample types based on tree health status, independent of site health status

Tree health status	Sample type	Positive samples %
Asymptomatic	Gq Spring leaves	35
	Bg Spring leaves	58
	Gq Autumn leaves	46
	Bg Autumn leaves	45
	Gq Litter	62
	Bg Litter	46
Symptomatic	Gq Spring leaves	20
	Bg Spring leaves	15
	Gq Autumn leaves	43
	Bg Autumn leaves	57
	Gq Litter	45
	Bg Litter	30

Results for asymptomatic trees

A higher percentage of spring leaf samples from asymptomatic trees, independent of site, were positive for *B.goodwinii* (58%) compared to *G.quercinecans* (35%) (Table 11). In autumn leaf samples a similar percentage from asymptomatic trees, independent of site, were positive for *G.quercinecans* (46%) and *B.goodwinii* (45%) (Table 11).

Gibbsiella quercinecans

At asymptomatic sites across the two sampling years and periods ten out of the 36 trees sampled were positive for *G.quercinecans* (Figure 25). In comparison at symptomatic sites across the three sampling years and periods 30 out of the 74 trees sampled were positive for *G.quercinecans* (Figure 25).

In symptomatic sites where both autumn and spring leaves were sampled the number of detections of *G.quercinecans* was higher in two autumn compared to one site in spring (Figure 25). At asymptomatic sites detections were equal at one site and higher in spring at the other (Figure 25).

Brenneria goodwinii

Twenty five out of the 36 trees sampled across the two sampling years and periods were positive for *B.goodwinii* at asymptomatic sites (Figure 26). In comparison at symptomatic sites across the three sampling years and periods 26 out of the 74 trees sampled were positive for *B.goodwinii* (Figure 26).

Where both autumn and spring leaves were sampled in symptomatic sites, the number of detections of *B.goodwinii* was higher in two autumn compared to one site in spring (Figure 26). At asymptomatic sites detections were greater at one site in autumn and one in spring (Figure 26) with the site in spring also having higher detections of *G.quercinecans*.

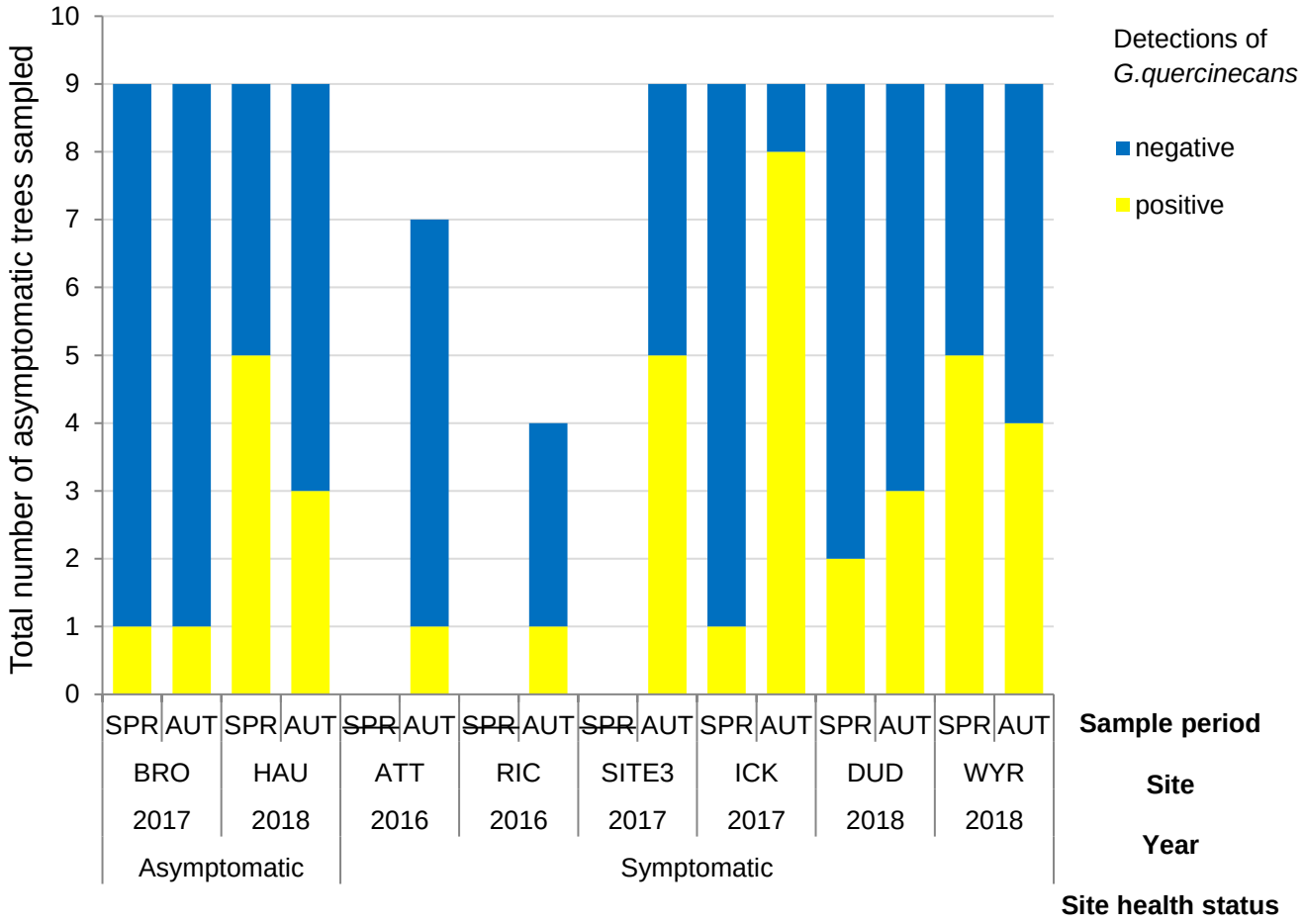


Figure 25. Positive and negative detections of *G.quercinecans* in the total number of asymptomatic trees sampled across three years 2016-2018 from asymptomatic and symptomatic sites in spring and autumn

The results presented for the overall number of trees in Figures 25 and 26 do not indicate whether the same tree was positive or negative in each sampling period. For example as Table 12 and Table 13 show at the 2017 asymptomatic site it was the same tree which was positive for *G.quercinecans* and *B.goodwinii* in both spring and autumn. At the 2018 asymptomatic site two of the nine trees were positive for *G.quercinecans* and *B.goodwinii* in both sample periods. Leaves sampled in both periods in 2018 at the two symptomatic sites resulted in four trees across these sites testing positive for *G.quercinecans*; three of these trees coming from one site (Table 13). However only one of these three trees also tested positive for *B.goodwinii* in spring and autumn (Table 13).

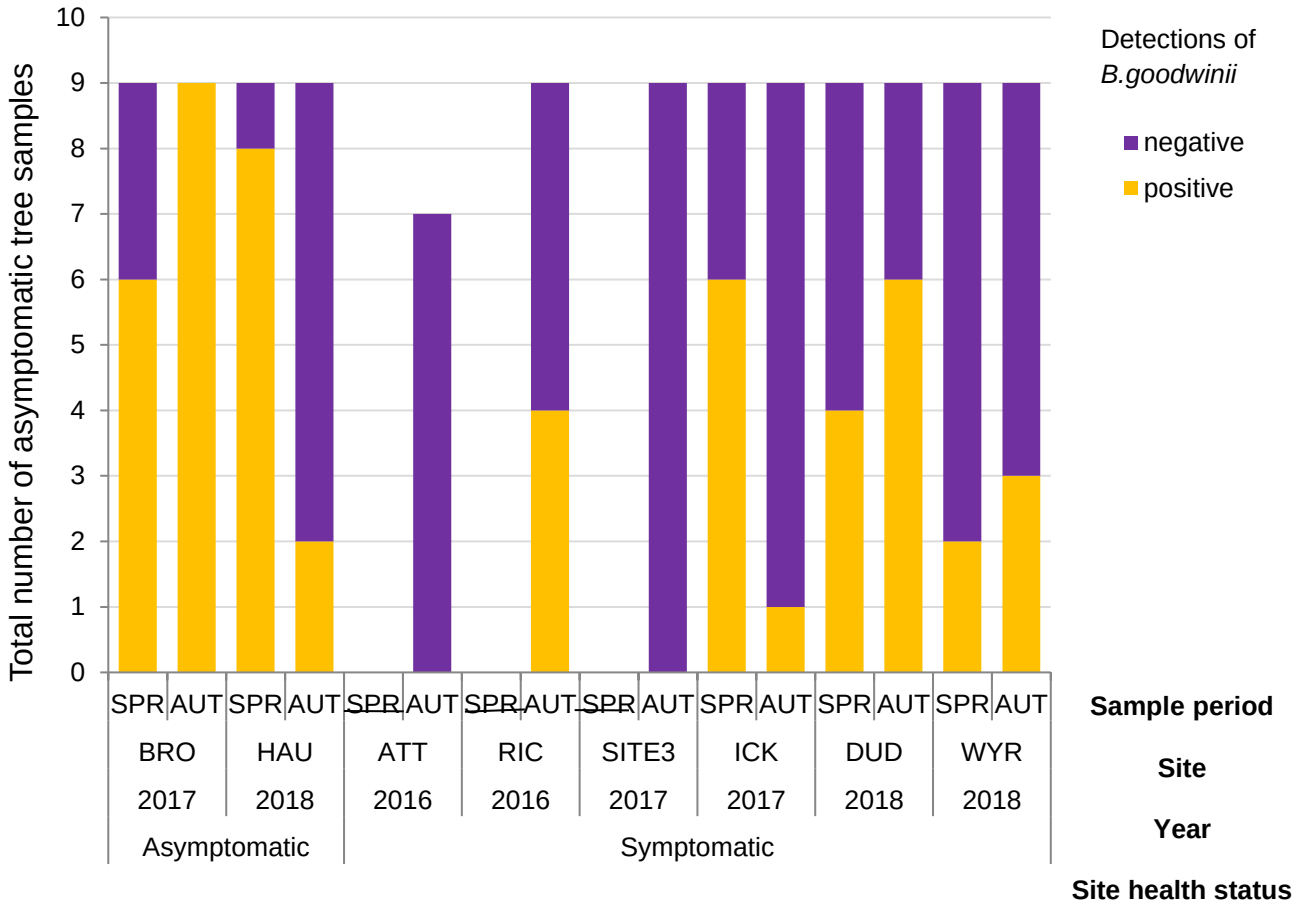


Figure 26. Positive and negative detections of *B.goodwinii* in the total number of asymptomatic trees sampled across three years 2016-2018 from asymptomatic and symptomatic sites in spring and autumn

Table 12. Positive results of the detection of *G.quercinecans* in spring and autumn leaf samples from asymptomatic trees in symptomatic and asymptomatic sites 2016-2018

Tree identification	**Site health status**	**Sample year**	***G.quercinecans* Spring leaves**	***G.quercinecans* Autumn leaves**
BRONB9	Asymptomatic	2017	positive	positive
HAUNB1	Asymptomatic	2018	positive	positive
HAUNB2	Asymptomatic		positive	negative
HAUNB3	Asymptomatic		positive	negative
HAUNB5	Asymptomatic		negative	positive
HAUNB6	Asymptomatic		positive	positive
HAUNB8	Asymptomatic		positive	negative
ATTNB1	Symptomatic	2016	not taken	positive
RICNB4	Symptomatic		not taken	positive
ICKNB1	Symptomatic	2017	negative	positive
ICKNB2	Symptomatic		positive	positive
ICKNB3	Symptomatic		negative	positive
ICKNB4	Symptomatic		negative	positive
ICKNB5	Symptomatic		negative	positive
ICKNB6	Symptomatic		negative	positive
ICKNB8	Symptomatic		missing	positive
ICKNB9	Symptomatic		negative	positive
SITE3NB2	Symptomatic		not taken	positive
SITE3NB4	Symptomatic		not taken	positive
SITE3NB5	Symptomatic		not taken	positive
SITE3NB6	Symptomatic		not taken	positive
SITE3NB7	Symptomatic		not taken	positive
DUDNB1	Symptomatic	2018	negative	positive
DUDNB2	Symptomatic		negative	positive
DUDNB4	Symptomatic		positive	positive
DUDNB8	Symptomatic		positive	negative
WYRNB1	Symptomatic		positive	negative
WYRNB2	Symptomatic		positive	negative
WYRNB3	Symptomatic		positive	positive
WYRNB4	Symptomatic		positive	positive
WYRNB5	Symptomatic		positive	positive
WYRNB6	Symptomatic		positive	negative
WYRNB7	Symptomatic		negative	positive
WYRNB8	Symptomatic		negative	positive

Table 13. Positive results of the detection of *B.goodwinii* in spring and autumn leaf samples from asymptomatic trees in symptomatic and asymptomatic sites 2016-2018

Tree identification	Site health status	Sample year	*B.goodwinii* Spring leaves	*B.goodwinii* Autumn leaves
BRONB1	Asymptomatic	2017	positive	positive
BRONB2	Asymptomatic		positive	positive
BRONB3	Asymptomatic		positive	positive
BRONB4	Asymptomatic		negative	positive
BRONB5	Asymptomatic		positive	positive
BRONB6	Asymptomatic		negative	positive
BRONB7	Asymptomatic		negative	positive
BRONB8	Asymptomatic		positive	positive
BRONB9	Asymptomatic		positive	positive
HAUNB1	Asymptomatic	2018	positive	positive
HAUNB3	Asymptomatic		positive	negative
HAUNB4	Asymptomatic		positive	negative
HAUNB5	Asymptomatic		positive	negative
HAUNB6	Asymptomatic		positive	positive
HAUNB7	Asymptomatic		positive	negative
HAUNB8	Asymptomatic		positive	negative
HAUNB9	Asymptomatic		positive	negative
SITE3NB4	Symptomatic		not taken	positive
SITE3NB5	Symptomatic		not taken	positive
SITE3NB6	Symptomatic		not taken	positive
ICKNB1	Symptomatic		positive	negative
ICKNB2	Symptomatic		positive	negative
ICKNB3	Symptomatic		positive	negative
ICKNB4	Symptomatic		positive	negative
ICKNB5	Symptomatic		positive	positive
ICKNB7	Symptomatic		positive	negative
ICKNB9	Symptomatic		negative	positive
DUDNB1	Symptomatic	2018	negative	positive
DUDNB2	Symptomatic		negative	positive
DUDNB3	Symptomatic		positive	positive
DUDNB4	Symptomatic		negative	positive
DUDNB7	Symptomatic		positive	positive
DUDNB8	Symptomatic		positive	positive
DUDNB9	Symptomatic		positive	negative
WYRNB5	Symptomatic		positive	negative
WYRNB6	Symptomatic		negative	positive
WYRNB7	Symptomatic		positive	positive
WYRNB8	Symptomatic		negative	positive

Results for symptomatic trees

For symptomatic trees there was a greater percentage of positive detections of *G.quercinecans* (20%) compared to *B.goodwinii* (15%) in spring leaf samples (Table 11). A greater percentage of autumn leaf samples from symptomatic trees had a positive detections of *B.goodwinii* (57%) compared to *G.quercinecans* (43%) (Table 11).

Gibbsiella quercinecans

In the 2016 and 2017 sample years at symptomatic sites only autumn leaves were analysed (Figure 27). Of the 20 samples collected 11 showed positive and nine negative for *G.quercinecans* (Figure 27). In 2018 both spring and autumn leaves were analysed from two symptomatic sites (Figure 27). Across both seasons of the 10 trees sampled this resulted in six positive and three negative for *G.quercinecans* (plus one lost autumn sample) one site and all 10 samples negative at the second site (Figure 27).

Brenneria goodwinii

In 2016 all nine autumn leaf samples from symptomatic sites (5ATT:4RIC) had a negative result for *B.goodwinii* (Figure 28). Autumn samples from both symptomatic sites in 2017 indicated four negative and seven positive *B.goodwinii* detections (Figure 27). Of the 19 spring and autumn samples analysed in 2019 (plus one autumn sample lost) 12 were negative and seven positive for *B.goodwinii* (Figure 27).

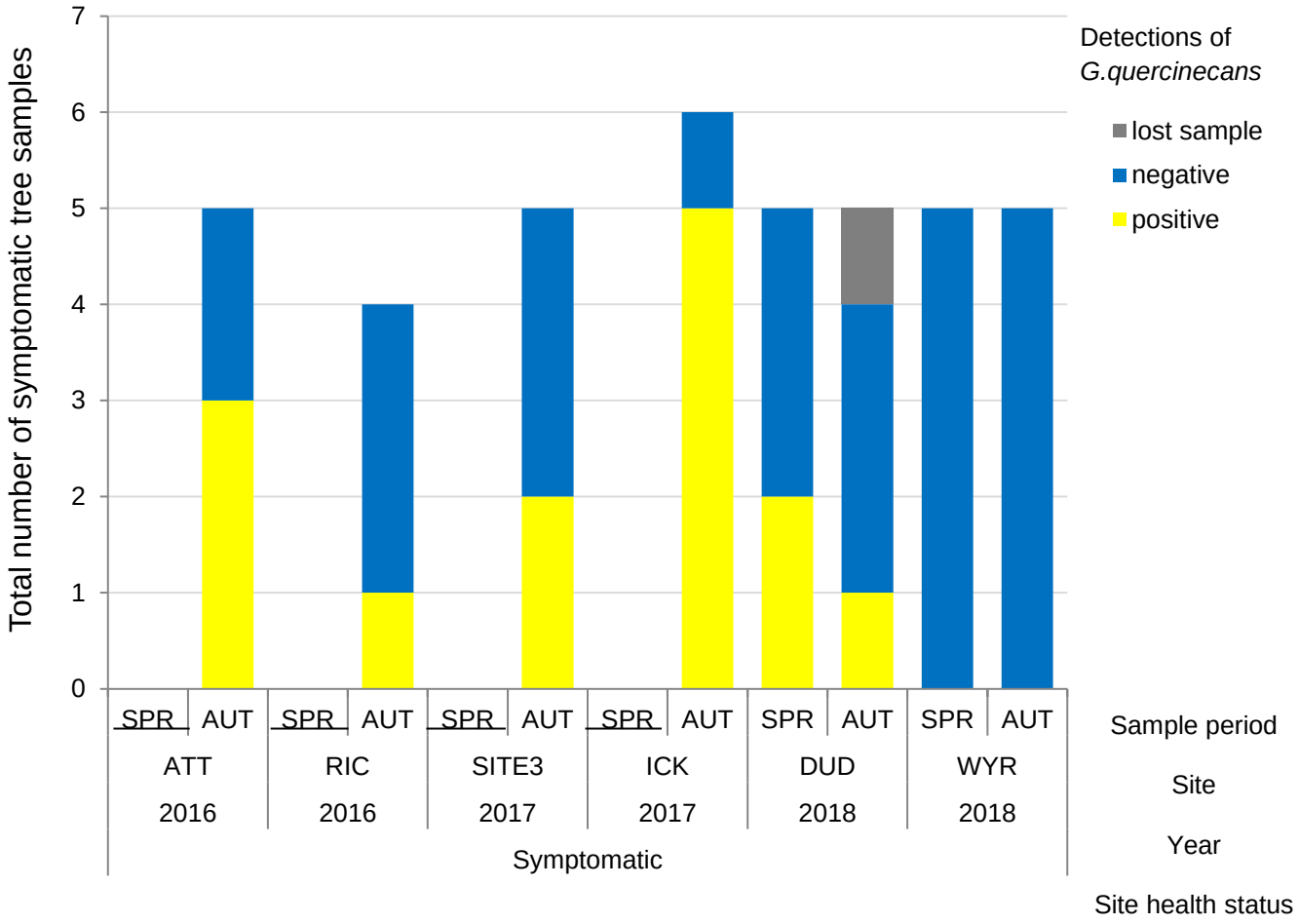


Figure 27. Positive and negative detections of *G.quercinecans* in the total number of symptomatic trees sampled across three years 2016-2018 from symptomatic sites in spring and autumn

The results presented for the overall number of trees at symptomatic site in Figures 27 and 28 do not show whether it is same tree positive and/or negative for both bacteria within the sampling period. In 2017 across both sites five trees were positive for both bacteria (1 SITE3 : 4 ICK) (Table 14, Table 15). In 2018 at site WYR no trees were positive *G.quercinecans* in either spring or autumn or *B.goodwinii* in spring with only two trees positive for *B.goodwinii* in autumn (Figure 27, Figure 28).

At the other symptomatic site (DUD) in 2018 a single tree was both positive for *B.goodwinii* and *G.quercinecans* in spring (Table 14, Table 15). A single, different tree was positive for both *B.goodwinii* and *G.quercinecans* in autumn (Table 14, Table 15)

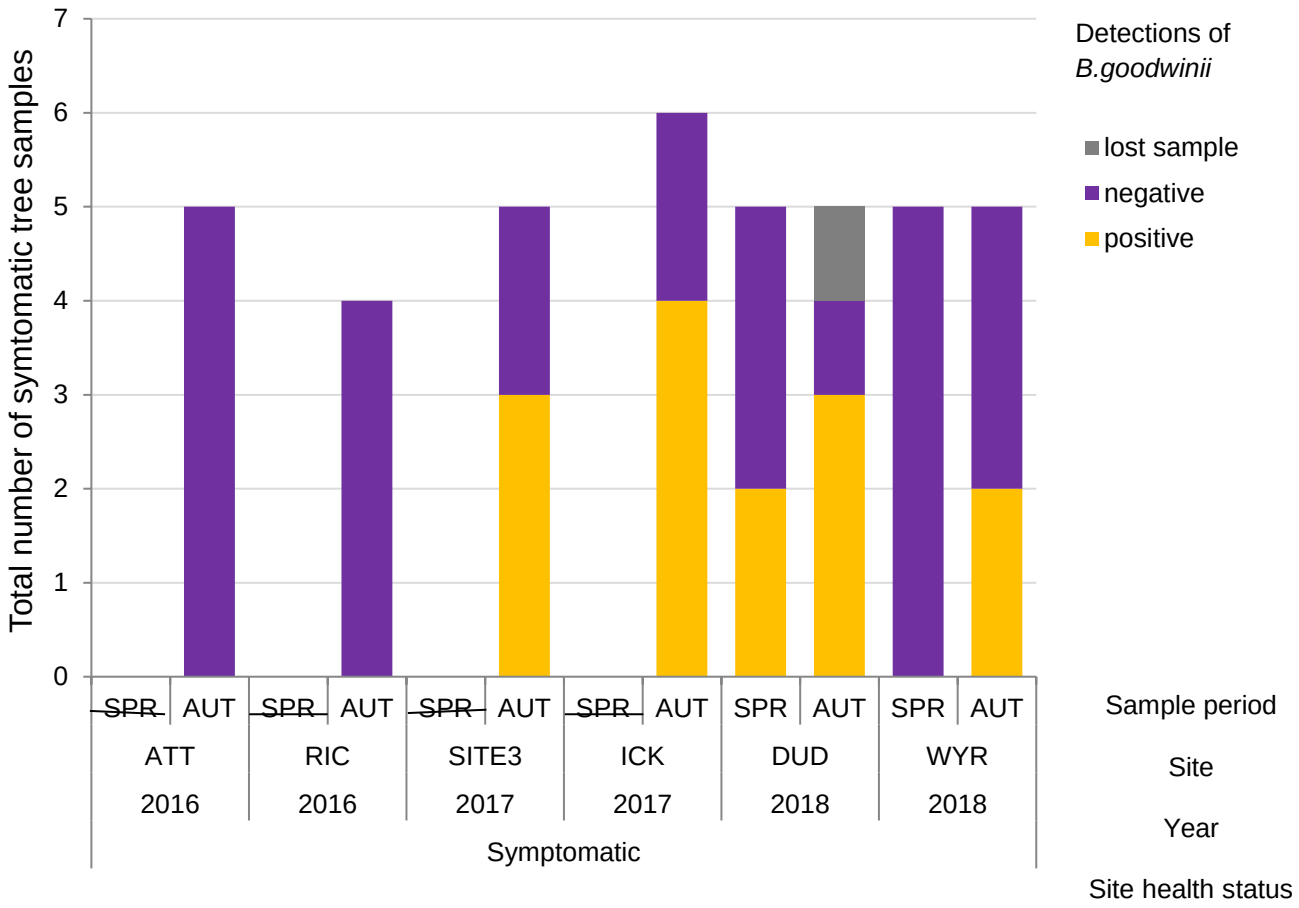


Figure 28. Positive and negative detections of *B.goodwinii* in the total number of symptomatic trees sampled across three years 2016-2018 from symptomatic sites in spring and autumn

Table 14. Positive results of the detection of *G.quercinecans* in spring and autumn leaf samples from symptomatic trees in symptomatic sites 2016-2018

Tree identification	**Sample year**	***G.quercinecans* Spring leaves**	***G.quercinecans* Autumn leaves**
RICB1	2016	not taken	positive
ATTB3		not taken	positive
ATTB4		not taken	positive
ATTB5		not taken	positive
SITE3B1	2017	not taken	positive
SITE3B2		not taken	positive
ICKB1		not taken	positive
ICKB2		not taken	positive
ICKB3		not taken	positive
ICKB5		not taken	positive
ICKB6		not taken	positive
DUDB3	2018	positive	negative
DUDB4		negative	positive
DUDB5		positive	missing sample

Table 15. Positive results of the detection of *B.goodwinii* in spring and autumn leaf samples from symptomatic trees in symptomatic sites

Tree identification	**Sample year**	***B.goodwinii* Spring leaves**	***B.goodwinii* Autumn leaves**
SITE3B1	2017	not taken	positive
SITE3B3		not taken	positive
SITE3B4		not taken	positive
ICKB2		not taken	positive
ICKB3		not taken	positive
ICKB5		not taken	positive
ICKB6		not taken	positive
DUDB1	2018	negative	positive
DUDB3		negative	positive
DUDB4		positive	positive
DUDB5		positive	missing sample
WYRB2		negative	positive
WYRB4		negative	positive

3.3.1.2 Litter

Independent of site, a higher percentage of litter samples from asymptomatic trees were positive for *G.quercinecans* (62%) compared to *B.goodwinii* (46%) (Table 11). For symptomatic trees there was a greater percentage of positive detections of *G.quercinecans* (45%) compared to *B.goodwinii* (30%) (Table 11).

In 2017 three litter samples from symptomatic trees and ten from asymptomatic trees were positive for *G.quercinecans* (Figure 29). In 2018 litter samples from ten asymptomatic and 6 symptomatic trees were positive for *G.quercinecans* (Figure 29).

Across the two symptomatic sites sampled in 2017 only one out of 18 litter samples from asymptomatic trees was positive for *B.goodwinii* (Figure 29). None of the 11 litter samples from symptomatic trees were positive for *B.goodwinii* (Figure 29). In 2018 litter samples from 13 asymptomatic and 6 symptomatic trees were positive for *B.goodwinii* (Figure 29).

In 2017 of the litter samples from symptomatic sites that tested positive *G.quercinecans* none were also positive for *B.goodwinii* (Table 16). In addition the single sample that was positive for *B.goodwinii* was negative for *G.quercinecans* (Table 16).

In 2017 all nine litter samples from the asymptomatic sites were positive for *G.quercinecans* with five also positive for *B.goodwinii* (Figure 30). In 2018 samples from four trees were positive for *G.quercinecans* (Figure 30). Of these four were also positive for B.goodwinii (Table 17).

Of the 2018 litter samples from symptomatic sites 10 from asymptomatic trees and six from symptomatic trees were positive for *G.quercinecans* (Table 16). Of these, 10 asymptomatic and five symptomatic trees were also positive for *B.goodwinii* (Table 16).

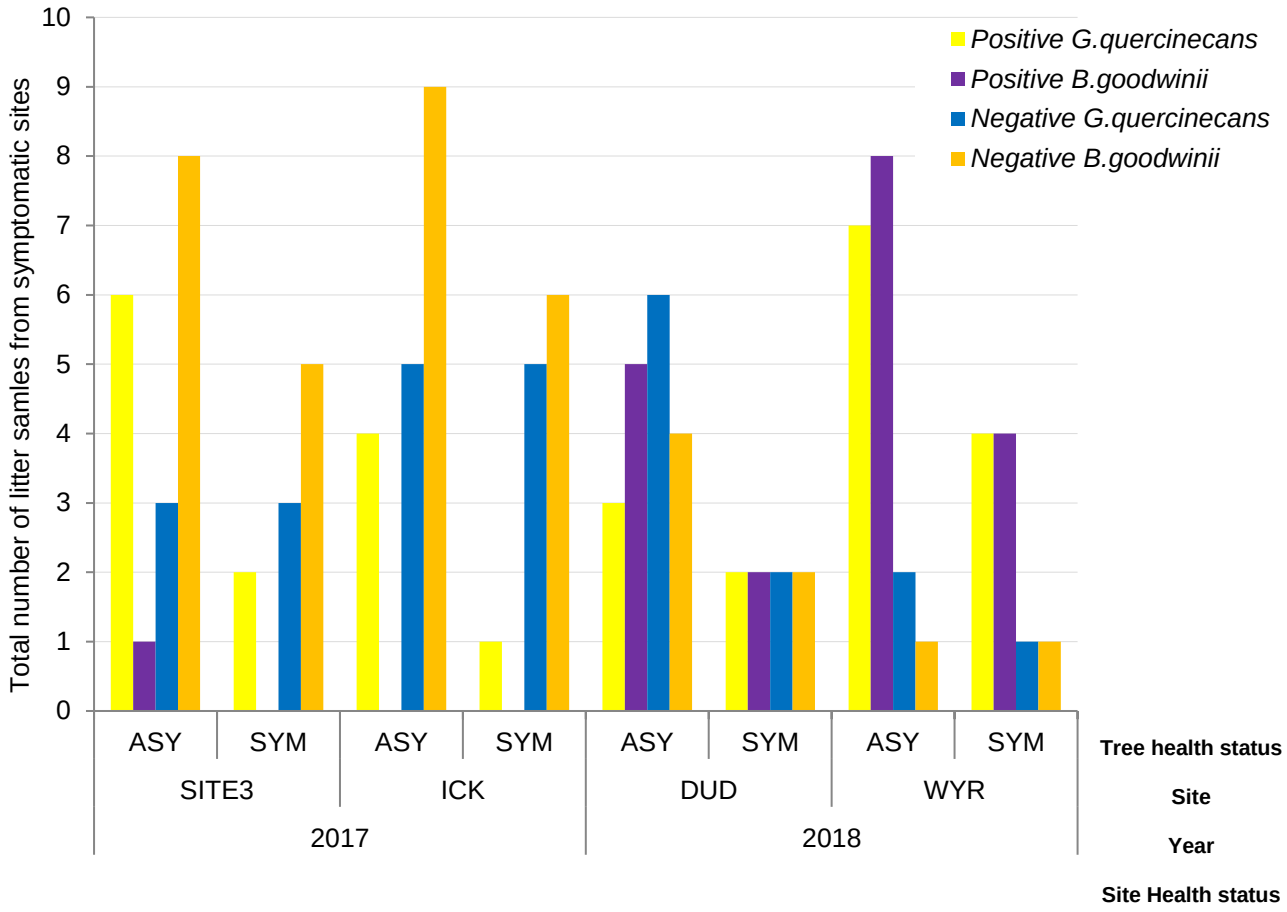


Figure 29. Positive and negative detections of *G.quercinecans* and *B.goodwinii* in the total number of litter samples from asymptomatic and symptomatic trees sampled across two years 2017-2018 from symptomatic sites in autumn

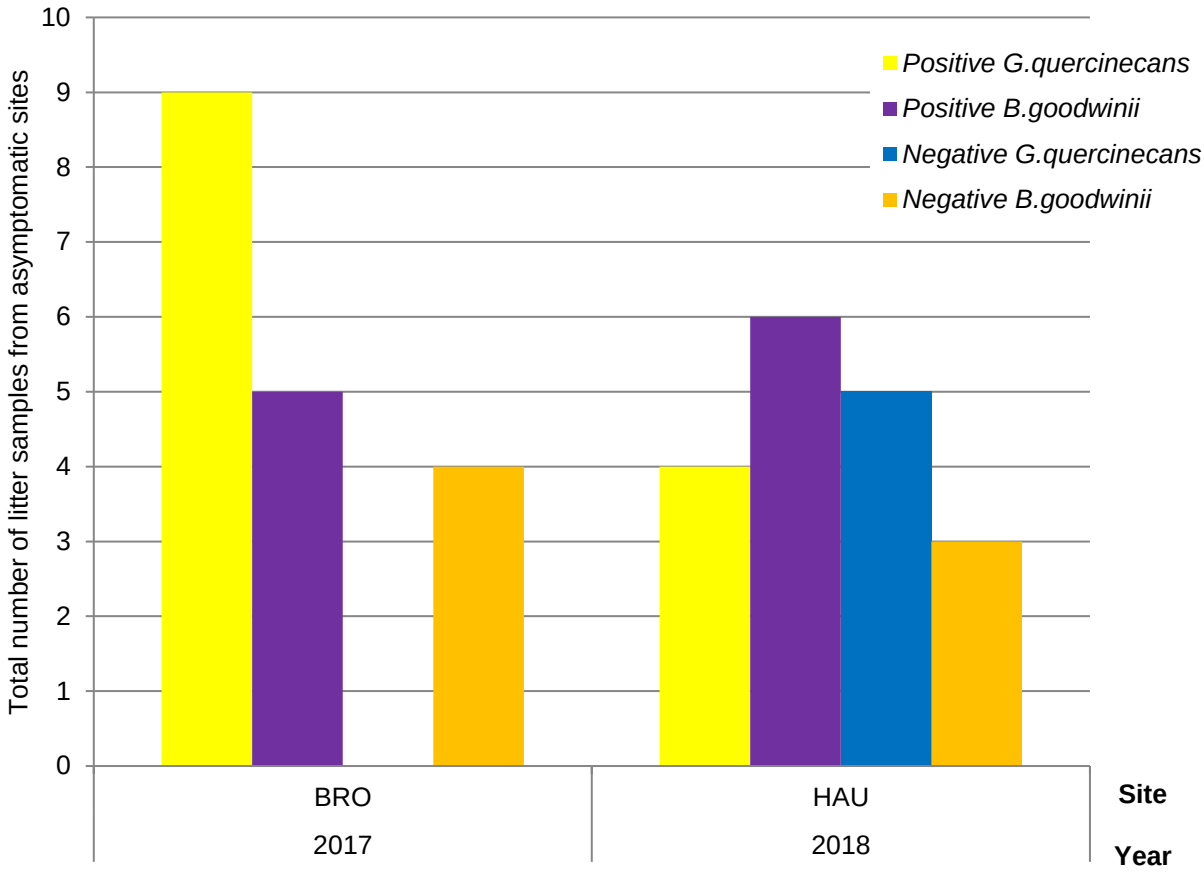


Figure 30. Positive and negative detections of *G.quercinecans* and *B.goodwinii* in the total number of litter samples from asymptomatic trees sampled across two years 2017-2018 from asymptomatic sites in autumn

Table 16. Positive results of the detection of *G.quercinecans and B.goodwinii* in litter samples from asymptomatic and symptomatic trees in symptomatic sites

Tree identification	**Tree health status**	**Sample year**	***G.quercinecans* Litter**	***B.goodwinii* Litter**
ICKB1	Symptomatic	2017	positive	negative
ICKNB3	Asymptomatic		positive	negative
ICKNB5	Asymptomatic		positive	negative
ICKNB6	Asymptomatic		positive	negative
ICKNB7	Asymptomatic		positive	negative
SITE3B1	Symptomatic		positive	negative
SITE3B5	Symptomatic		positive	negative
SITE3NB1	Asymptomatic		positive	negative
SITE3NB2	Asymptomatic		positive	negative
SITE3NB3	Asymptomatic		positive	negative
SITE3NB4	Asymptomatic		negative	positive
SITE3NB5	Asymptomatic		positive	negative
SITE3NB7	Asymptomatic		positive	negative
SITE3NB9	Asymptomatic		positive	negative
DUDNB1	Asymptomatic	2018	positive	positive
DUDNB2	Asymptomatic		negative	positive
DUDNB3	Asymptomatic		positive	positive
DUDNB4	Asymptomatic		negative	positive
DUDNB6	Asymptomatic		negative	positive
DUDNB7	Asymptomatic		positive	negative
DUDB1	Symptomatic		positive	positive
DUDB3	Symptomatic		positive	negative
DUDB4	Symptomatic		negative	positive
WYRNB1	Asymptomatic		positive	positive
WYRNB2	Asymptomatic		positive	positive
WYRNB4	Asymptomatic		positive	positive
WYRNB5	Asymptomatic		positive	positive
WYRNB6	Asymptomatic		positive	positive
WYRNB7	Asymptomatic		positive	positive
WYRNB8	Asymptomatic		positive	positive
WYRNB9	Asymptomatic		negative	positive
WYRB1	Symptomatic		positive	positive
WYRB2	Symptomatic		positive	positive
WYRB4	Symptomatic		positive	positive
WYRB5	Symptomatic		positive	positive

Table 17. Positive results of the detection of *G.quercinecans* and *B.goodwinii* in litter samples from asymptomatic sites

Tree identification	Tree health status	Sample year	*G.quercinecans* Litter	*B.goodwinii* Litter
BRONB1	Asymptomatic	2017	positive	positive
BRONB2	Asymptomatic		positive	negative
BRONB3	Asymptomatic		positive	positive
BRONB4	Asymptomatic		positive	positive
BRONB5	Asymptomatic		positive	positive
BRONB6	Asymptomatic		positive	positive
BRONB7	Asymptomatic		positive	negative
BRONB8	Asymptomatic		positive	negative
BRONB9	Asymptomatic		positive	negative
HAUNB2	Asymptomatic	2018	positive	positive
HAUNB5	Asymptomatic		positive	positive
HAUNB6	Asymptomatic		positive	positive
HAUNB7	Asymptomatic		negative	positive
HAUNB8	Asymptomatic		negative	positive
HAUNB9	Asymptomatic		positive	positive

3.3.1.3 Acorns

In 2016 acorns were collected from two symptomatic sites and in 2017 from one site (Figure 31, Table 18). Acorns were collected from asymptomatic and symptomatic trees with the exception of one site in 2016 where no acorns were collected from asymptomatic trees. Catkins were collected from a single symptomatic site in 2017 (Table 19). In 2016 one acorn sample from an asymptomatic tree was positive for *G.quercinecans* (Figure 31). The same sample was also positive for *B.goodwinii* (Table 18).

In 2017 there was a positive detection of *G.quercinecans* from the inner acorn tissue of two symptomatic and one asymptomatic tree (Figure 31). The outer acorn tissue of four asymptomatic and two symptomatic samples were positive for *G.quercinecans* (Figure 31). *Brenneria goodwinii* was detected in the inner acorn tissue of two asymptomatic and two symptomatic samples (Figure 31). Two asymptomatic and one symptomatic outer acorn samples were positive for *B.goodwinii* (Figure 31).

There were detections of both bacteria in two symptomatic inner acorn samples (Table 18). Of these one sample of outer acorn tissue was also positive for *G.quercinecans*; although it was negative for *B.goodwinii* (Table 18). Two inner acorn samples from asymptomatic trees were positive for both bacteria (Table 18). The inner and outer acorn tissue from one asymptomatic tree was positive for both bacteria (Table 18).

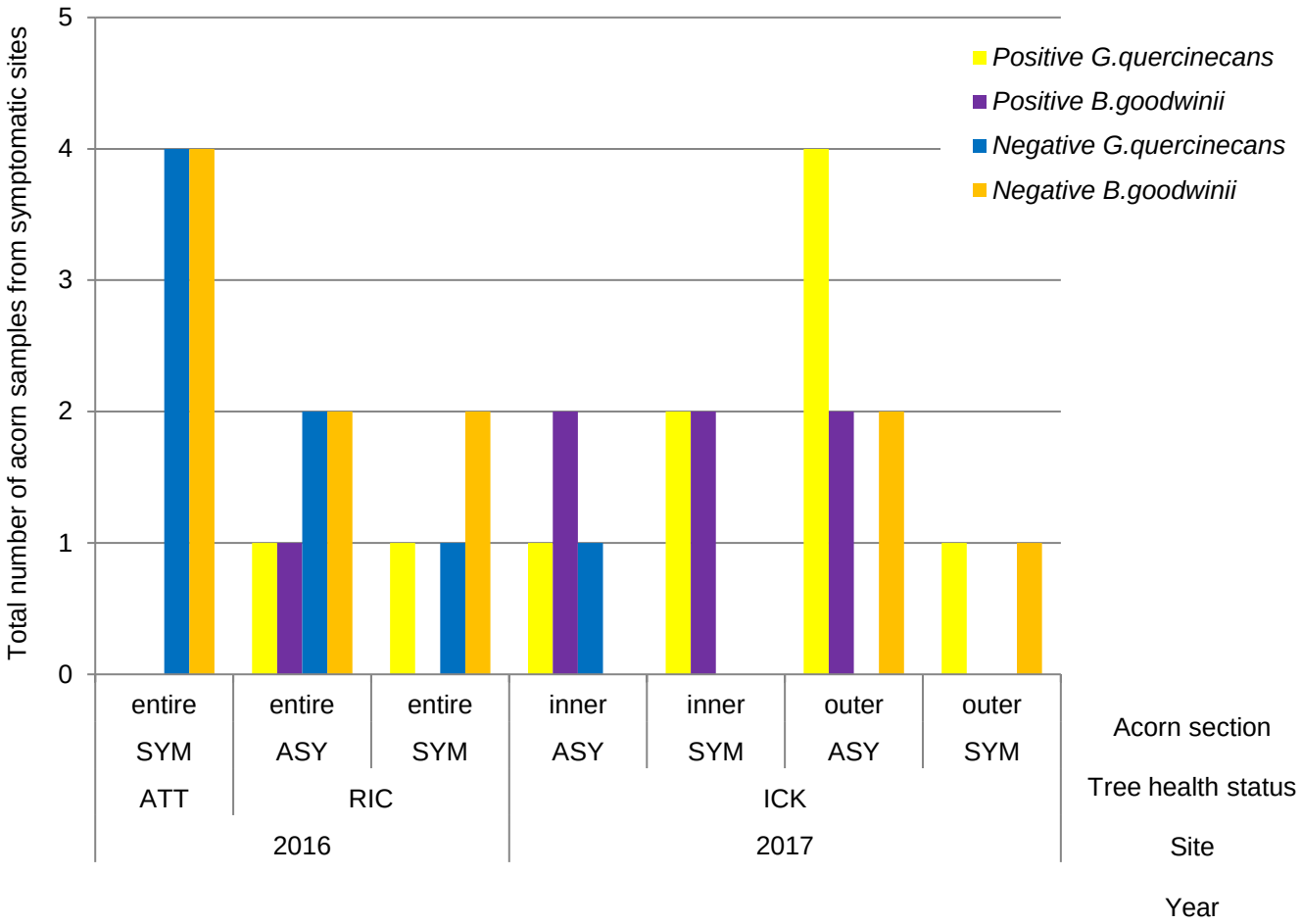


Figure 31. Positive and negative detections of *G.quercinecans* and *B.goodwinii* in the total number of acorn samples from asymptomatic and symptomatic trees sampled across two years 2016-2017 from symptomatic sites in autumn

Table 18. Acorn samples taken from symptomatic and asymptomatic trees in 2016 at AOD symptomatic sites with a positive detection *G.quercinecans* or *B.goodwinii*

Tree identification	Tree health status	Sample year	Acorn section	*G.quercinecans*	*B.goodwinii*
RICB3	Symptomatic	2016	Acorn (entire)	positive	negative
RICNB4	Asymptomatic			positive	positive
ICKB1	Symptomatic	2017	Acorn (inner)	positive	positive
ICKB6	Symptomatic			positive	positive
ICKNB2	Asymptomatic			positive	positive
ICKNB8	Asymptomatic			negative	positive
ICKB6	Symptomatic		Acorn (outer)	positive	negative
ICKNB2	Asymptomatic			positive	positive
ICKNB5	Asymptomatic			positive	negative
ICKNB6	Asymptomatic			positive	negative
ICKNB8	Asymptomatic			positive	positive

3.3.1.4 Catkins

Catkin samples from seven asymptomatic trees in 2017 were positive for *G.quercinecans* and six for *B.goodwinii* (Table 19). Both bacteria were detected in four catkin samples from asymptomatic trees and one sample from a symptomatic tree (Table 19).

Table 19. Catkin samples with a positive detection *G.quercinecans* and/or *B.goodwinii* taken from symptomatic and asymptomatic trees in 2017 from a single symptomatic site

Tree identification	Tree health status	*G.quercinecans*	*B.goodwinii*
ICKNB1	Asymptomatic	positive	positive
ICKNB2	Asymptomatic	positive	positive
ICKNB3	Asymptomatic	positive	negative
ICKNB4	Asymptomatic	positive	negative
ICKNB5	Asymptomatic	positive	positive
ICKNB7	Asymptomatic	positive	positive
ICKNB9	Asymptomatic	negative	positive
ICKNB10	Asymptomatic	positive	negative
ICKB1	Symptomatic	positive	negative
ICKB2	Symptomatic	positive	positive

3.3.2 Field investigation into the presence of *G.quercinecans* and *B.goodwinii* on leaves via culture of washings from leaves and litter

Two symptomatic and two asymptomatic leaf wash samples from the single symptomatic site (WYR) were positive for *G.quercinecans* (Table 20). Three symptomatic and four asymptomatic leaf was samples were positive for *B.goodwinii* (Table 20). Two asymptomatic and one symptomatic leaf wash sample were positive for both bacteria (Table 19). Washed symptomatic tree litter was positive for both bacteria however that from asymptomatic trees was only positive for *B.goodwinii* (Table 20).

Table 20. All washed leaf samples with positive and/or negative detections of *G.quercinecans* and/or *B.goodwinii* taken from symptomatic and asymptomatic trees in 2018 from a single symptomatic site (WYR)

Tree Identification	Tree health status	Sample type	*G.quercinecans*	*B.goodwinii*
WASHWB1	Symptomatic	Washed leaves	positive	positive
WASHWB2	Symptomatic		negative	positive
WASHWB3	Symptomatic		positive	negative
WASHWB4	Symptomatic		negative	positive
WASHWNB1	Asymptomatic		positive	positive
WASHWNB2	Asymptomatic		negative	positive
WASHWNB3	Asymptomatic		positive	positive
WASHWNB4	Asymptomatic		negative	positive
WASHWBLi	Symptomatic	Washed litter	positive	positive
WASHWNBLi	Asymptomatic		negative	positive

3.3.3 Glasshouse investigation into the survival of *G.quercinecans* and *B.goodwinii* on leaves after inoculation

Control trees had no detection of *G.quercinecans,* however two samples were positive for *B.goodwinii* (Table 21). Where leaves were inoculated with a combination of *G.quercinecans* and *B.goodwinii* no samples at any dilution factor were positive for *G.quercinecans*, whereas *B.goodwinii* was detected in all three samples of the highest dilution factor (Table 22).

There was no detection of *G.quercinecans* at any dilution factor on the surfaces of the inoculated oak sapling leaves (Table 23). Conversely *B.goodwinii* was detected on the leaf surface at all three applied dilution factors (Table 24).

Table 21. Positive detections of *G.quercinecans* and *B.goodwinii* on the surfaces of control oak leaf saplings inoculated with sterile distilled water

Tree identification	***G.quercinecans***	***B.goodwinii***
EXPCONTROL1	negative	negative
EXPCONTROL3	negative	positive
EXPCONTROL7	negative	positive

Table 22. Positive detections of *G.quercinecans* and/or *B.goodwinii* on the surfaces of oak leaf saplings inoculated with combination of three dilutions of *G.quercinecans* and *B.goodwinii*

Tree identification/dilution factor	***G.quercinecans***	***B.goodwinii***
Gq/Bg10-2 Tree4	negative	positive
Gq/Bg10-2 Tree5	negative	positive
Gq/Bg10-2 Tree3	negative	positive
Gq/Bg10-3 Tree7	negative	negative
Gq/Bg10-3 Tree1	negative	negative
Gq/Bg10-3 Tree6	negative	positive
Gq/Bg10-4 Tree1	negative	negative
Gq/Bg10-4 Tree7	negative	negative
Gq/Bg10-4 Tree6	negative	negative

Table 23. Positive detections of *G.quercinecan*s on the surfaces of oak leaf saplings inoculated with combination of three dilutions of *G.quercinecans*

Tree identification/dilution factor	***G.quercinecans***
GQ10-2 Tree7	negative
GQ10-2 Tree6	negative
GQ10-3 Tree 6	negative
GQ10-3 Tree 4	negative
GQ10-3 Tree 1	negative
GQ10-4 Tree 1	negative
GQ10-4 Tree 2	negative
GQ10-4 Tree 5	negative

Table 24. Positive detections of *B.goodwinii* on the surfaces of oak leaf saplings inoculated with combination of three dilutions of *B.goodwinii*

Tree identification/dilution factor	***B.goodwinii***
BG10-2 Tree 3	positive
BG10-2 Tree7	positive
BG10-2 Tree 2	positive
BG10-3 Tree 4	positive
BG10-3 Tree 1	positive
BG10-4 Tree 5	negative
BG10-4 Tree 6	positive
BG10-4 Tree 5	positive

3.4 Discussion

The aim of this chapter was to investigate whether there is an association between the oak phyllosphere and two bacteria associated with Acute Oak Decline; *B.goodwinii* and *G.quercinecans.* Their detection on live oak leaves, litter, acorns and catkins are new findings and are newly revealed by this study to exist outside of the stem bleeds. Evidence that they can occur either on or within the tissues of both symptomatic and asymptomatic trees is also a new discovery. These results support the suggestion that *G.quercinecans* and *B.goodwinii* have an association with the oak phyllosphere.

Differences in the number of positive detections in live leaves were observed between asymptomatic and symptomatic sites. At asymptomatic sites a greater number of spring and autumn samples were positive for *B.goodwinii* compared to *G.quercinecans*. At symptomatic sites, regardless of tree health status, samples had a similar number of positive detections for each bacteria in both spring and autumn leaf samples. When tree health status is taken into account at symptomatic sites, asymptomatic trees, with the exception of *G.quercinecans* in autumn leaves, had a higher percentage number of positive detections of both bacteria in all sample types.

There were differences in the percentage of positive detections in the leaves of asymptomatic trees (regardless of site). Asymptomatic trees regardless of site had a high percentage (58%) of positive detections of *B.goodwinii* in spring leaf samples followed by a consistent detection in the autumn (45%) and litter samples (46%). In contrast the percentage of positive detections of *G.quercinecans* rose with increasing leaf age from spring leaves (35%) to autumn leaves (46%) to litter (62%).

These results could indicate differences in prevalence of the two bacteria. It may be that *B.goodwinii* is the more widely occurring bacteria within the oak phyllosphere, accounting for its greater detection. The relatively constant detection of *B.goodwinii* at symptomatic sites and on asymptomatic trees regardless of site supports this. Detection of *B.goodwinii* but not *G.quercinecans* in asymptomatic bark tissue on AOD sites (Sapp *et al.,* 2016; Denman *et al*., 2016) also adds to this idea. It is however in contrast to Meaden *et al*. (2016) where no *Brenneria* sequences were identified in microcores of health *Q.robur* trees on a non AOD sites. This suggests that additional factors influence the presence of *B.goodwinii* on bark and potentially other oak tissues, such as an already declining tree or abiotic conditions.

At individual sites there were interesting patterns to be observed. At one asymptomatic site in 2017 (BRO) for example eight out of the nine trees sampled were negative in spring leaves for *G.quercinecans* however all nine were positive for this bacteria in litter. In 2018 at

one symptomatic site (WYR) no spring leaf samples were positive for *B.goodwinii* or *G.quercinecans* however both were detected in litter samples. Environmental factors are known to affect the presence of bacteria within the phyllosphere (Laforest-Lapointe *et al.,* 2016). Oak bacterial communities can also vary over geographic distance (Sapp *et al*., 2016). The results could therefore indicate site dependent abiotic effects on the appearance of *B.goodwinii* and *G.quercinecans*. Differences in temperature, moisture or nutrients could all influence the colonisation and establishment of both bacteria at the site level.

Soil water level has been shown to be a key factor influencing the likelihood of AOD being present on a site (Brown *et al.,* 2018). A high water content in leaf intercellular spaces may also suppress plant defences in response to pathogen secreted effector proteins (Beattie 2011). Experiments with *Xanthomamonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* indicate that low leaf water content may inhibit pathogen growth (Beattie 2011). Lower rainfall, clay rich and seasonally waterlogged soil were all found to be more prevalent on AOD sites (Brown *et al.,* 2018). Sites with a high level of soil nitrogen, a potential indicator of low tree health was also more likely to be higher at sites with AOD (Brown *et al.,* 2018).

It has been suggested that there is an interplay between the two bacteria and that *G.quercinecans* is the primary coloniser creating conditions for *B.goodwinii* (Doonan *et al*., 2019). When all samples from asymptomatic and symptomatic trees are considered together regardless of site there was no clear evidence that a positive detection of either bacteria in a spring leaf sample led to any subsequent detections of another in an autumn leaf sample. Indeed only one tree from an asymptomatic site was positive in both spring and autumn for both bacteria. This indicates a lack of evidence for interaction between *B.goodwinii* and *G.quercinecans* within leaves. Sample size, particularly for the spring samples, was small so the results are not conclusive.

Both bacteria were present in the leaf washings of symptomatic and asymptomatic trees at the symptomatic site (WYR). This implies that they do not specifically require a declining host. It also supports the suggestion that both bacteria can be non-pathogenic in the oak phyllosphere and that it is external factors which induce pathenogenicity. With the exception of a single symptomatic tree all leaf washing samples were positive for *B.goodwinii;* positive detections of *G.quercinecans* were not consistent.

It may be that *B.goodwinii* is more widespread than *G.quercinecans* and so had a higher detection rate. This further adds to the evidence from the leaf analysis where *B.goodwinii* was detected in a higher overall number of samples. Alternatively this may be evidence that

B.goodwinii is primarily an epiphyte and *G.quercinecans* primarily an endophyte. That *B.goodwinii* has the ability to exist as an epiphyte has been mentioned previously (Denman *et al*., 2018). A number of virulence factors have been identified in non-pathogenic bacteria and may be involved in host interactions unrelated to pathenogenicity (Niu *et al*., 2013). Both species may be opportunistic pathogens, becoming virulent in response to abiotic factors or changes to a host. The detection of *B.goodwinii* in asymptomatic bark from an AOD site (Sapp *et al*., 2016) may be additional evidence other factors are required to induce virulence.

Bacteria that can survive through gaps in host availability have an advantage over those that cannot (Allen *et al*., 2009). The detection of both bacteria in litter samples suggests they could have an overwintering life stage. Exudate appears from lesions during spring which could be a hypersensitive response triggered by bacterial presence. Additionally oak host defences have been shown to be triggered by the presence of both bacteria in lesions (Broberg *et al.,* 2018). The absence of exudate appearing from bleeds in winter (Denman *et al.,* 2014) is supportive of a period of reduced activity and may point to the use of litter as an alternative resource.

There was a greater number of positive detections of *B.goodwinii* in the litter washings at the symptomatic site (WYR). These show that *B.goodwinii* was detected alive in the litter from under symptomatic and asymptomatic trees whereas *G.quercinecans* was only detected in the litter from under symptomatic trees. This is interesting as *B.goodwinii* is considered the more pathogenic and is closely aligned with necrogenic phytopathogens (Doonan *et al*., 2019). It may be that *B.goodwinii* is more adapted to utilising litter as an alternative resource and so detection over time is relatively consistent. The lower pathogenic potential of *G.quercinecans* (Doonan *et al*., 2019) or a need for more time to become established could have resulted in the apparent increase in detection over time. The presence of a T3SS secretion system in *B.goodwinii* and not in *G.quercinecans* (Doonan *et al*., 2019) further supports the idea that host defences need to be compromised before *G.quercinecans* is able to effectively colonise. Alternatively, the results from leaves and litter may simply indicate differences in activity or abundance and therefore likelihood of detection.

The implications that *G.quercinecans* is an endophyte bacteria and *B.goodwinii* an epiphyte is further supported by evidence from the inoculation trial. Results suggests that whilst *B.goodwinii* can survive on the surfaces of leaves, *G.quercinecans* cannot. The negative results of *G.quercinecans* could be due to unviable colonies however previous attempts at serial dilutions had showed still viable colonies after the same time period. Another explanation could be that whilst *B.goodwinii* may not have entered the leaf at the point of sampling, *G.quercinecans* had indicating they both may be able to switch between lifestyles.

Saplings may have not been left for long enough after inoculation to look at independent survival from any lingering growth media. *Brenneria goodwinii* was also detected on control trees, which may indicate its presence already in oak tissues before inoculation.

At the single symptomatic site (ICK) there was evidence that trees with catkins that were positive for *B.goodwinii* or *G.quercinecans* later had a positive detection within an acorn. This may indicate a continuum of colonisation from an initial presence on a catkin although a prior detection on a spring leaf was not necessarily positive.

Differences in the number of detections on the various tissues may indicate niche partitioning or that there are differences in the ability of the bacteria to colonise different tissues. Niche partitioning may further explain the greater presence of *G.quercinecans* on acorns and catkins. This may be evidence to suggest that the bacteria are acting in different ways and are potentially adapted to different oak tissue types. The increasing number of detections of *G.quercinecans* in asymptomatic trees as leaf age increases is suggestive of this bacteria requiring a greater level of leaf senescence before colonisation can take place. Where the bacteria are detected is an important aspect in understanding how the bacteria act. This may point towards whether they are epiphytes or endophytes or able to switch between both lifestyles and influence any future attempts at control or prevention. Bacteria are known to utilise existing openings such as stomata or wounds to enter into a hosts tissue and that they can be transferred from flowers to seeds (Frank *et al.,* 2017). The results presented here hint that these may be potential colonisation roots for *B.goodwinii* and *G.quercinecans*.

The presence of *G.quercinecans* and *B.goodwinii* at asymptomatic and symptomatic sites challenges the idea that these bacteria are specific to oak. This discovery hints at the possibility the bacteria may be present in the wider woodland environment and as Ottesen *et al. (*2016) and Telias *et al. (*2011) have suggested are managing to establish themselves on oak trees. The findings were across both sites which suggests local abiotic factors were influential and that the presence of the bacteria on these tissues was further due to the suitability of the tissue itself for colonisation.

Considered a causal agent of stem bleeds (Brady *et al.,* 2010; Brady *et al.,* 2017) a management approach for is likely to be challenging if both bacteria are widespread in the environment and can exist on multiple types of oak tissue. In addition their presence on external tissues indicates indirect transmission via wind and water could be possible. Direct or indirect movement of bacteria by vectors is also a possibility. Management

may therefore be better directed at improving overall tree and site health for example managing soil water conditions since this appears to be a primary factor in AOD.

An association of *A.biguttatus* with AOD has been suggested in several papers e.g. Denman *et al.,* 2014; Denman *et al.,* 2018, but, as previously discussed there has been no evidence to support its implied status as a vector. The evidence presented here of the presence of both *B.goodwinii* and *G.quercinecans* on a variety of oak tissues brings this assumption further into question. With detections on leaves, acorns, litter and catkins a number of movement pathways are possible. Two of those possible, vectoring by any potential insects and rainsplash, are discussed further in Chapters 4 and 5 respectively.

3.5 Conclusion

The recent rise in oak decline in Britain and in Europe is a cause for concern. The identification of Acute Oak Decline has led to questions over its epidemiology and spread both within sites and across the country. One of these key questions is the origin of the two bacteria associated with bleeds and the means by which they colonise oak tissues. This study has demonstrated the presence of the bacteria in asymptomatic and symptomatic sites and on the live oak leaves, litter, acorns and catkins of asymptomatic and symptomatic trees. These findings indicate a likely widespread presence of both bacteria in the woodland environment and offer an explanation as to their entry routes and movement pathway to new hosts.

Chapter 4

Investigating the relationship between xylophagous Coleoptera and two bacteria associated with Acute Oak Decline; *B.goodwinii* and *G.quercinecans*

4.1 Introduction

Insect-bacteria interactions can be defined as commensalism, mutualism or parasitism. In commensalism one organism benefits whilst the other is neither positively nor negatively impacted. Mutualism benefits both organisms and parasitism benefits one to the detriment of the other. Inherited symbionts may be obligate, unable to exist without each other, or facultative, choosing to exist together (Engel and Moran, 2013). There is further a differentiation to be made between permanent or indigenous (autochonous) bacteria of an insect microbial community and those that are transient (allochthonous) and ingested via food intake or acquired from the environment (Dillon and Dillon, 2004).

Many insects have evolved to accommodate microorganisms such as protists, fungi, archaea, and bacteria within their digestive system (Engel and Moran, 2013). The role of bacteria found within intestinal organs can be to aid and improve food digestion through the provision of nutrients or digestive enzymes (Dillon and Dillon, 2004; Douglas, 2009; Klepzig *et al.,* 2009; Engel and Moran, 2013). Bacteria can aid in the digestion of lignin and cellulose through carbohydrate fermentation, nitrogen fixing (Rizzi *et al.,* 2013) and via the production of amino acids and vitamins (Dillon and Dillon, 2004). Other beneficial associations include bacteria providing resistance to pathogens (Eleftherianos *et al.,* 2013), resistance to parasites and aiding in defence against predation (Engel and Moran, 2013).

The presence of indigenous bacteria is unknown for most species as is the function of the bacteria themselves (Dillon and Dillon, 2004). Microorganisms present within the guts of insects have also been found to be widespread within the environment (Engel and Moran, 2013). Rather than being transferred between individuals or between generations this suggests many are obtained from the environment and colonisation of insects by bacteria may be more opportunistic (Engel and Moran, 2013). This has led to the proposal by Engel and Moran (2013) that many bacteria may be ingested and only those required by the insect retained. Alternatively it may be only those bacteria able to colonise successfully that remain (Orlovskis *et al.,* 2015).

Discussed in section 1.2.3.1 insects can have associations with phytopathogens. Bacteria acquired via feeding can be spread via 'circulative propogative' (bacteria acquired replicate within the insect) or 'circulative non-propogative' (bacteria acquired do not replicate within the insect) means Orlovskis *et al*. (2015).

Many microorganisms are unable to automatically colonize the gut and may only become established when circumstances are favourable (Engel and Moran, 2013). Colonisation success can be determined by pH or influenced by gut structure or nutrients (Engel and Moran, 2013). Bacteria, through quorum sensing, respond to population density; accordingly regulating gene expression (Bassler, 1999). These modifications can in turn determine which microorganisms are present and may limit the niches which the insect can exploit (Engel and Moran, 2013). Insects may conversely impact nutrient cycling by influencing bacterial colonisation; damaged oak leaves for example had more bacterial isolates able to utilize ammonia and nitrate than those on damaged beech leaves (Müller *et al.,* 2003).

A number of obstacles to the study of the insect microbiome have been expressed in a review by Dillon and Dillon (2004). They highlight the difficulty of laboratory studies in replicating interactions that may be present in reality and difficulties in distinguishing between permanent or transient microorganisms (Dillon and Dillon, 2004). An additional implication from Dillon and Dillon (2004) was that the functional ability of a bacterial community is independent of its composition. A community can therefore appear stable since those bacteria required by the insect are both part of its physiology as well as acquired transiently when needed from the environment.

4.1.1 Bacteria and associations with xylophagous insects

The presence of stem lesions and tissue necrosis symptomatic of Acute Oak Decline has led to suggestions a xylophagous (wood boring) insect may be involved (Denman *et al.,* 2014; Denman *et al.,* 2018). Species whose adults or larvae which burrow into wood are a potential contributing factor to decline diseases and as discussed in section 1.1.

A meta-analysis of 62 insect species reported two classes of bacteria, Proteobacteria (57.4%) and Firmicutes (21.7%), as the main components (Colman *et al.,* 2012). Although the bacterial communities of few individual xylophagous species have been studied, Proteobacteria also accounted for 97.8% of the community across all life stages of the bark beetle *Dendroctonus rhizophagus* (Briones-Roblero *et al.,* 2017). Bacteria within the *Enterobacteriaceae* were however found to be the primary constituents of the gut bacteria of *Anoplophora chinensis* (citrus long-horn); an invasive pest in Europe whose larvae feed on tree cambium, phloem and xylem (Rizzi *et al.,* 2013).

There are indications that variation in bacterial communities exists between different xylophagous insects and there have also been observed differences between individuals of the same species (Engel and Moran, 2013). When cultured, the gut bacterial community of *Ips pini* (pine engraver) was low in species richness, consisting of only six bacterial genera (Delalibera Jr. *et al.,* 2007). In comparison a higher species richness was found in a study of various life stages of the bark beetle *D.rhizophagus* with 23 genera identified via 454 pyrosequencing of 16S rRNA. The genera were however dominated by *Rahnella* species which accounted for 91 percent (Briones-Roblero *et al.,* 2017).

Diet may be an important influence on the composition of the community present (Colman *et al.,* 2012). In a meta-analysis of 62 insects their diet was significantly linked to their gut bacterial communities (Colman *et al.,* 2012). For xylophagous insects this study showed that species with a dead wood habitat had higher average levels of operational taxonomic units[6] than those with a live wood habitat (Colman *et al.,* 2012). This could therefore potentially be a reflection of the habitat and the bacteria necessary to utilise a dead wood resource.

Host plant may influence the bacterial communities found and gut bacteria may also mean insects have the capacity to exist on a host that is of poorer quality (Dillon and Dillon, 2004). Adult *A.chinensis* fed on *Alnus* and *Acer* had different gut bacterial communities to larvae fed on *Liquidambar* and *Salix* (Rizzi *et al.,* 2013). A similar change in diversity was found for *A.glabripennis* (Asian long-horn) larvae reared on different hosts suggesting an explanation for the capacity of *Anoplophora* to exploit a range of hosts (Geib *et al.,* 2009). An ability to adapt to a host is advantageous in generalist feeders, allowing unrestricted availability of food. In the case of invasive insects there is the potential for this to pose a greater threat to the invaded plant community.

As insects go through developmental stages bacteria can be lost during transformations and there is not a clear indication in many cases of how, or indeed whether, bacteria can be transferred from female to egg or between adults or larvae (Engel and Moran, 2013). Often insect adult and larval stages have different diets so it may be possible that at different life stages the bacteria change to suit the food source (Duan *et al.,* 2015). When looking at differences in gene expression between *A.planipennis* moulting larvae and during metamorphosis Duan *et al*. (2015) identified an enzyme that expressed 22 times higher in adult mid guts compared to larva. This enzyme is involved in lignin digestion and the concentration therefore may reflect the different diets of larvae and adult (Duan *et al.,* 2015).

[6]Operational Taxonomic Units (OTU's) are short sequences of 16S RNA used to characterise bacterial species based on the similarity of the sequence.

4.1.2 *Agrilus biguttatus* and its connection to oak

As mentioned in section 1.1 several studies have implied an association between Acute Oak Decline and *A.biguttatus.* The UK reports of AOD (Figure 32) and the geographical distribution of *A.biguttatus* (Figure 33) cover a similar range and the high level of association has led to concerns it could be a potential factor in the disease (Brown *et al.,* 2015). *A.biguttatus* has been implicated as having a causal role (Denman *et al.,* 2018) however there is no evidence to date to support this or identify it as a vector.

It is possible to make a visual comparison between the geographical distribution of AOD and other insects with oak as a host (Figure 33). As with *A.biguttatus* the apparent correlation without evidence does not indicate a causal role and the distribution may match habitat requirements of these insects.

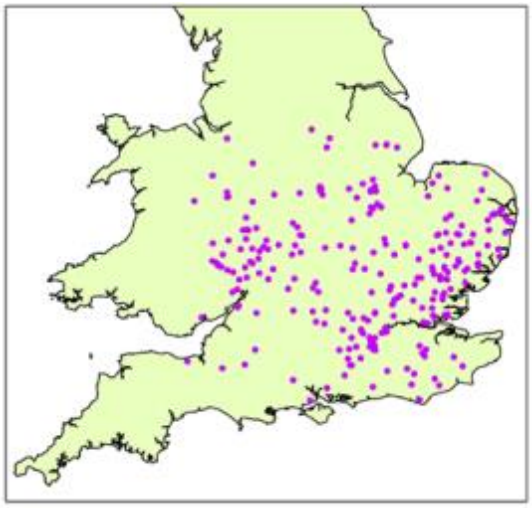


Figure 32. Distribution of positive sites to March 2016 (Forestry Commission, 2016)

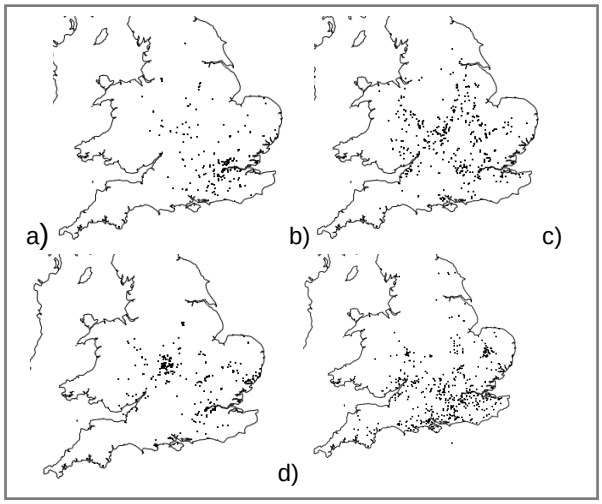


Figure 33. Distribution comparison of a) *A.biguttatus* b) *A.mysticus* c) *C.glandium* and d) *S.melanura* (NBN, 2016)

There are 3,069 *Agrilus* species and subspecies recorded worldwide, only 22 percent of which have a recorded plant association (Jendek and Poláková, 2014). Six species of *Agrilus* are found in the UK which have associations with several different plant species (Jendek and Poláková, 2014). These are summarised in Table 25 with full details in Appendix 1, Table 1. *Agrilus biguttatus* is a native UK species associated with ancient and declining oaks. Females are thought to seek out bark crevices in which to lay their eggs and after hatching the larvae bore into the bark and develop within the vascular tissue (Brown *et al.,* 2014).

Adults upon emerging are assumed to move up to the canopy to feed and mate. Based on observations *Agrilus* species are believed to use visual cues to locate ovipositing sites, food plants and for mating (Domingue *et al.,* 2011). Adults of *A.biguttatus* have however also been shown in laboratory experiments to respond to the volatile organic compounds (VOC's) of the leaves and bark of oak (Vuts *et al.,* 2016).

Table 25. Summary of host plant associations of six *Agrilus* species found in the UK as reported in Jendek and Poláková (2014). See Appendix 1, Table 1 for full details

Species	Summary of host plant associations
A.angustulus	Records of larva and adults on *Castenea* (Sweet chestnut), *Corylus* (Hazel), Fagus (Beech) and *Quercus* species.
A.biguttatus	The primary larval host are *Quercus* species. Larvae have also been reared from *Castenea* and *Fagus* species.
A.cuprescens	*Rosa* and *Rubus* (*Rosaceae*) are the primary larval host plant.
A.cyanescens	*Lonicera* species are the primary larval host plants. Adults have also been recorded on other *Caprifoliaceae*.
A.laticornis	Primary larval hosts are species of *Quercus*. Larvae have also been reared from *Castenea* species. Associations are also reported on *Corylus* and *Rosa*.
A.sinuatus	Larvae develop in various species in the *Rosaceae*.
A.sulcicollis	Primary larval host are *Quercus* species. Larvae have also been found in species of *Fagus* and *Castenea*.
A.viridis	Associations reported on *Acer*, *Alnus* (Alder), *Betula* (Birch), *Fagus* and *Salix* (Willow) species.

The attraction of a specialist insect to its specific host is to be expected however whether individuals are more attracted to infected or weakened trees or what may attract individuals to infected trees has not yet been established (Vuts *et al.,* 2016). There are indications that leaf loss has to reach a certain threshold before *A.biguttatus* move in with exit holes only observed for trees with over 90 percent leaf loss (Vansteenkiste *et al.,* 2004). Canopy condition may therefore be important to *A.biguttatus* when selecting hosts or it may be that the condition of the canopy is a reflection of the health of the tree. A percentage leaf loss over 90 per cent indicates a severely weakened tree and the presence of *A.biguttatus* at this point is suggestive of a secondary pest (Vansteenkiste *et al.,* 2004).

For another *Agrilus* species, *A.planipennis*, girdled ash trees had an altered chemical composition of their phloem, with increases in sesquiterpene compounds, which could be detected and used by *A.planipennis* when seeking host trees (Crook *et al.,* 2008). A similar effect could be occurring with *A.biguttatus*.

Larval galleries of *A.biguttatus* have been found when actively searched for behind lesions in over 95 percent of cases (Brown *et al.,* 2015). On this evidence the conclusion that *A.biguttatus* may be acting as a secondary coloniser or vector is understandable however there are difficulties with this inference. Although larval galleries were found alongside sites of lesion formation the identification of these is based on the fact that larvae found at one site were identified as *A.biguttatus* (Denman *et al.,* 2014). All subsequent similar looking galleries were then interpreted as belonging to *A.biguttatus* (Denman *et al.,* 2014) without further corroboration e.g. via molecular analysis of frass or swabs of the gallery surface.

In a comparison of the bacterial communities found in different bark layers of symptomatic and asymptomatic oak trees (10 AOD: 5 non AOD trees) Sapp *et al*. (2016) also investigated the larval galleries found although the results were not reported. Insect galleries may nevertheless be a potential pathway of spread through the tree as bacteria can colonise both damaged tissue (Underwood *et al.,* 2007) and frass (Mitchell and Hanks, 2009). Entry and exit holes created by insects may also provide an ingress point that allows initial bacterial colonisation or a mechanism by which bacteria can be 'moved' as the insect leaves the tree.

In an experiment to test whether *B.goodwinii* and *G.quercinecans* singly, together and in combination with *A.biguttatus* eggs were able to initiate stem bleeding results indicated that all combinations, including a control application of water, were able to induce necrosis (Denman *et al*., 2018). The assumed involvement of *A.biguttatus* led to no other wood boring insects being investigated, neither were tunnels created artificially to see whether the bacteria were utilising already degrading tissues. Without eliminating other possibilities there is still no clear evidence for any involvement of *A.biguttatus.*

A.biguttatus has previously been considered solely as attracted to weakened oaks (Evans *et al.,* 2004) and whose galleries may kill the trees via girdling (Moraal and Hilszczanski, 2000). These support the view that *A.biguttatus* may be a contributing factor to a decline syndrome. The correlation found between the necrotic lesions and larval galleries however does not mean that there is a causal relationship. Furthermore the evidence obtained by Brown (2014) on the composition of the adult gut bacteria was not supportive of *A.biguttatus* as a vector. The question of whether *A.biguttatus*, or other insects associated with oak, are a causal agent, vector or a contributing factor in the decline syndrome remains unanswered.

4.1.3 Chapter Aims

The aim of this chapter is to investigate whether there is any association between xylophagous coleoptera and *B.goodwinii* and *G. quercinecans.*

4.2 Methodology

Xylophagous Coleoptera were collected from two sites in 2016 and six sites in 2017 (Table 4). Insects were collected according to the protocol set out in section 2.2.3 with subsequent sample preparation and DNA extraction following the protocol outlined in section 2.2.5.1. In order to maximise the potential for detection of *G.quercinecans* and *B.goodwinii* DNA was extracted from several individuals of the same species where possible. Individuals of the same species were grouped together by date, up to a maximum of four individuals and DNA extracted from the group.

Detection of the presence of *G.quercinecans* and *B.goodwinii* was via real-time PCR as covered in section 2.2.5.2. Additional *A.biguttatus* were provided by Forest Research in 2016 and 2018. No cage caught adults were available for this project in 2017 due to poor emergence. Individual adults were collected from sections of symptomatic oak from AOD sites which had been placed in emergence cages (Figure 34). See Reed *et al*. (2018) for methodology of rearing *A.biguttatus*. Adults were stored without preservative at -26°C.

Figure 34. Sections of symptomatic oak from AOD sites placed in emergence cages

Five field collected xylophagous Coleoptera, which have associations with oak, were selected for analysis (Table 26). A single adult *A.biguttatus* was field collected in 2016. These species were found to be the most consistently caught across all sites.

Table 26. The five xylophagous Coleoptera selected to analyse for the presence of *G.quercinecans* and *B.goodwinii*

Species	Family	Ecology
Agrilus laticornis[1]	*Buprestidae*	Larvae develop in bark; adults feed on oak leaves
Curculio glandium[2]	*Curculionidae*	Larvae develop in acorns
Curculio venosus[2]	*Curculionidae*	Larvae develop in acorns
Glischrochilus quadripunctatus[3]	*Nitidulidae*	Predatory on *Buprestidae*, utilises existing tunnels
Platypus cylindrus[4]	*Curculionidae*	Adults bore into bark to lay eggs

Identification keys: [1]Vorst, O. (2009); [2]Morris, M.G. (2012); [3]Hackston, M. (2012); [4]Duffy, E.A.J. (1953)

4.3 Results

Agrilus biguttatus

Neither *G.quercinecans* nor *B.goodwinii* were detected in the single 2016 field caught *A.biguttatus*. In 2016 no *A.biguttatus* from the emergence cages were positive for either bacteria.

In 2018 five emergence cage samples from three different sites were positive for *B.goodwinii* compared to two, from the same site, for *G.quercinecans* (Table 27).

Table 27. The total number of *A.biguttatus* analysed in the emergence cage samples which were positive or for *G.quercinecans* or *B.goodwinii* in 2016 and 2018

Sample	Site	Year	Number of individuals analysed	*G.quercinecans*	*B.goodwinii*
I1	Site A	2016	1	negative	negative
I2			1	negative	negative
I3			1	negative	negative
E1	Site B	2018	4	positive	negative
E4			1	negative	positive
E5			1	negative	positive
E6			1	negative	negative
E7			1	positive	positive
E2	Site C		1	negative	positive
E3	Site D		1	negative	positive

Symptomatic and asymptomatic sites

In 2016 and 2017 *A.laticornis* was collected from all sites (Figures 35-38). At only one symptomatic site in 2017 (ICK) were all five xylophagous Coleoptera collected (Figure 35, Figure 36). At this site (ICK) all five species also had a grouped sample with a positive detection of *B.goodwinii* (Figure 36).

Agrilus laticornis

A higher number of sites had a positive grouped sample detection of *B.goodwinii* compared to *G.quercinecans* (Table 28). Both bacteria were detected in samples from three symptomatic sites (Table 28). A single grouped sample from an asymptomatic site was positive for *B.goodwinii* with no samples from either asymptomatic site positive for *G.quercinecans* (Figure 37, Figure 38).

Curculio glandium

Overall a greater number of grouped samples from a higher number of sites had a positive detection of *B.goodwinii* compared to *G.quercinecans* (Table 29). Only two grouped samples from symptomatic sites had positive detections of both bacteria (Table 29). The sample collected from one asymptomatic site (NOR) was positive for *B.goodwinii* (Figure 38) but not for *G.quercinecans* (Figure 37).

Curculio venosus

A single grouped sample from a symptomatic site in 2017 had a positive detection of *B.goodwinii* with *G.quercinecans* not detected in any samples (Table 30). No *C.venosus* were caught at asymptomatic sites (Table 30).

Glischrochilus quadripunctatus

There was no detection of *G.quercinecans* in any samples from asymptomatic or symptomatic sites and only a single grouped sample from a symptomatic site in 2017 was positive for *B.goodwinii* (Table 31).

Platypus cylindrus

Two grouped samples from symptomatic sites, one in 2016 and one in 2017, were positive for *G.quercinecans.* Of these one was also positive for *B.goodwinii* (Table 32). No *P.cylindrus* were collected from asymptomatic sites (Table 32).

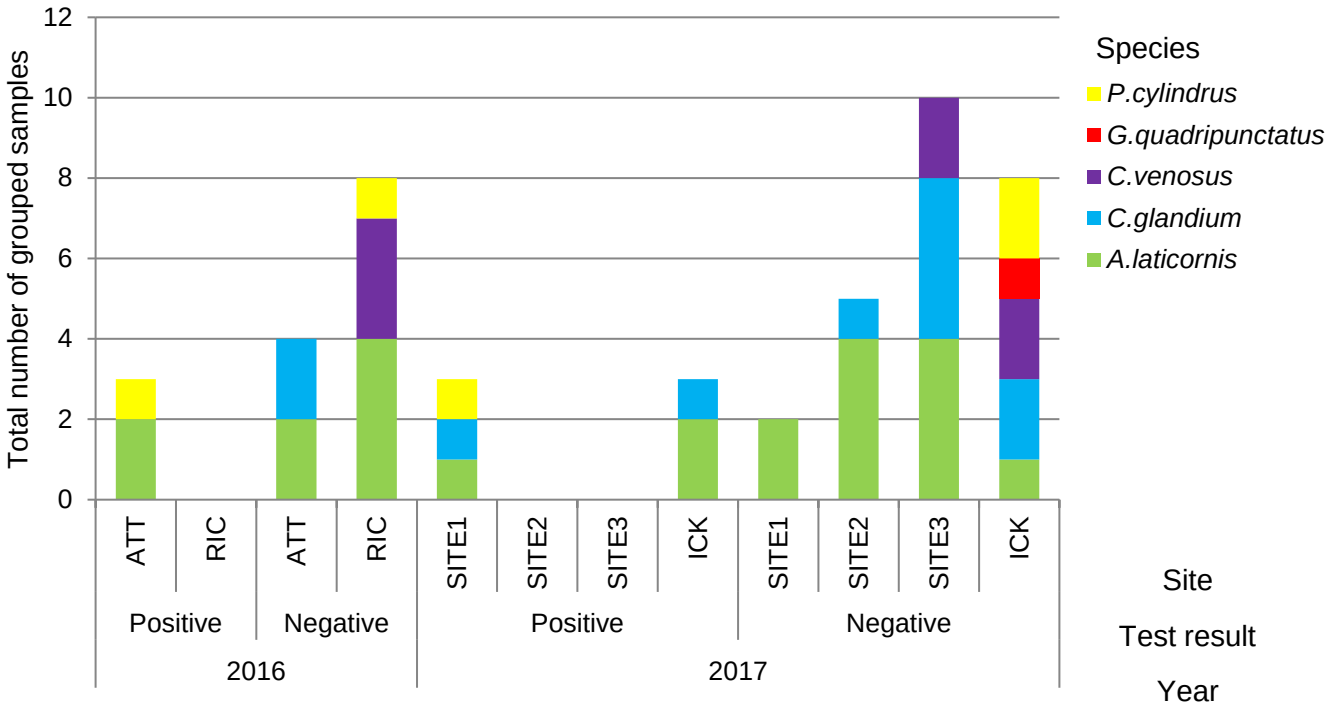


Figure 35. Total number of grouped xylophagous Coleoptera species samples with a positive or negative test result for *G.quercinecans* at symptomatic sites in 2016 and 2017

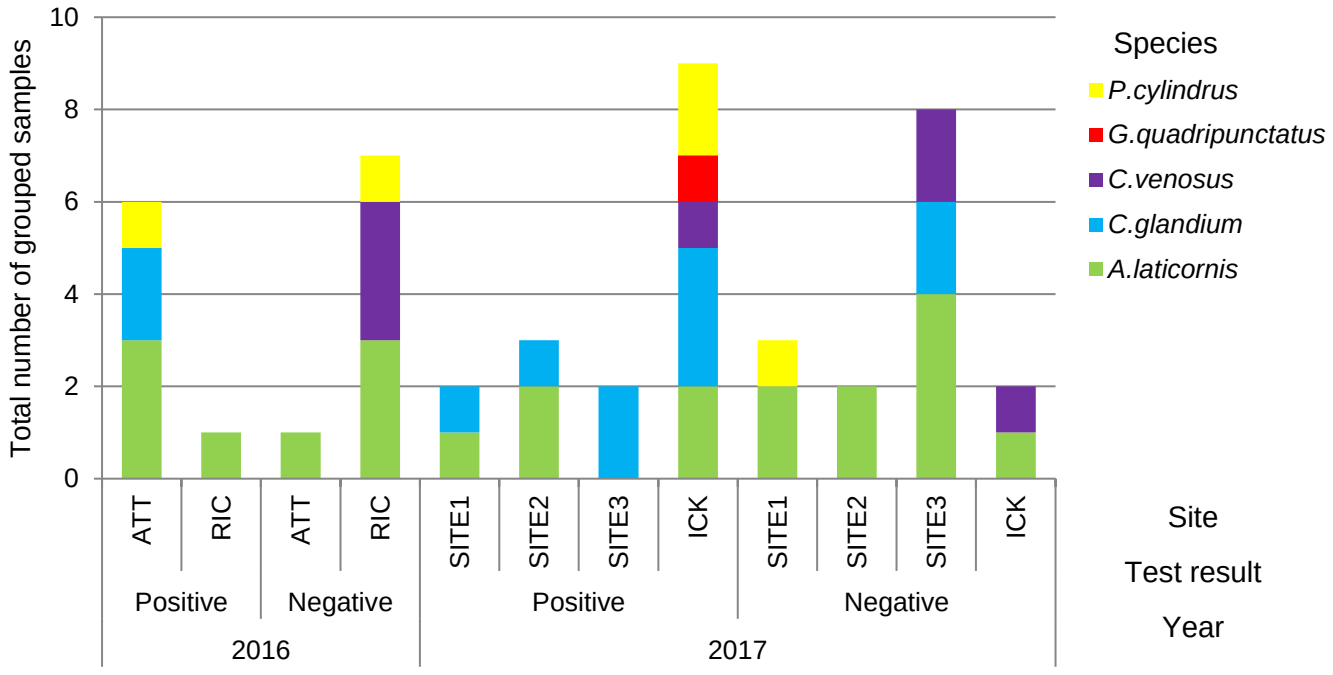


Figure 36. Total number of grouped xylophagous Coleoptera species samples with a positive or negative test result for *B.goodwinii* at symptomatic sites in 2016 and 2017

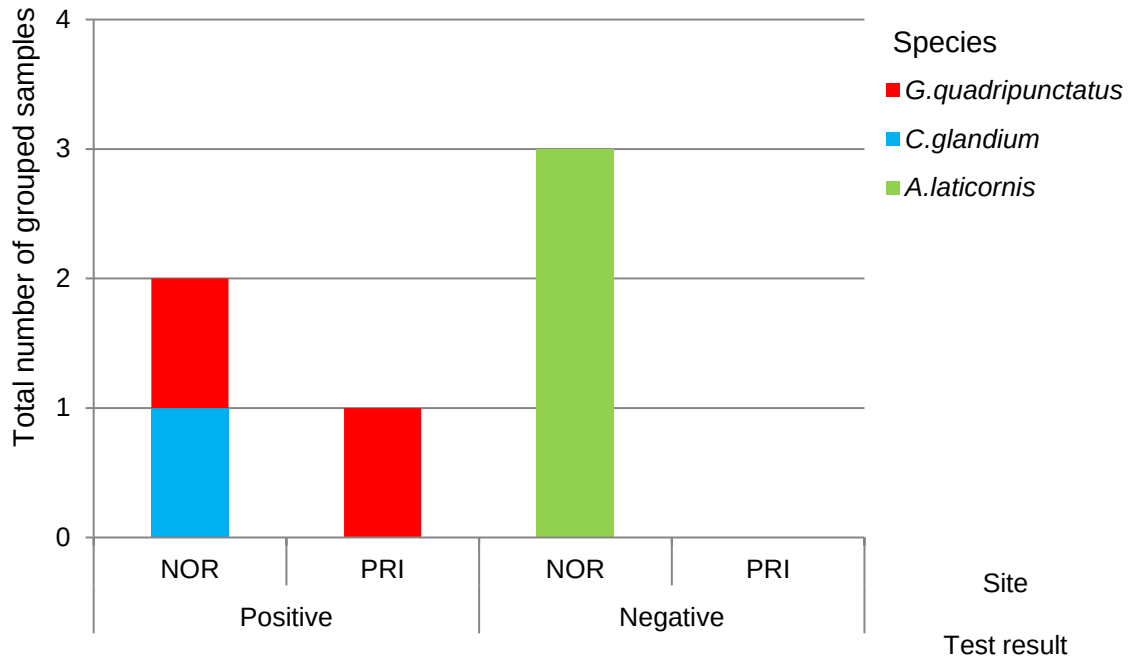


Figure 37. Total number of grouped xylophagous Coleoptera species samples with a positive or negative test result for *G.quercinecans* at asymptomatic sites in 2017

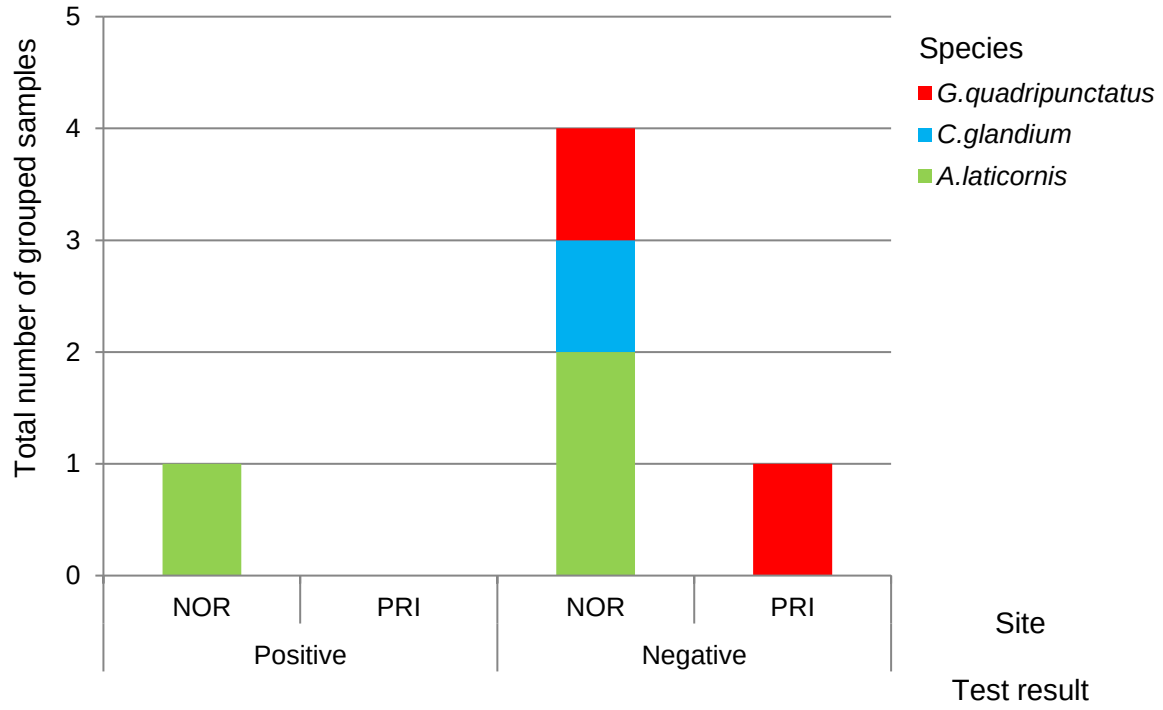


Figure 38. Total number of grouped xylophagous Coleoptera species samples with a positive or negative test result for *B.goodwinii* at asymptomatic sites in 2017

Table 28. The total number of individual adult *A.laticornis* analysed in 2016 and 2017 and the number of grouped samples which were positive for *G.quercinecans* or *B.goodwinii*

						Number of groups positive		Number of groups negative	
Site	**Year**	**Site health status**	**Total caught**	**Total individuals analysed**	**Number of groups by sample date**	***G.quercinecans***	***B.goodwinii***	***G.quercinecans***	***B.goodwinii***
NOR	2017	Asymptomatic	7	5	3	0	1	3	2
PRI			0	0	0	0	0	0	0
ATT	2016	Symptomatic	8	8	4	2	3	2	1
RIC			54	14	4	0	1	4	3
SITE1	2017		12	10	3	1	1	2	2
SITE2			34	15	4	0	2	4	2
SITE3			15	11	4	0	0	4	4
ICK			17	10	3	2	2	1	1

Table 29. The total number of individual adult *C.glandium* analysed in 2016 and 2017 and the number of grouped samples which were positive for *G.quercinecans* or *B.goodwinii*

						Number of groups positive		Number of groups negative	
Site	**Year**	**Site health status**	**Total caught**	**Total individuals analysed**	**Number of groups by sample date**	***G.quercinecans***	***B.goodwinii***	***G.quercinecans***	***B.goodwinii***
NOR	2017	Asymptomatic	5	4	1	1	0	0	1
PRI			0	0	0	0	0	0	0
ATT	2016	Symptomatic	8	4	2	0	2	2	0
RIC			0	0	0	0	0	0	0
SITE1	2017		1	1	1	1	1	0	0
SITE2			4	4	1	0	1	1	0
SITE3			8	8	4	0	2	4	2
ICK			9	9	3	1	3	2	0

Table 30. The total number of individual adult *C.venosus* analysed in 2016 and 2017 and the number of grouped samples which were positive for *G.quercinecans* or *B.goodwinii*

						Number of groups positive		Number of groups negative	
Site	**Year**	**Site health status**	**Total caught**	**Total individuals analysed**	**Number of groups by sample date**	***G.quercinecans***	***B.goodwinii***	***G.quercinecans***	***B.goodwinii***
NOR	2017	Asymptomatic	0	0	0	0	0	0	0
PRI			0	0	0	0	0	0	0
ATT	2016	Symptomatic	0	0	0	0	0	0	0
RIC			3	3	3	0	0	3	3
SITE1	2017		0	0	0	0	0	0	0
SITE2			0	0	0	0	0	0	0
SITE3			2	2	2	0	0	2	2
ICK			6	6	2	0	1	2	1

Table 31. The total number of individual adult *G.quadripunctatus* analysed in 2016 and 2017 and the number of grouped samples which were positive for *G.quercinecans* or *B.goodwinii*

						Number of groups positive		Number of groups negative	
Site	**Year**	**Site health status**	**Total caught**	**Total individuals analysed**	**Number of groups by sample date**	***G.quercinecans***	***B.goodwinii***	***G.quercinecans***	***B.goodwinii***
NOR	2017	Asymptomatic	1	1	1	0	0	1	1
PRI			3	3	1	0	0	1	1
ATT	2016	Symptomatic	0	0	0	0	0	0	0
RIC			0	0	0	0	0	0	0
SITE1	2017		0	0	0	0	0	0	0
SITE2			0	0	0	0	0	0	0
SITE3			0	0	0	0	0	0	0
ICK			3	3	1	0	1	1	0

Table 32. The total number of individual adult *P.cylindrus* analysed in 2016 and 2017 and the number of grouped samples which were positive for *G.quercinecans* or *B.goodwinii*

						Number of groups positive		**Number of groups negative**	
Site	**Year**	**Site health status**	**Total caught**	**Total individuals analysed**	**Number of groups by sample date**	***G.quercinecans***	***B.goodwinii***	***G.quercinecans***	***B.goodwinii***
NOR	2017	Asymptomatic	0	0	0	0	0	0	0
PRI			0	0	0	0	0	0	0
ATT	2016	Symptomatic	1	1	1	1	1	0	0
RIC			1	1	1	0	0	1	1
SITE1	2017		1	1	1	1	0	0	1
SITE2			0	0	0	0	0	0	0
SITE3			0	0	0	0	0	0	0
ICK			5	3	2	0	2	2	0

4.4 Discussion

The association of *G.quercinecans* and *B.goodwinii* with insects is a new finding. The results presented here indicate that both bacteria may have relationships with a variety of insects, including those analysed in this chapter. It may be that as these coleoptera use oak as a habitat, they have incidentally come into contact with the bacteria. Alternatively their relationship may be closer. The lifestyles of these five Coleoptera; *A.laticornis*, *C.glandium*, *C.venosus*, *G.quadripunctatus* and *P.cylindrus* also suggest potential entry routes for the bacteria.

Overall, low numbers of Coleoptera were caught. This might be due to the trap types used, abundance at each site or a reflection of insect activity during the trapping period. The only species to be collected at all seven sites was *A.laticornis* with both asymptomatic and symptomatic site samples positive for *B.goodwinii* and *G.quercinecans*. This adds to the evidence from Chapter 3 that the bacteria are present at both symptomatic and asymptomatic sites. Only three species were collected from both asymptomatic sites; *A.laticornis*, *C.glandium* and *G.quadripunctatus*. This may be a reflection of the age of the sites with symptomatic sites generally containing older oaks or due to site conditions and available resources. Collection within fewer sites with a greater number of traps may have been a better strategy for maximising numbers caught.

Bacteria rely on transmission pathways to move to new hosts (Frank *et al*., 2017) and insect vectors are a common mechanism by which they can do this (Kannan and Bastas, 2015). The results of Chapter 3 show that *B.goodwinii* and *G.quercinecans* are present on/within various oak tissues. Four symptomatic sites used for phyllosphere sampling (2016: ATT & RIC; 2017 SITE3 & ICK) were also used for insect trapping. At all four sites autumn samples were collected and at one (ICK) spring leaf samples were also collected. All sites had positive detections of *G.quercinecans* and *B.goodwinii* within leaf, acorn and litter samples.

The presence of *G.quercinecans* and *B.goodwinii* in bark tissue (Sapp *et al*., 2016) and oak leaf samples shows that they have the opportunity to come into contact with insects; an important component in any insect-bacteria relationship (Nadarash and Stravindes, 2011). Adults of *A.laticornis*, *C.glandium* and *C.venosus* are all known to feed on oak leaves and individuals could have ingested bacteria through feeding. Another possibility is incidental contact whilst moving over the canopy or, in the case of *G.quadripunctatus* and *P.cylindrus*, through tunnels burrowed into bark. The results of Chapter 3 also indicate both bacteria are present within acorns and could further account for the presence on *C.glandium* and *C.venosus* via larvae feeding within the acorn or contact as adults emerge.

Ingestion of *G.quercinecans* and *B.goodwinii* via feeding does not explain the detection from *A.biguttatus* emergence cage samples as there were no leaves for the adults to feed on. The presence of the bacteria could therefore be accounted for by incidental contact moving round the cage or contact within tunnels or bark as adults emerged.

Incidental contact and feeding are both viable modes of bacterial transmission between hosts (Frank *et al*, 2017). Bacteria can enter plant tissues during feeding as they are regurgitated from within an insects gut (Orlovskis *et al*., 2015). In order for this strategy to be successful bacteria need to persist through the digestive tract (Orlovskis *et al*., 2015) and three different methods have been highlighted in section 1.2.3.1. Wounds created by the chewing feeding action and burrowing of beetles is also a means by which bacteria can enter into plant tissue (Orlovskis *et al*., 2015).

Questions remain over the exact nature of the insect-bacteria relationship. As discussed in section 4.1 commensalism, mutualism and parasitism are all potential interactions (Engel and Moran, 2013). Any of these five Coleoptera, or other insects, could have a close association with both or either *G.quercinecans* or *B.goodwinii* however it is not possible to draw any firm conclusions from the results in this chapter.

The plant cell wall degrading enzymes identified within the genomes of *G.quercinecans* and to a greater extent in *B.goodwinii* (Broberg *et al*., 2018; Doonan *et al*., 2019) may confer advantages onto both the larvae and adults of the insects discussed in this chapter all of which have a life stage that either feeds on or excavates out plant tissue. When all the species are considered *B.goodwinii* was identified in a greater number of samples and this was also reflected in the leaf sampling. This may be a reflection of bacterial abundance within the oak phyllosphere or could indicate *B.goodwinii* is more adapted to colonise and survive within insects.

Both bacteria have the capacity to utilise decayed tissue (Kraepiel and Barny, 2015) and could be in turn be used by xylophagous insects. In addition *B.goodwinii,* through its ability to suppress host defences (Doonan, 2016), may be why it is present in a greater number of samples. Although the number of individuals caught and analysed was low, both *Curculio* species samples for example had positive detections of *B.goodwinii.* In comparison in *A.laticornis* samples both bacteria were positively detected. This could reflect the bacteria required for the different habitat niches used by these insects. Any bacteria with a specific or non-specific association with one or many insects either as hosts or vectors will though need to overcome insect defences (Nadarash and Stavrinides, 2011) and it is not clear *G.quercinecans* or *B.goodwinii* have the capacity to do this.

A model developed to look at insect-pathogen relationships showed that there are many influences on vector-host relationships as well as on rate of pathogen spread (Crowder *et al*., 2019). The Coleoptera discussed in this chapter will have interactions as described by Crowder *et al*. (2019) such as competition for resources and predation. For example in the case of *A.laticornis*, *C.glandium* and *C.venosus* there will be competition for food resources. Between *C.glandium* and *C.venosus* for egg laying opportunities and interactions with *G.quadripunctatus* may involve predation. Interactions affecting vector movement are a key influence on the spread of plant pathogens and a greater understanding of these factors is therefore important in understanding plant disease ecology (Crowder *et al*., 2019).

The seasonal appearance of *G.quercinecans* or *B.goodwinii* has been discussed previously. Drought induced changes to plant metabolism and defences which result in altered vector behaviour have also been found increase the risk of disease and reduction in tree health (Szczepaniec and Finke, 2019). Climate change is predicted to increase the frequency of drought and heat waves (Sallé *et al*., 2014). Temperature will also influence the geographic range of species (Sallé *et al*., 2014). A modelling study of *A.biguttatus* for example showed that the thermal requirement of *A.biguttatus* is the limiting factor to its current UK distribution (Reed *et al*., 2018). The predicted increase in summer temperatures as a result of climate change was therefore found to predict an increase in *A.biguttatus* range (Reed *et al*., 2018). Bacteria with multiple movement pathways may be less affected by environmental changes which could explain the lack of a clear pattern to detection on phyllosphere and insect samples.

A review on the plant responses to the combined effects of herbivory, drought and pathogen transmission has highlighted the lack of studies into plant responses to multiple stresses (Szczepaniec and Finke, 2019). Particularly understudied are three way plan host-vector-pathogen relationships (Szczepaniec and Finke, 2019). The effects of drought will influence insect behaviour and host plant resistance simultaneously (Szczepaniec and Finke, 2019). One key impact is a drought induced decrease in plant resistance to phytopathogens with a concurrent increase in insect movement to new, more palatable, hosts (Szczepaniec and Finke, 2019).

Drought may already be linked to incidences of AOD (Brown *et al*., 2018) and is known to be a predisposing factor in insect damage (Sallé *et al*., 2014). Insect activity and wood boring insects are considered contributing factors to decline diseases (Sinclair, 1965; Manion, 1991) and this disease model has been discussed in section 1.1. It is possible that all or some of the insects mentioned here could be contributing to oak decline. In Europe both *A.biguttatus* and *P.cylindrus* are considered to be associated with oak declines as secondary

pests of trees in poor health (Sallé *et al*., 2014) and defoliations in particular have been implicated in previous AOD outbreaks (Denman *et al*., 2010). If the exudate from bark cracks is considered an induced hypersensitive response to bacterial colonisation it appears that current outbreaks of AOD are more severe than previous ones. This could indicate that other factors, such as insect activity or abiotic conditions are exacerbating the situation.

Although much has been made of the association of Acute Oak Decline with *A.biguttatus* (Denman *et al.,* 2014; Denman *et al.,* 2018). Detection of both bacteria in samples from the five Coleoptera do not support the idea that it is a sole vector or causal agent. This is supported by the evidence of Brown (2014) which indicated the composition of the adult gut bacteria was not supportive of *A.biguttatus* as a vector. None of the trap types used proved effective at capturing *A.biguttatus* despite being a colour and height that has proved effective in other studies (Brown, 2014). The addition of a lure based on volatiles attractive to *A.biguttatus* (Vuts *et al*., 2016) may offer a more effective solution in future. The use of emergence cages has proved successful however the limited numbers of adults available for analysis restricted the data collection. It is also not possible to eliminate contact between the emerging adults and exudate on the logs, although this incidental contact would still be a viable transmission mechanism.

Insects are habitually thought of solely in terms of a vector based relationship with phytopathogens (Nadarash and Stavrinides, 2011). There is evidence that insects can act as alternate hosts to aid bacterial persistence through gaps in host availability (Allen *et al*., 2009). Phytopathogens can also exploit insects, as they would plants, as additional hosts (Nadarash and Stavrinides, 2011) and can induce chemical changes to volatile emissions that manipulate insects (Szczepaniec and Finke, 2019).

The association of insects with phytopathogens can be coincidental but could prove to be an evolutionary stage on the way to becoming a closer relationship (Nadarash and Stavrinides, 2011) e.g. vector-pathogen or mutualistic. Infection of a host plant can still occur in the absence of a particular insect vector (Orlovskis *et al*., 2015) through the use of multiple pathways (Orlovskis *et al*., 2015). Several other phytopathogens use several different insects as well as rain and wind to encounter new hosts (Orlovskis *et al*., 2015). These include *Ralstonia solanacearum*, cause of various plant wilts and *Erwinia amylovora*, cause of fireblight (Orlovskis *et al*., 2015). The presence of *G.quercinecans* and *B.goodwinii* on multiple insects and external plant tissues does not rule out potentially multiple transmission mechanisms.

4.5 Conclusion

How *G.quercinecans* and *B.goodwinii* colonise oak and their potential movement pathways to new hosts has been an unanswered question. The results presented here indicate that both bacteria could be moved via multiple insects either as vectors or through incidental contact. Other potential routes to the colonisation of new hosts could be via feeding damage or burrowing through bark.

The exact relationship *G.quercinecans* and *B.goodwinii* have with insects does still however remain unknown. It may be that the detection is a result of incidental contact or there may be benefits conferred by the bacteria such as aiding in digestion of oak tissues and suppressing host defences. Multiple transmission mechanisms of *G.quercinecans* and *B.goodwinii* cannot be discounted and another potential movement pathway is the subject of the next chapter.

Chapter 5

Investigating the potential of rain as a transmission mechanism for two bacteria associated with Acute Oak Decline; *B.goodwinii* and *G.quercinecans*

5.1 Introduction

Airborne dispersal is an important means by which bacteria colonise new hosts (Frank *et al.,* 2017) and wind and rain have previously been highlighted in section 1.2.3.2 as key transference mechanisms. The presence of both *G.quercinecans* and *B.goodwinii* in lesions and in phyllosphere samples, particularly leaves (Chapter 3) indicates that this may be a potential movement pathway.

Lesions can be a source of bacteria in the atmosphere (Pruvost *et al*., 2002; Bock *et al*., 2005). A greater amount of bacteria was collected from trees with newer lesions on leaves and twigs (Bock *et al*., 2005). Bacteria was also more easily released from younger lesions of citrus trees with *Xanthomonas axonopodis* pv. *citri* cankers (Pruvost *et al*., 2002). Aerial movement can be an effective disperser of bacteria between leaves of the same plant (Lindemann and Upper, 1985), over short distances (Butterworth and McCartney, 1991) and has also been suggested as an explanation for the long distance spread of pathogens to new areas (Viljanen-Rollinson *et al*., 2007). Biogeography, studying species geographic distribution through time, has however been highlighted as an understudied influence on bacterial dispersal (Baltrus, 2020). The practical difficulties of sampling at a large enough scale being a limiting factor for research (Baltrus, 2020).

Bacteria able to survive transport are likely to be more successful colonising new areas (Šantl-Temkiv *et al*., 2018). In an experiment by Pettifor *et al*. (2020) *G.quercinecans*, but not *B.goodwinii* was able to produce viable colonies up to 28 days after inoculation into sterile rainwater. *Erwinia amylovora* (fire blight) has been shown to be able to survive and remain pathogenic in rainwater and is also able to survive at low temperature (4°C) (Biosca *et al*., 2019). For another phytopathogen, *Pseudomonas syringae*, the water cycle may be important for transport to new habitats and hosts (Morris *et al*., 2008). Detections in rain, snow, mountain streams and lakes as well as non-crop wild plants highlight the potential for travel over large distances and the ability to adapt in order to colonise the different niches sampled (Morris *et al*., 2008).

The formation of bio aerosols, small airborne particles released into the atmosphere, can be initiated via rain splash and is a means by which bacteria accumulate on foliar surfaces (Frank *et al.,* 2017). Rainsplash has been experimentally shown to displace bacteria from both leaves (Butterworth and McCartney, 1991; Bock *et al.,* 2005) and soil (Joung *et al.,* 2017) and an increase in bacterial growth has been observed in the days following rain (Lindemann and Upper, 1985). The number of phytopathogenic sequences has also been shown to increase following rain (Jang *et al*., 2018). Duration of rainfall was important with a shorter rainfall event found to be more effective for bacterial dispersal (Pruvost *et al*., 2002). This was concluded to be because a longer timeframe resulted in bacteria being washed off rather than becoming aerosolised (Pruvost *et al*., 2002).

Rain can be an influence on phyllosphere bacterial communities through several means (Allard *et al*., 2020). Rain can bring new bacteria, increase the water available to existing species and remove weakly attached cells thereby creating space for colonisation (Allard *et al*., 2020). An additional explanation suggested by Champoiseau *et al*. (2009) for increase in *X.albineans* was due to stomata opening in the high humidity associated with rainfall.

In a study of bacteria associated with snap bean plants, significant increases in airborne bacteria were seen during rain indicating that they had been dislodged from leaves (Lindemann and Upper, 1985). This displacement can be very effective. Up to 90 percent of three bacteria applied to the leaves of two crop plants was washed off through artificially generated rain splash (Butterworth and McCartney, 1991). Wind driven rain has been linked to the spread of *Erwinia amylovora* (fire blight) in a nursery setting (McManus, 1994) and an increase in *Xanthomonas albineans*, (sugar cane leaf scald) was also linked to greater rainfall with infection rate simultaneously increasing (Champoiseau *et al*., 2009).

Significant increases in viable airborne bacteria, measured by Colony-Forming Units (CFU) counts on agar, have been observed to occur at the hottest part of a dry, sunny day (Lindemann and Upper, 1985). Relative humidity and sample season were linked to differences in airborne bacterial communities after rain (Jang *et al*., 2018) and a high humidity level similarly resulted in increased growth of endophytic bacteria (Xin *et al.,* 2016).

The survival of aerosolised *P.syringae* was on the other hand found to be influenced by lower humidity although this was in conjunction with temperature (Walter *et al*., 1990). Bacteria were detected at all distances (up to 15 m) from source, however over 1 m survival rate did not differ from controls whereas over 3 m relative humidity and temperature became more influential on the rate of bacterial decline (Walter *et al*., 1990). An increase in temperature also increased the amount of bacteria collected from citrus canker infected trees following a simulated wind/rain event (Bock *et al*., 2005). The development of

symptoms associated with *E.amylovora*, was dependent on temperature appearing earlier at the higher temperature of 28°C followed by 14°C, with the slowest development occurring at 4°C (Biosca *et al*., 2019).

Leaf structure is an influence on bacterial colonisation and establishment (Muller et al., 2003). Leaf structure was a factor in determining whether or not newly arrived bacteria successfully colonised a leaf surface suggesting different areas of a leaf are more conducive to colonisation than others (Monier and Lindow, 2005). Bacteria landing on veins or glandular tricomes, were more likely to survive when compared to normal epidermal cells or bases of hooked tricomes (Monier and Lindow, 2005). The effect was observed when trees were under drought stress so it is not clear whether the same effect would be observed in unstressed plants. It may be that these areas are less prone to drying or are slower to dry than other leaf areas (Monier and Lindow, 2005).

When large, more rigid leaves, were compared to light flexible ones both leaf size and flexibility were found to influence the type of dispersal mechanism and the 'pathogen load' released (Gilet and Bourouiba, 2015). An increase in leaf flexibility reduced the range of the splashed droplets however once a leaf reached a certain rigidity threshold the ejection mechanism changed to favour larger droplets which had a greater range (Gilet and Bourouiba, 2014). For larger, stiffer leaves the droplet on droplet means was effective at displacing the contaminated droplet greater distances and could result in a more localised pathogen spread (Gilet and Bourouiba, 2015). Larger water droplets were further found to be more likely to infect nearby plants (Gilet and Bourouiba, 2014). Oak leaves are fairly rigid so this mechanism is potentially the more effective. The evidence of 'clustering' of AOD infected trees has previously been taken to indicate a biotic agent rather than an environmental agent, such as rain, was responsible for pathogen movement (Brown, 2014). The evidence from Gilet and Bourouiba, (2015) suggests a rain dispersal mechanism would result in a local clustering of infected trees.

A number of experiments have been carried out to demonstrate the efficacy of the airborne movement of various plant associated bacteria by simulated wind and rain. When an agar plate inoculated with *Azospirillum brasilense*, a nitrogen fixing bacterium, was placed near glasshouse ventilation, colonies were subsequently detected on agar plates 1.5 m and 6 m away (Bashan, 1991). Inoculated plants were also able to act as a source of bacteria (Bashan, 1991). Uninoculated plants and agar plates placed in the same glasshouse were both found to have *A.brasilense* present with the amount collected decreasing with increasing distance from the inoculum source (Bashan, 1991).

In another greenhouse experiment *P.syringae* and *Erwinia herbicola* were aerosolized using a fan placed near plants sprayed with the two bacteria; bacteria were also directly applied to leaves as a control (Walter *et al*., 1990). Directly applied bacteria survived up to eight days however those that had drifted onto the leaves via air only survived 48 hours on the plants (*Phaseolus vulgaris* var. *humilis* and *Avena sativa* var. *Cayuse*) (Walter *et al*., 1990).

A slightly different experiment was carried out with trees infected with *Xanthomonas axonopodis* pv. *citri*, cause of citrus canker (Bock *et al*., 2005). Infected trees were exposed to simulated wind driven rainsplash and bacteria subsequently collected up to 12 m from the source (Bock *et al*., 2005). The quantity of bacteria collected was highest in the first few minutes following simulated wind/rain, declining over the first hour (Bock *et al*., 2005).

The results of these studies show experimentally that bacteria can be aerially transported to new hosts in several different ways. In the case of *A.brasilense* it was via air alone (Bashan, 1991), *P.syringae* and *E.herbicola* were displaced by wind after arriving on leaves in water droplets (Walter *et al*., 1990), whilst *X.axonopodis* pv. *citri* was transferred in wind driven rain (Bock *et al*., 2005). All of these methods proved effective as movement mechanisms for bacteria and could apply to *G.quercinecans* and *B.goodwinii.* The amount of bacteria collected was dependent on the distance from the source (Bashan, 1991) as well as the time elapsed since a dispersal event was initiated (Walter *et al*., 1990; Bock *et al*., 2005) and wind speed (Bock *et al.,* 2005). This indicates that environmental differences have an influence on aerial dispersal. At a local level microclimates are considered to have an impact on the survival of bacteria (Baltrus, 2020).

The type of dispersal mechanism and timing of dispersal is additionally thought to influence the structure of plant microbial communities, including bacteria (Baltrus, 2020). When trees were under water stress the successful survival of new bacteria was strongly influenced by the bacteria already present on a leaf surface (Monier and Lindow, 2005). Using bean leaves with previously established colonies of *Pantoea agglomerans* (299R) the survival of immigrant cells of *Pseudomonas fluorescens* (A506), *P.syringae* (B728a) and *P.agglomerans* was then investigated (Monier and Lindow, 2005). Aggregates formed by *P.agglomerans* influenced the survival of newly deposited bacteria and either aided or hindered survival under drought stress (Monier and Lindow, 2005). Whilst survival of *P.syringae* was similar whether landing on aggregates or clear leaf areas, the chance of survival for *P.fluorescens* and *P.agglomerans* was almost doubled when landing on aggregations compared to a clear area (Monier and Lindow, 2005). Single resident cells were additionally found to have a higher death rate under dry conditions compared to resident aggregated bacteria (Monier and Lindow, 2005).

An investigation into the dynamics of rainsplash on leaf surfaces indicated water applied to the leaves remained as small droplets rather than fully wetting the leaf as a film (Gilet and Bourouiba, 2014). Droplets of rain on the leaf surface could move bacteria, resulting in their accumulation at certain points (Monier and Lindow, 2005). This may encourage the formation of localised bacterial aggregations (Monier and Lindow, 2005) or biofilms (Morris and Monier, 2003) strategies thought to increase survival (Nongkhlaw and Joshi, 2014). Bacteria with the ability to adhere more effectively to a leaf will benefit from resistance to dislodgment by rain and any advantage in adhesion increases the chance of colonisation (Suoniemi *et al*., 1995). Structures such as pili, fimbriae and flagella act as anchoring mechanisms for bacteria on a plant surface (Suoniemi *et al*., 1995; Haiku and Westerlund-Wikström, 2013; Rossez *et al.,* 2015).

The development of biofilms has been suggested as a strategy to enhance the rain transmission of bacteria (Monier *et al.,* 2003). The positioning of biofilms e.g. on trichomes, raises the bacteria above the leaf surface thereby exposing them to the wind speeds and sheer force necessary to break the biofilm and release cells (Morris and Monier, 2003). A type three secretion system (T3SS) such as that found in the genomic analysis of *B.goodwinii* can be an indication of the capacity to form biofilms (Doonan, 2016).

Chapter 3 has already presented evidence that *G.quercinecans* and *B.goodwinii* are associated with the oak phyllosphere with detections on leaves, litter, acorns, catkins and bark. Both bacteria are also present in stem lesions. Together these results indicate that airborne transmission of *G.quercinecans* and *B.goodwinii* is a viable and potential means by which these bacteria can encounter new hosts.

5.1.1 Chapter Aims

Although both wind and rain separately are able to move bacteria it is likely that under field conditions both together will play a part. Direct movement by air is also not considered a common mechanism (Kannan and Bastas, 2015). Bacteria found to be present in rainwater will give an indication of their potential to be moved by various aerial mechanisms including wind, rainsplash and wind driven rain.

The aim of this chapter is to explore the potential of *B.goodwinii* and *G.quercinecans* to be transmitted though aerial movement by investigating rainwater for their presence.

5.2 Methodology

As detailed in section 2.2.4 in spring and autumn 2018 rain traps were placed in the lower canopies of symptomatic and asymptomatic trees in two symptomatic and one asymptomatic sites. Three additional traps were placed in open ground.

Table 33 indicates the sites used and number of traps which successfully collected rainwater. At WYR three autumn traps did not collect due to lack of rain. At DUD one ground trap in autumn and one ground and one canopy in spring were trampled and two spring canopy traps failed to collect rain.

Variable amounts of rainfall were collected so that whilst all the rain was filtered according to the protocol in 2.2.4, only 20ml was used for analysis to reflect the lowest volume collected.

Table 33. Sites used in spring and autumn 2018 for the collection of rainwater and the number of traps which successfully collected rain

Site	**DUD**[2]	**WYR**[1]	**HAU**[3]
Site health status	Symptomatic	Symptomatic	Asymptomatic
Year	2018	2018	2018
Number of raintraps deployed (Number of raintraps successful)			
Spring			
Symptomatic	3 (2)	3 (3)	N/A
Asymptomatic	3 (1)	3 (2)	3 (3)
Ground	3 (2)	3 (3)	3 (3)
Autumn			
Symptomatic	3 (3)	3 (0)	N/A
Asymptomatic	3 (3)	3 (0)	3 (3)
Ground	3 (2)	3 (0)	3 (3)

[1]Wyre; [2]Dudmaston; [3]Harper Adams. For site descriptions see section 2.1

Sample preparation and DNA extraction following the protocol outlined in section 2.2.5.1 Species specific PCR assays were then used for the detection of *G.quercinecans* and *B.goodwinii* as detailed in section 2.2.5.2.

5.2.1 16S rDNA PCR assay

A complementary universal 16S rDNA bacterial PCR assay was carried out on a supplementary set of samples subsampled from the rain water collected (Table 34). Although Nanodrop™ results would indicate the presence of DNA, this assay would confirm whether bacterial DNA was being successfully extracted via the technique detailed in section 2.3.4, providing a greater level of confidence to any subsequent positive detections of *B.goodwinii* and *G.quercinecans* in rain samples.

The use of 16S rDNA assay is widely used as a way of identifying and separating bacteria (Clarridge, 2004; Woo *et al.,* 2008) since it is highly conserved across bacterial species. The successful amplification of the 16S region via PCR would determine whether there is bacterial DNA in the sample.

Table 34. Number of raintrap samples subsampled to be used in a bacterial 16S rDNA PCR

Site	**DUD**[2]	**WYR**[1]	**HAU**[3]
Site health status	Symptomatic	Symptomatic	Asymptomatic
Year	2018	2018	2018
Number of raintrap samples used			
Spring			
Symptomatic	2	3	N/A
Asymptomatic	1	2	3
Ground	2	3	3
Autumn			
Symptomatic	1	0	N/A
Asymptomatic	3	0	2
Ground	2	0	2

[1]Wyre; [2]Dudmaston; [3]Harper Adams. For site descriptions see section 2.1

A search of literature related to either AOD or any of the bacteria *G.quercinecans* or *B.goodwinii* was undertaken to see if a 16S universal assay had been used in any previous research in order to identify potential primers. The only study to detail the primers used was that by Meaden *et al*. (2016) whose study successfully used primers to amplify the V4 region of bacteria extracted from bark microcores including *B.goodwinii*.

The forward and reverse universal primer GTGCCAGCMGCCGCGGTAA (5'-3') and GGACTACHVGGGTWTCTAAT (5′-3′) used were identified as primer sequences F515 and R806 respectively. A further literature search was then undertaken to find details of any PCR assay setup using F515 and R806. The most detailed setup was that referenced in the highly cited Caporaso *et al*. (2011). This protocol was therefore selected for use with an alteration of extending the denaturing for 12 minutes, as recommended for the Master Mix used, and the addition of a cooling step. The reaction mix was prepared for 100 25 μl reactions using two 2 ml reaction tubes. Each reaction contained:

10.0 µl Hot FirePol EvaGreen (Soils BioDyne) Master Mix (Newmarket Scientific)
13.0 µl PCR grade water (Merck)
0.5 µl forward primer (Eurofins Genomics) 10µM final concentration
F515 [5' GTGCCAGCMGCCGCGGTAA 3']
0.5 µl reverse primer (Eurofins Genomics) 10µM final concentration
R806 [5' GGACTACHVGGGTWTCTAAT 3']
1.0 µl template DNA

A 96 well plate was used for the subsequent PCR reaction. The plate was kept on ice within a U. V sterilised cabinet and primers were kept on ice whilst being used. 24μl of the mix was pipetted into each well. 1μl of sample (template) DNA was then added with two replicate wells used per sample. Positive controls of a mix of *B.goodwinii* (Strain FRB 21), *G.quercinecans* (Strain FRB 31) and *Rahnella victoriana* (Strain FRB 1) were used along with a negative control of molecular grade water. A cover was attached to the plate which was then placed in A BioRad CFX96 which was set up for a 25 μl volume reaction (Table 35). A positive detection was one out of the two wells.

Table 35. BioRad CFX96 programme set up for 16S amplification

Stage	**Cycles**	**Time (mm:ss)**	**Temperature (°C)**
Initial denaturation	1	12:00	95
qPCR	40	00:45	95
		0:50	60
		00:90	72
Extension		10. 00	72
Cooling	1	00:30	40

5.3 Results

5.3.1 16S rDNA PCR assay

Of the spring canopy rainwater collected, five asymptomatic and four symptomatic canopy samples had a positive detection of bacterial DNA. In the autumn, rain canopy samples from two asymptomatic and two symptomatic trees had a positive detection of bacterial DNA. Four spring and two autumn ground raintrap samples had a positive detection of bacterial DNA.

5.3.2 Spring and autumn raintrap samples

5.3.2.1 Spring and Autumn canopy raintrap samples

Asymptomatic site

In spring one sample was positive for *G.quercinecans* with all three positive for *B.goodwinii* (Figure 39; Table 36). In autumn no samples were positive for *G.quercinecans* with two remaining positive for *B.goodwinii* (Figure 39; Table 36).

Symptomatic sites

Across both sites and sample periods only one asymptomatic raintrap at one site was positive in autumn for *G.quercinecans* (Figure 39; Table 36). In contrast *B.goodwinii* was present in rain samples from both sites and sample periods (Figure 39; Table 36). A similar result was seen for symptomatic traps; one autumn trap in one site was positive for *G.quercinecans* whereas *B.goodwinii* was detected at both sites and in both sample periods (Figure 40; Table 36).

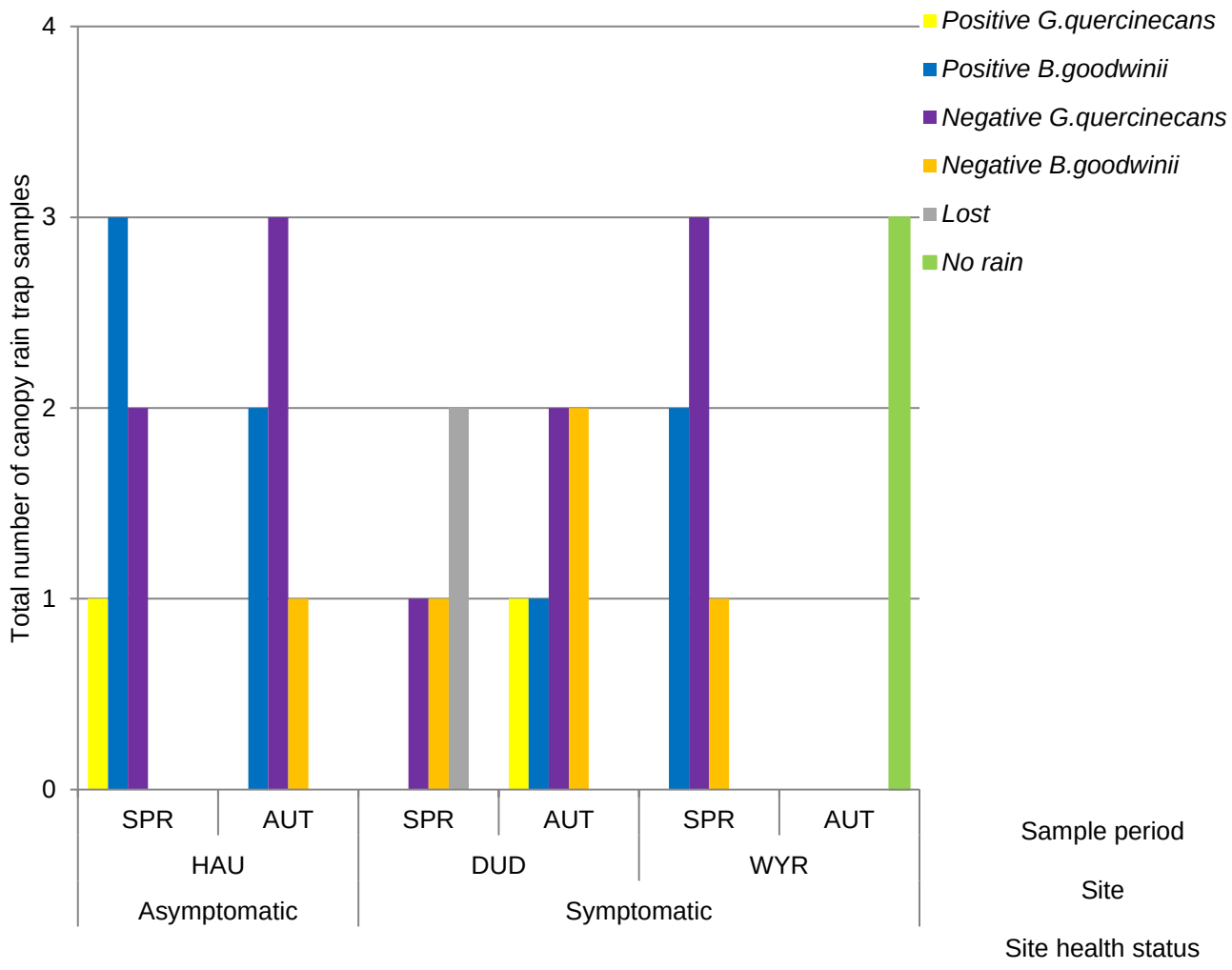


Figure 39. Positive and negative detections of *G.quercinecans* and *B.goodwinii* in the total number of canopy rain trap samples from asymptomatic trees sampled in spring and autumn in 2018 at asymptomatic and symptomatic sites

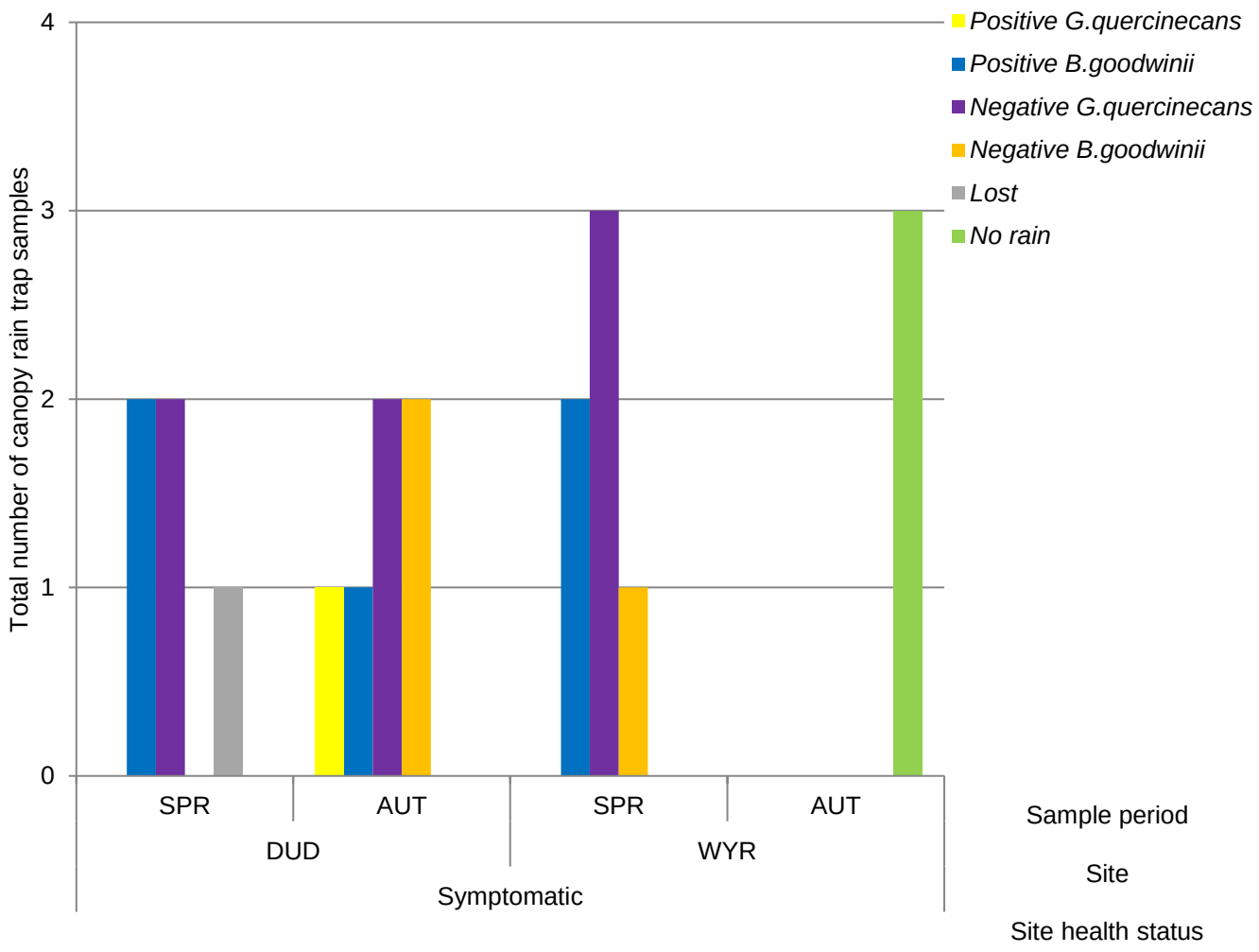


Figure 40. Positive and negative detections of *G.quercinecans* and *B.goodwinii* in the total number of canopy rain trap samples from symptomatic trees sampled in spring and autumn in 2018 at symptomatic sites

Table 36. Positive and negative detections of *G.quercincans* and *B.goodwinii* in canopy rain trap samples from asymptomatic and symptomatic trees at asymptomatic and symptomatic sites collected in spring and autumn 2018

Site health status	**Sample period**	**Site**	**Tree health status**	**Trap ID**	***G.quercinecans***	***B.goodwinii***
Asymptomatic	Spring	HAU	ASYM	HAUC1	negative	positive
				HAUC2	negative	positive
				HAUC3	positive	positive
		DUD	ASYM	DUDC1	negative	negative
				DUDC2	lost	lost
				DUDC3	lost	lost
Symptomatic			SYMP	DUDC1	negative	positive
				DUDC2	lost	lost
				DUDC3	negative	positive
		WYR	ASYM	WYRC1	lost	lost
				WYRC2	negative	positive
				WYRC3	negative	positive
			SYMP	WYRC1	negative	positive
				WYRC2	negative	positive
				WYRC3	negative	negative
Asymptomatic	Autumn	HAU	ASYM	HAUC1	negative	positive
				HAUC2	negative	positive
				HAUC3	negative	negative
		DUD	ASYM	DUDC1	positive	negative
				DUDC2	negative	positive
				DUDC3	negative	negative
Symptomatic			SYMP	DUDC1	positive	positive
				DUDC2	positive	positive
				DUDC3	negative	positive
		WYR	ASYM	WYRC1	no rain	no rain
				WYRC2	no rain	no rain
				WYRC3	no rain	no rain
			SYMP	WYRC1	no rain	no rain
				WYRC2	no rain	no rain
				WYRC3	no rain	no rain

5.3.2.2 Spring and Autumn ground raintrap samples

Spring ground rain samples at the asymptomatic site had a positive detection within them of *G.quercinecans* and *B.goodwinii* (Figure 41; Table 37). At one symptomatic site two spring samples at one site had a positive result for *G.quercinecans* (Figure 41; Table 37). In autumn two samples from the asymptomatic site were positive for *B.goodwinii* with *G.quercinecans* not detected at all (Figure 41; Table 37). Rain was only collected at one symptomatic site in autumn with *B.goodwinii* an *G.quercinecans* both detected (Figure 41; Table 37).

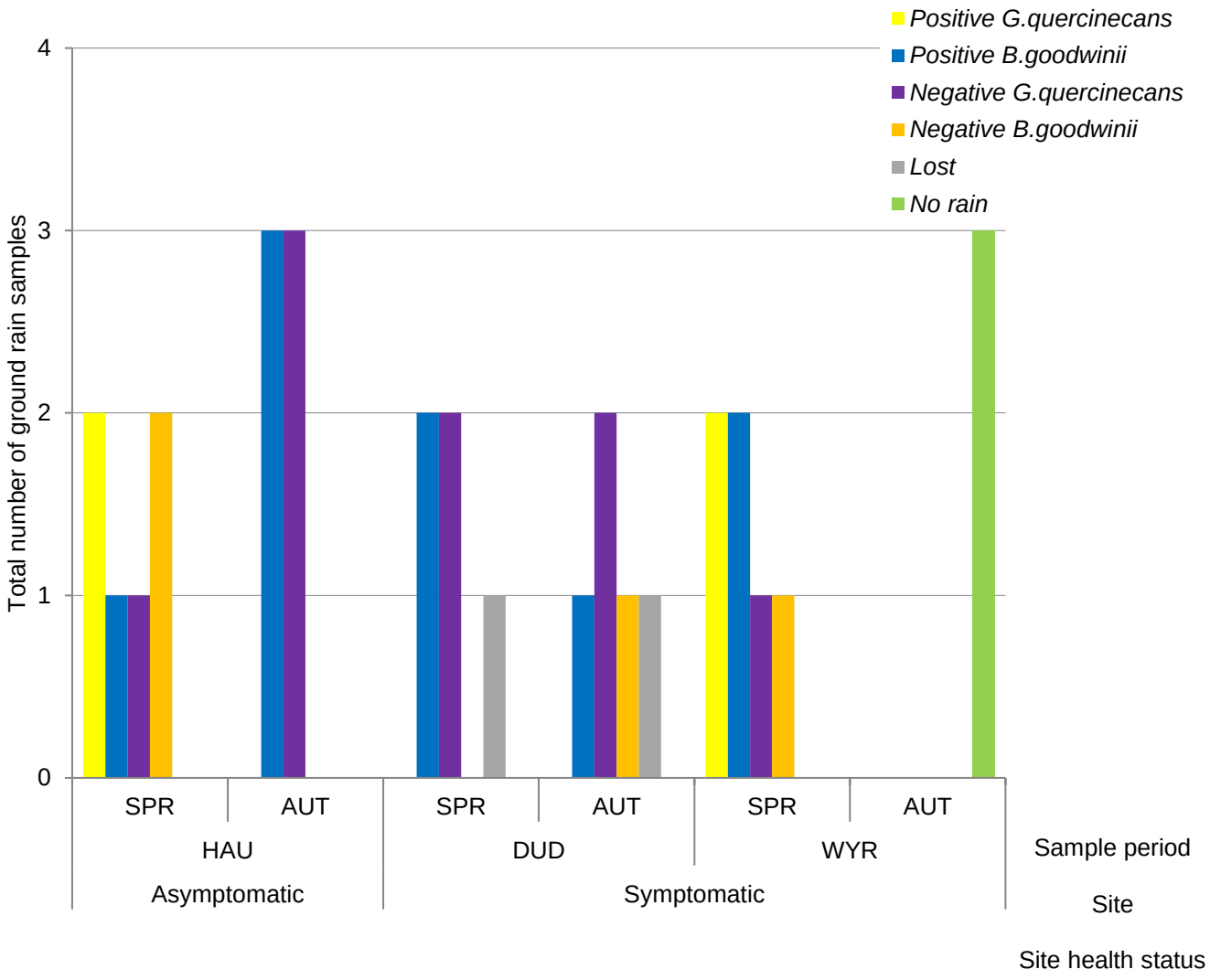


Figure 41. Positive and negative detections of *G.quercinecans* and *B.goodwinii* in the total number of ground rain trap samples from asymptomatic and symptomatic trees sites sampled in spring and autumn in 2018

Table 37. Positive and negative detections of *G.quercinecans* and *B.goodwinii* in ground rain trap samples from asymptomatic and symptomatic sites collected in spring and autumn 2018

Site health status	**Sample period**	**Site**	**Trap ID**	***G.quercinecans***	***B.goodwinii***
Asymptomatic	Spring	HAU	HAUG1	positive	negative
			HAUG2	negative	negative
			HAUG3	positive	positive
Symptomatic		DUD	DUDG1	negative	positive
			DUDG2	negative	positive
			DUDG3	lost	lost
		WYR	WYRG1	negative	positive
			WYRG2	positive	negative
			WYRG3	positive	positive
Asymptomatic	Autumn	HAU	HAUG1	negative	positive
			HAUG2	negative	positive
			HAUG3	negative	positive
Symptomatic		DUD	DUDG1	negative	positive
			DUDG2	negative	negative
			DUDG3	lost	lost
		WYR	WYRG1	no rain	no rain
			WYRG2	no rain	no rain
			WYRG3	no rain	no rain

5.4 Discussion

The association of *G.quercinecans* and *B.goodwinii* with rainwater is a new finding. The results presented in this chapter indicate aerial transport is a viable movement pathway for these bacteria and that they have the potential to be transported via rain and transferred to new hosts.

The presence of *G.quercinecans* and *B.goodwinii* on various external oak tissues indicates their availability for transport and detection in leaf washings highlights their capacity to be dislodged by water (Chapter 3). Rain collected from both symptomatic and asymptomatic sites were positive for *G.quercinecans* and *B.goodwinii*. This agrees with the results from Chapter 3 that the bacteria are present at both symptomatic and asymptomatic sites.

It also provides further evidence that both bacteria could be present within the wider environment and exist without causing harm to a plant host. Oak may therefore be one of many plant hosts utilised by *G.quercinecans* and *B.goodwinii*. Multiple hosts can be used by phytopathogens, not necessarily causing detrimental effects (Leben, 1974; Vanette, 1982). One such phytopathogen is *P.syringae* for which non-host plants can provide alternative hosts (Morris *et al*., 2008). The capacity of *G.quercinecans* and *B.goodwinii* to become pathogenic may consequently be prompted by environmental conditions or changes to host susceptibility (Broberg *et al.,* 2018) such as a decline in host resistance (Lucas, 1998).

Bacteria are also able to regulate their populations via quorum sensing (Bassler, 1999) and this may be occurring for *G.quercinecans* and *B.goodwinii*. In a study on *P.syringae* pv. *syringae* the capacity of the phytopathogen to initiate symptom expression was correlated to population size present on leaves (Rouse *et al.,* 1994). It remains unknown what population size of *G.quercinecans* and *B.goodwinii* is needed to initiate a host response, however it is possible a certain threshold needs to be reached before pathogenicity is triggered. Investigation of the population levels required to initiate a host defence response e.g. through controlled application of different bacterial concentrations onto various oak tissues would add to the understanding of AOD progression.

Abiotic conditions influence bacterial colonisation and communities (Laforest-Lapointe *et al*., 2016; Ottesen *et al*, 2016). For example, increased bacterial growth following rain has been recorded in several experiments (Lindemann and Upper, 1985; Champoiseau *et al*., 2009; Jang *et al*., 2018; Allard *et al*., 2020). The amount of collected airborne bacteria has also been shown to increase during hot sunny days (Lindemann and Upper, 1985). Relative humidity (Xin *et al.,* 2016; Jang *et al*., 2018) and temperature (Walter *et al*., 1990; Biosca *et al*., 2019) may also have an effect.

Geographical distance and abiotic factors were an influence on the bacterial communities of oak (Sapp *et al*., 2016) and site conditions have been shown to be a predisposing factor in determining the susceptibility of a location to AOD (Brown, 2014). Sites with lower recorded rainfall and seasonally waterlogged soil were found to be more likely to have AOD present (Brown *et al*., 2018). Remission from AOD infection is possible (Brown *et al*., 2016) and a linear disease progression of AOD was not found over four years at eight monitored woodland plots (Brown, 2014). Evidence from studies on bacterial colonisation and growth as well those on the epidemiology of AOD suggest that site conditions and abiotic factors influence the presence AOD. The results from this chapter indicate that rainfall potentially plays a key role in disease development.

Raintraps placed in the lower canopy and on the ground were a successful means of collecting rainwater. Collected rain may have been directly captured as it fell, runoff from leaves or wind driven rain. As observed from other studies rain can dislodge bacteria from leaf surfaces (Butterworth and McCartney, 1991) and along with wind driven rain transport them over various distances (Bashan, 1991; Bock *et al*., 2005). In a study on the aerial dispersal of Arctic bacteria both long distance movement and local sources were found to contribute to atmospheric bacteria available for deposition (Šantl-Temkiv *et al*., 2018). The presence of *G.quercinecans* and *B.goodwinii* in ground and canopy caught rain could therefore indicate they are able to be transferred between leaves of the same tree and over short distances.

Oak leaves are relatively stiff and evidence from rainsplash dynamics shows that this type of leaf favours a droplet on droplet dispersal (Gilet and Bourouiba, 2015) and the displacement of larger water droplets (Gilet and Bourouiba, 2014). This was found to be effective at displacing droplets over greater distances (Gilet and Bourouiba, 2014) and could result in a more localised pathogen spread (Gilet and Bourouiba, 2015). As both bacteria have been detected after inoculation in soil (Pettifor *et al*., 2020), it cannot be discounted that the traps may have contained cells displaced from the soil by rain. Trees with AOD have been observed to 'cluster' (Brown, 2014), so a localised spread via rain is possible. Evidence from Gilet and Bourouiba (2014) also suggest transport of *G.quercinecans* and *B.goodwinii* over longer distances could be possible.

No rain collection in autumn at one site and lost samples have limit the ability to draw definitive conclusions from the results. In addition it remains uncertain whether detections of *G.quercinecans* and *B.goodwinii* in rainwater were of live or dead cells. Using sterile rainfall inoculated with cultures of *G.quercinecans* and *B.goodwinii* Pettifor *et al.* (2020) found that *B.goodwinii* did not produce viable colonies at initial inoculation or at any time point after.

On the other hand *G.quercinecans* colonies were countable throughout the experiment. A similar approach with collected rainwater would give an indication of their survival potential. Raintraps at increasing distances from a tree and across a site could also reveal whether long distance movement is possible. Alternately there are several methods for the collection of airborne bacteria (Lundholm, 1982; Després *et al*., 2012; Mbareche *et al*., 2017) which could be utilised to investigate transport of *G.quercinecans* and *B.goodwinii.*

There was little consistency to the positive and negative detections from the same traps; a result in spring not correlating to the result in autumn. Detection of *G.quercinecans* was higher in autumn canopy samples compared with spring samples. Compared to *B.goodwinii*, fewer samples were also positive for *G.quercinecans* in spring. Within symptomatic tree canopy samples *G.quercinecans* was only detected in autumn rain whereas *B.goodwinii* was recorded in spring and autumn canopy traps. The same pattern was observed in ground traps at asymptomatic and symptomatic sites.

These differences suggest seasonal changes in environmental conditions affects bacterial survival or might be simply a reflection of abundance. Seasonal changes to leaves (Lindow and Brandl, 2003) and leaf structure can determine successful colonisation and establishment of deposited bacteria (Muller et al., 2003; Monier and Lindow, 2005). Results from Chapter 3 however identified both *G.quercinecans* and *B.goodwinii* in spring, autumn and litter leaf samples. Greater detection of *B.goodwinii* may be due to it being present more consistently on leaf surfaces; adding support to the suggestion of Chapter 3 that *G.quercinecans* is endophytic and *B.goodwinii* epiphytic. Bacteria can shift from an epiphytic to an endophytic state (Beattie and Lindow, 1995) therefore it cannot be discounted that *G.quercinecans* has an epiphytic phase.

The overall greater number of detections of *B.goodwinii* at symptomatic sites could lend support to the proposal that it has a primary role in AOD (Broberg *et al.,* 2018). The apparent capacity of *B.goodwinii* to form biofilms (Doonan, 2016), a potential mechanism that increases the aggregation of cells at certain places on a leaf and enhances the likelihood of cells being dislodged by rain (Monier *et al*., 2003; Morris and Monier, 2003) could also be responsible for a greater level of detection.

Two culture methods have been compared by Yadav *et al.,* (2010); leaf imprint - whereby a leaf was pressed into nutrient agar and serial dilution plating - where leaves were homogenized and spread on nutrient agar in a serial dilution. This comparison found serial dilution plating more effective for looking at the total abundance of epiphytic bacterial populations although the use of leaf imprints had a higher ability to detect bacteria on a leaf surface (Yadav *et al.,* 2010).

Using methods like these the locations of *G.quercinecans* and *B.goodwinii* on leaves could be found along with population density. This would both give an indication of whether either species forms aggregations and help answer the question of the threshold population needed to initiate host responses.

The use of a 16S universal assay to detect whether any bacterial DNA was present within the rain samples indicates that the method of DNA extraction used was effective. Filtering and centrifuging the rainwater to concentrate bacterial cells allowed a greater proportion of the collected rain to be analysed and potentially resulted in a higher number of detections. An increased length of time in the centrifuge and further filtering would potentially yield a higher amount bacterial cells.

5.5 Conclusion

The mechanism by which *G.quercinecans* and *B.goodwinii* colonise new hosts has been an unanswered question. The results of this chapter has highlighted aerial transference e.g. rain splash and airborne distribution as potential movement pathways. There is also evidence to suggest that whilst both bacteria are found together they may have different lifestyles and survival strategies.

Chapter 6

Overall discussion and recommendations for further study

6.1 Overall discussion

The association of *Brenneria goodwinii* and *Gibbsiella quercinecans* with the oak phyllosphere has been an under investigated area in Acute Oak Decline research. How *G.quercinecans* and *B.goodwinii* might be transported to new hosts was also an unanswered question in the epidemiology of AOD. The focus of this thesis was therefore to:

1) Identify the associations of *B.goodwinii* and *G.quercinecans* with the oak phyllosphere, focusing on live oak leaves, leaf litter, acorns and catkins.

2) Investigate two potential transmission mechanisms
    i. movement via insect vectors to focus on xylophagous coleoptera.
    ii. airborne movement via rain.

6.1.1 The oak phyllosphere, *B.goodwinii* and *G.quercinecans*

The plant phyllosphere is known to harbour a diverse array of microorganisms, including bacteria, so the detection of *G.quercinecans* and *B.goodwinii* is a valid one. For foliar bacterial endophytes and epiphytes the surface of the leaves is where initial colonisation is likely to take place (Vanette, 1982; Beattie and Lindow, 1999; Lindow and Brandl, 2003; Frank *et al.,* 2017). Detection of *G.quercinecans* and *B.goodwinii* on leaves (Chapter 3) indicates this may be a point of initial colonisation. Other phyllosphere bacterial communities utilise flowers and seeds (Aleklett *et al*., 2014; Rosenbleuth and Martinez-Romero, 2006). Both *G.quercinecans* and *B.goodwinii* have been identified on acorns and catkins (Chapter 3); together with leaves indicating they may be part of the oak bacterial community.

The inability of bacteria to actively create openings through which they can move into a plant hosts tissues means they need to exploit existing openings (Manion, 1991) such as wounds or stomata. Stomata in particular are an established entry point for pathogens and this may be a means by which they enter oak tissue. Feeding wounds created by insect leaf feeding (Orlovskis *et al*., 2015) or through excavating exit holes are another possibility and wounds on bark have already been shown experimentally to facilitate entry of *G.quercinecans* and *B.goodwinii* into vascular tissues (Denman *et al*., 2018).

The possibility of *B.goodwinii* or *G.quercinecans* being epiphytic or endophytic has been previously alluded to (Denman *et al*., 2018) and phylogenetic analysis indicates *B.goodwinii* is closely associated with necrotrophs and *G.quercinecans* with saprophytes (Doonan *et al*., 2019). Necrotrophs utilise dead or dying tissue of declining hosts, are opportunistic and non obligate whilst saphrotrophs use only dead tissue (Lucas, 1998, Glazebrook, 2005). Unlike *G.quercinecans,* which was identified in all samples, the detection of *B.goodwinii* via qPCR but not culturing after soil inoculation has additionally led to the proposal it is an oak obligate endosymbiont and unable to exist outside the host environment (Pettifor *et al*., 2020); its presence in qPCR a result of entering a viable but non-culturable state in the soil (Pettifor *et al*., 2020). The presence of *B.goodwinii* in litter samples (Chapter 3) could however mean that it requires this resource and is unable to exist alone in soil. The results of Chapter 3 have also added to the wider suggestion that *G.quercinecans* and *B.goodwinii* have different lifestyles and niches or are able to switch between epiphytic and endophytic states.

The presence of virulence factors in *G.quercinecans* and *B.goodwinii* (Brown *et al*., 2018) may therefore not necessarily be an indication of pathogenicity. There is evidence virulence factors in opportunistic pathogens can confer advantages to the bacteria e.g. enhancing survival (Brown *et al*., 2012). Most plant pathogens, bacteria included, are also not usually obligate on a single host (Morris *et al.,* 2008; Frank *et al.,* 2017). UK populations of *B.goodwinii* have been shown in phylogenetic analysis to be highly diverse with individual sequence types dominating different sites and individual trees (Kaczmarek *et al*., 2017). The ability of *G.quercinecans* to survive in soil also supports the suggestion it is a generalist bacteria and potentially widely distributed in a forest environment (Pettifor *et al*., 2020). Evidence from Chapters 3-5 indicates both bacteria within leaves, litter, acorns and catkins, insect samples and rainwater adding to the suggestion both bacteria are opportunistic taking advantage of a weakened or stressed host.

Bacteria able to survive host gaps have an obvious advantage over those that cannot (Allen *et al*., 2009). Survival strategies include using overwintering resources, persistence in additional hosts or alternative hosts, aggregation and reducing metabolism (Leben, 1974; Vanette, 1982, Brown, 1997; Allen *et al.,* 2009). Several of these may be applicable to *G.quercinecans* and *B.goodwinii.* Results from Chapter 3 suggest litter could act as an overwintering resource in the period December to February when exudate dries up (Denman *et al*., 2014) whilst Chapter 4 shows various oak associated Coleoptera may act as additional or alternate hosts. The presence of both bacteria in rainwater (Chapter 5) also hints at the use of aggregation and the potential for other hosts besides oak.

One bacterial adaptation that may be applicable to AOD is persister cells. Phytopathogens use this strategy to survive periods of stress and is potentially a reason why they are not eliminated by the biotic and abiotic stresses to which they are subjected (Martins *et al*., 2018). Persister cells can be activated by changes in temperature, toxicity and oxidative stress (Martins *et al*., 2018). Although there are few known mechanisms of persister formation for phytopathogenic bacteria, in *P.syringae* the MarR (Multiple antibiotic resistance regulator) and LysR regulators are stimulated and in *E.amylovora* the RpoS (RNA polymerase, sigma S) regulator is involved (Martins *et al*., 2018). These regulators repress virulence factors and the TCA (Tricarboxylic Acid Cycle) cycle within the bacteria ultimately leading to ceasing of cell growth and finally persistence (Martins *et al*., 2018). *G.quercinecans* and *B.goodwinii* have MarR and RpoS and *G.quercinecans* LysR (Doonan, 2016). The use of insects as alternative hosts (Nadarash, 2011) is also a possibility given the evidence presented in Chapter 4.

The bacterial communities of oak have been shown to vary with site, potentially due to abiotic conditions (Sapp *et al.,* 2016) and seasonal changes have been observed in the bacterial communities of other plant species (Ercolani, 1978; Thompson *et al.,* 1993). These findings are reflected in the results from this study where individual sites showed different patterns in bacterial detection as well as seasonal differences. Over time it has been observed that available nutrient resources and competition become limiting factors to bacterial population density on plant surfaces (Suoniemi *et al*., 1995). Site abiotic and biotic differences could therefore play a key role in determining bacterial presence; an aspect of AOD that has already been highlighted (Brown *et al.,* 2018).

In an agricultural setting it has been noted that the continuous growing of a monoculture in the same place can lead to the soil and surrounding environment becoming biased towards a selective group of bacteria (Baltrus, 2020). The bacteria that are detected have adapted over time to survive alongside the crop and could be said, in effect, to have been selected for by the plants (Baltrus, 2020). This could be happening in the case of AOD within the oak dominated woodlands and parklands used in this study.

Exudates and VOC's released by plants have been shown to attract microbes (Baltrus, 2020) and are thought to prime plant defences to mitigate against future stresses (Brilli *et al*., 2009, Liu and Brettel, 2019). They are important for plant to plant communication (Moreira and Abdala-Roberts) and can act either to prevent colonisation of bacteria that may negatively impact a plant or to selectively enhance the growth of beneficial bacteria (Baltrus, 2020). In addition Orlovskis *et al.* (2015) noted that bacteria can alter plant signals to attract

insects. For AOD, VOC's have only been studied in relation to *A.biguttatus* (Vuts *et al*., 2016) however it may be that the VOC's emitted by oak have a more wide reaching purpose.

Co-evolution between plants and microbes is important in understanding long term interactions, geographical differences and biogeographic patterns however its influence is largely unknown (Baltrus, 2020). An increased understanding of microbial biogeography could further lead to predictions of future changes and their roles in ecosystems (van der Gast, 2015).

6.1.2 Potential movement pathways of *B.goodwinii* and *G.quercinecans*

In order to move to and successfully colonise new hosts bacteria must utilise movement pathways since they are unable to actively move themselves (Frank *et al.,* 2017). Several dispersal mechanisms for phytopathogenic bacteria are by now widely established including wind, rain, seeds and vectors (Esker *et al.,* 2007; Frank *et al.,* 2017). Colonization can be described as vertical, whereby bacteria survive through successive host generations or horizontal with each host generation acquiring the bacteria anew from their environment (Bright and Bulgheresi, 2010). A combination is also possible (Bright and Bulgheresi, 2010). Multiple dispersal routes for *G.quercinecans* and *B.goodwinii* has already been proposed (Brown, 2014) and the results of Chapters 3-5, support this suggestion.

One example of a horizontal transmission route is via insects and this has been highlighted in Chapter 4 as a potential path for *G.quercinecans* and *B.goodwinii*. For insect transmission to be possible there must be contact between the two (Nadarash and Stavrinides, 2011). Multiple insects are known to be involved in transmission (Orlovskis *et al.,* 2015) and the five Coleoptera that were analysed all have associations with oak. The positive detections on Coleoptera with differing lifestyles is suggestive of incidental contact rather than a specific host-vector association.

Microorganisms found within the environment can also be present in the guts of insects (Engel and Moran, 2013) and these Coleoptera may have acquired bacteria via feeding on oak tissues. In order to persist through the digestive tract (Nadarash, 2011, Orlovskis *et al*., 2015) both *G.quercinecans* and *B.goodwinii* would have to overcome insect defences however further evidence is needed on whether these bacteria are able to do this. Nevertheless incidental contact is still a viable route for *G.quercinecans* and *B.goodwinii* to come into contact with new hosts.

Both *G.quercinecans* and *B.goodwinii* have been found on acorns and catkins (Chapter 3) which could indicate the potential use of a vertical transmission pathway. Bacteria on seeds

may be beneficially selected for by parents, for example if they aid germination, and transferred to the next generation. (Truyens *et al*., 2014). This transference from parent to seed has been shown for several crop species (Truyens *et al*., 2014).

Detecting bacteria in seed and flowers does not necessarily mean the parent plant is the original source since seeds and flowers can acquire bacteria from the environment (Frank *et al.,* 2017). One mechanism through which seeds can attract bacteria is via chemical secretion (Truyens *et al*., 2014). In a review study of 131 bacterial genera from 25 different plants most were reported as similar to soil strains indicating this as the likely place of colonisation (Truyens *et al*., 2014). The number of acorn samples in this study was low so strong conclusions cannot be drawn, however *G.quercinecans* has been shown to survive in soil (Pettifor *et al*., 2020) and therefore this transmission pathway should not be ruled out.

The involvement of *A.biguttatus* is mentioned continuously in AOD studies (Denman *et al.,* 2010; Denman *et al.,* 2014; Brown *et al.,* 2015; Vuts *et al.,* 2016; Broberg *et al.,* 2018; Denman *et al*., 2018). Evidence from a review on European oak declines suggests bark and wood boring beetles, including *A.biguttatus*, are mainly secondary colonisers of trees in poor health (Sallé *et al*., 2014). The larval galleries found behind necrotic tissue and exit holes on trees with AOD (Brown *et al.,* 2015) also suggest a secondary impact. The results of Chapter 4 do not support the idea of *A.biguttatus* as a vector in agreement with Brown (2014) although incidental contact with *G.quercinecans* and *B.goodwinii* is clearly possible.

Climate change will affect interactions between pathogens, hosts and environment (Sturrock *et al*., 2011). Host susceptibility, phenological changes and the geographic distributions of host and pathogens are all predicted to change (Sturrock *et al*., 2011). As pathogens are able to adapt to new conditions and move to more favourable areas faster than a long lived stationary tree the impacts could be uncertain and complex (Sturrock *et al*., 2011). Research has already focussed on the temperature range of *A.biguttatus* and its potential shift due to climate change (Reed *et al*., 2018) however the temperature range of *G.quercinecans* and *B.goodwinii,* established as factors in Acute Oak Declines, may be of importance for predicting future outbreaks. Additional factors such as vectors and external reservoirs (Page *et al.,* 1995), potentially identified for AOD as the oak phyllosphere and soil (Pettifor *et al*., 2020), may prove relevant to the movement of AOD under climate change scenarios.

6.1.3 Is management of Acute Oak Decline possible?

Acute Oak Decline is currently identified as a decline disease (Denman *et al.,* 2014) with biotic and abiotic factors sequentially contributing to a decline in tree health (Manion, 1991). These components have been separated into predisposing, inciting and contributing factors (Table 1) and this model can be applied to Acute Oak Decline (Table 38).

Table 38. Predisposing, inciting and contributing factors of the decline disease concept (Manion, 1991) applied to Acute Oak Decline

	Predisposing	**Inciting**	**Contributing**
Mode of action			
	Long term slow changing	Short term	Long and short term
	Soil moisture[1]	Drought[1]	*G.quercinecans* and *B.goodwinii* [3]
	Clay rich soil[1]	Seasonally waterlogged soil[1]	Insect activity[4,5,6]
	High soil nitrogen[1]	Defoliation[2,3]	

[1]Brown *et al.,* 2018; [2]Denman *et al.,* 2010; [3]Brady *et al.,* 2010; [4]Vansteenkiste *et al.,* 2004; [5]Sallé *et al*., 2014; [6]Moraal and Hilszczanski, 2000

Not all decline syndromes however fit a single model (Oliva *et al*., 2013) with Sinclair and Hudler (1988) presenting four scenarios; i) decline by continuous effect of one factor ii) decline by single major factor and secondary stress iii) interchangeable predisposing, inciting and contributing factors and iv) group decline. Earlier decline disease concepts interpreted and advanced by Manion (1999) have also evolved (Manion, 2003). Earlier ideas of i) disease is not healthy and ii) decline disease affects weakened trees (Manion, 1999) have developed into i) a sustainable amount of disease is needed and ii) decline diseases remove dominant trees to stabilize the forest (Manion, 2003). This is expanded to explain that whilst dominant trees within the canopy have succeeded competitively it is potentially at the expense of genetic diversity and stress tolerance (Manion, 2003). The function of decline is therefore to naturally remove a portion of these mature trees so other individuals can mature and increase genetic diversity (Manion, 2003).

The multiple interacting components of the decline disease model make a clear management strategy challenging leading to questions over its place as a distinct disease category (Ostry *et al*., 2010). The main points against its use are that tree diseases are naturally complex and even those caused by a single damaging agent will have associated symptoms and be affected by environmental conditions, host susceptibility and interacting factors e.g. climate, insect damage (Ostry *et al*., 2010). Whilst a decline model can illustrate the complexity of tree disease, an equally applicable model to use for AOD is an

epidemiological triangle whereby interactions between host-environment-agent are required for disease to occur (Ostry *et al*., 2010, Oliva *et al*., 2013). When this is applied to AOD (Figure 42) the most likely factors involved can be clearly seen.

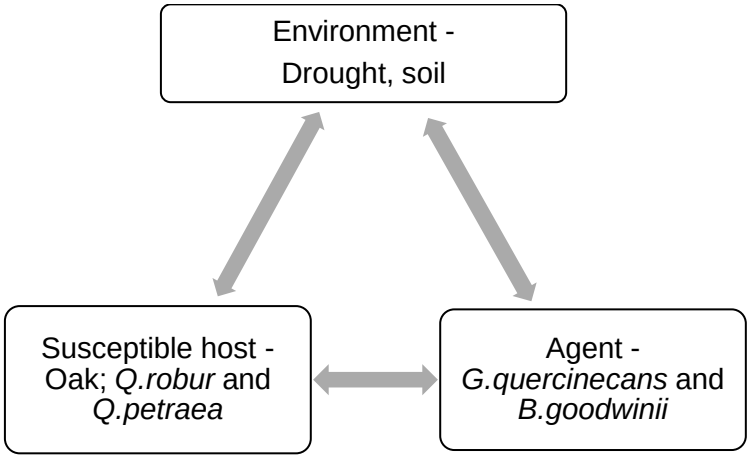


Figure 42. The epidemiological triangle disease model applied to AOD

Using this model it is easier to see where management interventions can be used (Ostry *et al.*, 2010). Under the supposition of decline being a natural phenomenon the role of disease in forest health should be thought of in terms of its impact and the extent of the effect on individual sites since differing levels of negative impacts will be sustainable (Manion, 2003). By establishing a baseline tolerance to AOD as suggested by Manion (2003), management can be aimed at preventing the baseline from being exceeded and an unhealthy level of disease and tree loss occurring.

Although plant responses to multiple stresses still require more investigation (Szczepaniec *et al*., 2019, Crowder *et al*., 2019) by predicting future outbreaks mitigation measures can be applied (Oliva *et al*., 2013). The developing field of precision arboriculture using remotely piloted aircraft to survey large areas, modelling and mapping tree health (de Petris *et al*., 2019) has potential to help with assessing AOD in the field. Genetic, population and age uniformity all increase vulnerability to disease epidemics (Oliva *et al*., 2013). To reduce host and site susceptibility the use of resistant oak varieties and increasing genetic variability on a site could help prevent an unsustainable level of disease being reached.

Low rainfall was associated with AOD presence on sites and appears to be an environmental, inciting factor in disease development (Brown *et al.,* 2018). Restricting water availability and localising desiccation at the infection site are a plant defence mechanism to restrict bacterial growth (Beattie, 2011). Drought can decrease plant resistance to

phytopathogens through changes to metabolism and defences (Szczepaniec *et al*., 2019) and is a recognised factor in increased risk of insect damage (Sallé *et al*., 2014). The effects of drought on plants impact insect behaviour and could increase vector movement to new hosts (Szczepaniec *et al*., 2019). Plants have also been shown to prioritise water stress over pathogen infection (Beattie, 2011).

Climate change is predicted to increase the frequency of drought events (Sallé *et al*., 2014). This has implications for AOD as an increase could to lead to a higher risk of it developing on a site. Water retention in a forest can be influenced by different site features and where retention is enhanced can mitigate the effects of drought (European Environment Agency, 2015). Influential features can include the area and density of tree cover, species composition, age and extent of lower vegetation layers (European Environment Agency, 2015). Management practices can impact all of these at a site level for example multiple storey vegetation enhances water interception whilst mixed aged stands ensure continuous cover (Schüler, 2006).

Other environmental factors, all related to soil, appear to provide a favourable environment for AOD establishment (Brown *et al.,* 2018). Seasonally waterlogged soil, high soil clay content, high soil nitrogen and low sulphur were all correlated with the presence of AOD on a site (Brown *et al.,* 2018). These soil parameters all have effects on tree health. High soil nitrogen and low sulphur are both associated with poor tree health (Brown *et al.,* 2018). A high clay content and waterlogging both leave soils vulnerable to compaction which has negative impacts on plant growth, soil microorganisms (Nawaz *et al*., 2013), reduces aeration and available water for plant uptake (Sekwakwa and Dikinya, 2012).

A uniformity of crop can facilitate the build up of a pathogen due to the high availability of potential hosts and may perpetuate its survival (Schuster and Coyne, 1974). Diversifying the tree species on site could therefore help reduce the number of potential hosts and the establishment of *G.quercinecans* and *B.goodwinii.* Diversification of species could also have additional benefits. One management strategy suggested to disrupt the splash dynamics of rainfall dissemination of bacteria was to alternate plants with varying leaf structures (Gilet and Bourouiba, 2014). By interspersing trees that have light flexible leaves in between oaks, with more rigid leaves, the contamination range of droplets carrying *G.quercinecans* and *B.goodwinii* could be reduced. Encouraging mixed species woodland or stands could therefore be advantageous in mitigating the spread of *G.quercinecans* and *B.goodwinii* via rain. In addition increasing spacing between plants beyond that of the minimum distance required for rain splashed water droplets to travel could reduce contamination risk by rain (Gilet and Bourouiba, 2014).

Conclusion

Acute Oak Decline is currently affecting *Q.robur* and *Q.petraea* in the UK. This study has newly shown that *G.quercinecans* and *B.goodwinii,* bacteria associated with stem bleeds, are also present in the oak phyllosphere. Several potential vectoring mechanisms have in addition been highlighted. These new findings contribute to the existing research on AOD and can feed into management strategies, several of which have been discussed here.

6.2 Recommendations for further research

As a result of the conclusions presented in this thesis two areas of further research are suggested.

i) Although part of the research in Chapter 3 showed that *G.quercinecans* and *B.goodwinii* were alive samples of oak leaves and litter since they could be cultivated there still remains the question of whether all the detections from the phyllosphere samples were of live or dead bacteria. The use of culturing techniques for samples of leaf washes and rainwater immediately after collection could show the viability of cells. Collections of air samples could also be taken to look at this transmission pathway.

ii) There are suggestions that *G.quercinecans* and *B.goodwinii* have different lifestyles. Examining the genetic composition of *G.quercinecans* and *B.goodwinii* was beyond the scope of this study however further study into their lifestyles would be beneficial. For example: if these bacteria are opportunistic, what triggers a change to pathogenic? Are both or one of the bacteria epiphytic or endophytic or able to switch between the two? Knowing what may trigger potential transitions could help with disease management.

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Appendix 1

Six species of *Agrilus* are found in the UK. Their associations as reported in Jendek & Poláková (2014) are shown in Table 1. Information up to the end of February 2014 was included. A confidence rating increasing from 0-3 was used to assess the reliability of the records found. For this reason only records with a confidence of 1-3 are included. Plant varieties have been excluded and species with the same confidence level for one or more *Agrilus* have been grouped together for simplicity.

0. Misidentification or misinterpretation of the association
1. Evidence from adult host record. No larval host record available.
2. Supported by adult host record where the source is considered the most reliable. Also includes host plants where larval associations with the plant genus are available as supporting evidence.
3. The most reliable associations supported by larval host records. Also includes records where the adults are commonly collected from the same plant species but larval host is unknown.

Table 1. Host associations as referenced in Jendek & Poláková (2014) Host Plants of World *Agrilus*

Agrilus species	*A.angustulus*	*A.biguttatus*	*A.cuprescens*	*A.cyanescens*	*A.laticornis*	*A.sinuatus*	*A.sulcicollis*	*A.viridis*
Confidence rating								
Plant association								
Acer sp.; A.campestre; A.opalus; A.platanoides; A.tataricum; A.pseudoplatanus								3
Alnus sp.					1			3
A.cordata; A.viridis								2
A.glutinosa	1							3
A.incana								3
Betula sp.	1				1			3
B.nana								2
B.pendula	1							3
B.pubescens								3
Carpinus sp.; C.betulus	1				1			3
C.orientalis								3
Castenea sp.	3						1	
C.sativa	3	3			3			2
Corylus sp.; C.avellana	3				3	1		3
Cotoneaster						3		
Crateagus sp.; C.azarolus; C.curisepala; C.laevigata; C.monogyna						2		
Cydonia								
Fagus sp.	3	3			1		3	3
F.sylvatica	3	3					3	3
F.orientalis								3
Lonicera sp; L.implexa; L.nigra; L.periclymenum; L.tatarica; L xylosteum				3				
L.caerulea; L.involucrata; L.morrowii				2		3		

Malus sp; M.domestica; M.pumila							**3**			
Mespilus sp.; M.germanica										
Myrica gale										3
Ostrya carpinifolia	3									1
Populus sp.							3			
Prunus sp.; P.davaicata							3			
Pyrus sp; P.amygdaliformis; P.communis; P.pyraster										
Quercus sp.; Q.petraea; Q.pubescens; Q.robur	3	3			3			3		
Q.cerris	3	3			2			2		
Q.suber	3	3			2					
Q.coccifera; Q.rubra	3									
Q.delachampii	3	3								
Q.faginea	2	3								
Q.frainetto	3							3		
Q.ilex	2	3			2					
Q.macranthera	3									
Q.rotundifolia	2									
Rhamnus sp.; R.carthartica				3						
R.alaternus										1
Rosa sp; R.acicularis; R.arkansana; R.blanda; R.canina; R.carolina; R.damascena; R.jundzillii; R.laxa; R.multiflora; R.nitida; R.rubiginosa; R.rubrifolia; R.silverhjelmii; R.spaldingii; R.spinosissima; R.woodsii			3							
R.alpina			2							
R.hugonis			2	3						
R.rugosa;			3	3						
Rubus sp.; R.caesius; R.fruiticosus			3							

Salix sp.; S.alba; S.aurita; S.caprea; S.cinerea; S.purpurea; S.sachalinensis; S.schwerinii; S.viminalis				**3**
S.auriculata; S.lanata			2	2
S.elaeagnifolia;			3	
Sorbus sp.; S.aria; S.acuparia; S.hardeggensis; S.intermedia				
Symphoria racemosa		1		
Symphoricarpos sp.; S.orbiculats		1		
Tilia sp.; T.cordata				3
Ulmus laevis	1			
U.minor				1