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

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

Bethany Pettifor

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A thesis submitted to Bangor University in candidature for the degree

Master of Science by Research

School of Natural Sciences

Bangor University, Deiniol Road, Bangor, LL57 2UW

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Summary

Acute oak decline (AOD) is a decline disease affecting oak species native to the UK (*Quercus robur* and *Q. petraea*). Part of the wider decline complex, AOD causes tree health decline, causing mortality in as little as five years. Of the four symptoms expressed by AOD-symptomatic trees, the most obvious are black weeping lesions on the stem. The cause of these lesions has been linked to two bacteria, *Brenneria goodwinii* and *Gibbsiella quercinecans*, however there is no knowledge on the ecological and environmental reservoirs of these phytopathogens. Rainwater and forest soils are common reservoirs of plant pathogens in a forest environment. Therefore, the aim of this study was to investigate the survival rates of *B. goodwinii* and *G. quercinecans* in rainwater and forest soil using a combination of agar-based colony counts and quantitative PCR (qPCR) detection methods. *Brenneria goodwinii* lost viability in both soil and rainwater at the time of inoculation. *Gibbsiella quercinecans*, however, could be isolated from rainwater for the entire duration of the experiment (84 days) and was isolated from forest soil up to day 28 of the experiment. These results demonstrate that *G. quercinecans* has the capacity to survive in environmental forest reservoirs outside of the tree host. qPCR analysis revealed that detection of the *G. quercinecans* GyrB gene declined in a similar trend to the culture-based study in forest soil. Detection of the *B. goodwinii* GyrB gene, however, was present up to day 28 of the soil survival experiment. Although further study would be required to understand this, *B. goodwinii* appears to have a limited ability to persist outside of the host, potentially in a viable but non-culturable (VBNC) state. These data provide evidence that *B. goodwinii* is an endosymbiont of oak trees, whereas the ability of *G. quercinecans* to remain viable in soil and rainwater biomes suggests a broad ecological distribution. These data advance understanding of the potential epidemiology of AOD-associated bacteria and their ecological reservoirs, thus increasing the overall knowledge of the pathology of AOD, which could aid in the development of future management strategies.

CHAPTER 1
Introduction

1.1 Introduction

Forests and woodlands cover approximately thirty percent of the Earth's land surface (Carlowicz, 2012), and include both farmed and naturally occurring forests, which are present at a range of latitudes. These include sub-polar temperate woodlands, areas of alpine vegetation, coniferous forests, deciduous broadleaf forests (the most common forest type in the UK), through to tropical rainforests near the equator (European Environment Agency, 2006; Pautasso, 2016). Forests are comprised not only of various tree species, but a multitude of interdependent plant and animal species, as well as microbial communities (Andreote and Pereira E Silva, 2017; Gamfeldt et al., 2013). It is this species richness that makes forests some of the most biodiverse ecosystems on the planet and makes them vital for the provision of key ecosystem services for the general functioning of the Earth (Boyd et al., 2013; Freer-Smith and Webber, 2017).

1.2 Ecosystem Services

Services provided by forests are also known as "ecosystem services" and can be divided into four primary categories (Figure 1.1); support, provision, culture and regulation, all of which contribute to human well-being (Zhang et al., 2007).

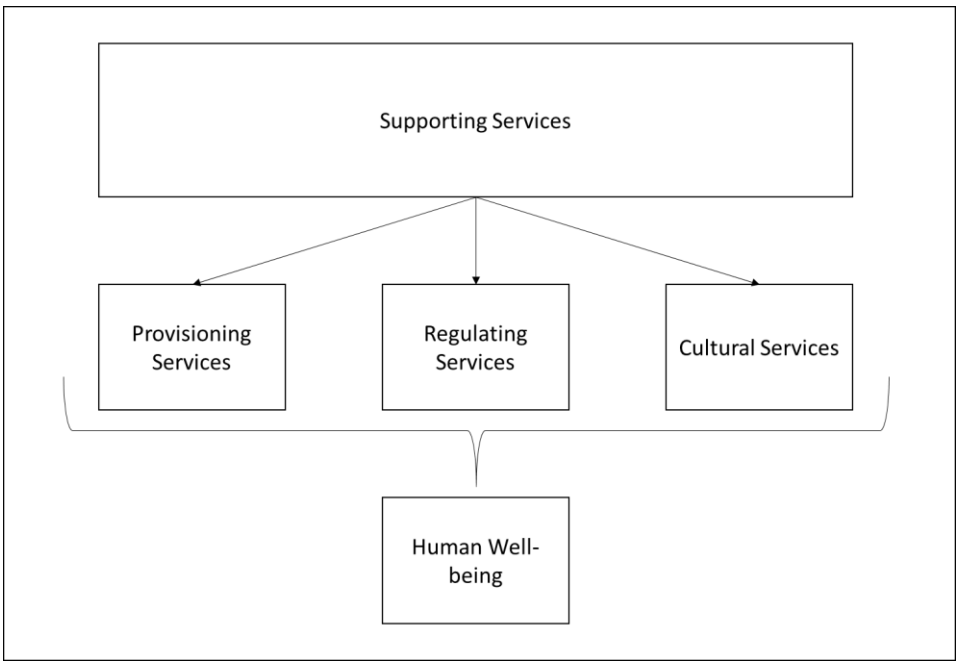

Figure 1.1. Flow diagram of ecosystem services and how they link with and lead to human well-being. Edited from (Carnol et al., 2014; Zhang et al., 2007)

Supporting services can include processes that do not directly benefit humans but are extremely important for everyday life, supporting services also regulate the other services in the system. These include processes such as carbon storage, soil formation and structure, and nutrient cycling (Benayas et al., 2009), which can often be linked to regulating services such as flood prevention, climate control and pollination (Trumbore et al., 2015; Zhang et al., 2007), which regulate the functioning of the biosphere.

Provisioning services include any service provided by the tree, that directly benefits humans, such as the provision of food, fuel and timber (Cazorla and Mercado-Blanco, 2016; Freer-Smith and Webber, 2017). These can also be described as economical services, as the profits obtained from the global trade of products derived from forests (whether this be food, timber, or whole plants) can be significant. For example, the value of forest derived products is estimated to have an annual worth of approximately $120 billion (Boyd et al., 2013). Wood products exported from the UK alone in 2017 were estimated to be worth approximately £1.9 billion, which was a 26% increase on the previous year (Forest Research, 2018a), indicating that the global trade and demand is increasing annually.

Cultural services provided by forests and natural ecosystems include recreational activities and the general experience of the aesthetics of forests and nature (Carnol et al., 2014). In recent years, studies have been undertaken to determine how much of an impact to human health and well-being the experiences of being in nature can have on individuals. This includes Sandifer et al. (2015) who, after conducting a large scale review of the relevant literature, concluded that being exposed to nature does in fact result in favourable effects on the mental and emotional wellbeing of individuals.

Awareness of these services has increased dramatically since the early 2000's (Costanza et al., 2014; Fisher et al., 2009). Understanding the benefits humans can obtain from simply being around these biodiverse forests and experiencing the natural world (Sandifer et al., 2015) has led to an increase in interest and therefore trade of whole plants and trees, as well as products derived from trees and forests on a global scale (Freer-Smith and Webber, 2017). This increase in international plant trade combined with climate change, increasing temperatures and altered weather conditions (Sturrock et al., 2011), has resulted in a higher risk of forest diseases being spread across the globe (Brown et al., 2017b).

1.3 Current Threats to Tree Health

Global plant trade and climate change have resulted in increased outbreaks of disease, causing public concern and an increased awareness of forest health (Freer-Smith and Webber, 2017). Table 1.1 summarises the major diseases currently affecting trees in the UK (Forestry Commission, 2017a). Tree

disease outbreaks, such as Dutch Elm Disease, Ash Dieback and Chestnut Blight have increased in incidence over the past century resulting in the loss of substantial densities of global forest (Stenlid and Oliva, 2016).

Table 1.1. Major tree diseases currently affecting forests and woodlands in the UK according to Forest Research (Forestry Commission, 2017a)

Name of Disease	Hosts Affected	Microbial Cause
Acute Oak Decline (AOD)	UK's Native Oak (*Quercus robur* & *Q. petraea*)	Polybacterial
Ash Dieback	Ash (*Fraxinus* spp.)	Fungus
Sweet Chestnut Blight	Sweet Chestnut (*Castanea sativa)*	Fungus
Dothistroma Needle Blight	Pine (*Pinus* spp.)	Fungus
Dutch Elm Disease	Elm (*Ulmus* spp.)	Fungus
Horse Chestnut Bleeding Canker	Horse Chestnut (*Aesculus hippocastanum*)	Bacteria
Massaria disease of plane trees	Plane (*Platanus* spp.)	Fungus
Phytophthora alni	Alder (*Alnus* spp.)	Oomycetes
Phytophthora austrocedri	Juniper bushes/Chilean Cedar (*Juniperus* spp./ *Austrocedrus chilensis*)	Oomycetes
Phytophthora kernoviae	Beech/Oak (*Fagus* spp. / *Quercus* spp.)	Oomycetes
Phytophthora lateralis	Lawson Cypress (*Chamaecyparis lawsoniana*)	Oomycetes
Phytophthora ramorum	Larch/Rhododendron (*Larix* spp. / *Rhododendron* spp.)	Oomycetes
Sirococcus Blight	Cedar/Hemlock (*Cedrus* spp./ *Conium maculatum*)	Fungus

Several diseases currently affect economically and ecologically important tree species such as oak, pine and cedar in woodlands across the UK (Table 1.1, (Forestry Commission, 2017a). Although a baseline level of tree death is required for the effective functioning of a healthy forest (Manion, 2003; van Lierop et al., 2015), as death and decay are necessary for various services including nutrient recycling, significant loss of certain species could result in a dramatic loss of profits (Holmes et al., 2009) and the loss of keystone species in an ecosystem. Loss of species can lead to habitat fragmentation and reduced gene flow, both important issues facing the conservation community (Holdenrieder et al., 2004).

Tree diseases are complex and intricate and are affected by a multitude of biotic and abiotic factors (Manion, 1981). An individual's susceptibility to disease is also influenced by these dynamics. Biotic factors include bacteria, fungi, viruses, or insects, and abiotic factors include environmental factors such as temperature, moisture and soil composition that can have an effect on the host organism's

health (Sturrock et al., 2011). These elements can act sequentially or in parallel and result in a major detrimental impact on the host tree.

1.4 Primary Pathogen Diseases

A majority of the diseases affecting trees in the UK are caused by a single primary pathogen, as seen in Table 1 (Forestry Commission, 2017a). Primary tree pathogens include bacteria such as *Pseudomonas syringae* pv. *aesculi*, which causes Horse Chestnut Bleeding Canker (Green et al., 2009), and fungal pathogens, such as *Sirococcus tsugae*, which is the cause of *Sirococcus* Blight (Pérez-Sierra et al., 2015), and *Dothistroma septosporum* the cause of *Dothistroma* needle blight (Fraser et al., 2015). Single pathogen diseases are conceptualised using the "disease triangle" (Figure 1.2), although this model can be altered depending on the specific disease being explained, and the interactions between the host and environment as well as the pathogen involved (Francl, 2001; Holdenrieder et al., 2004).

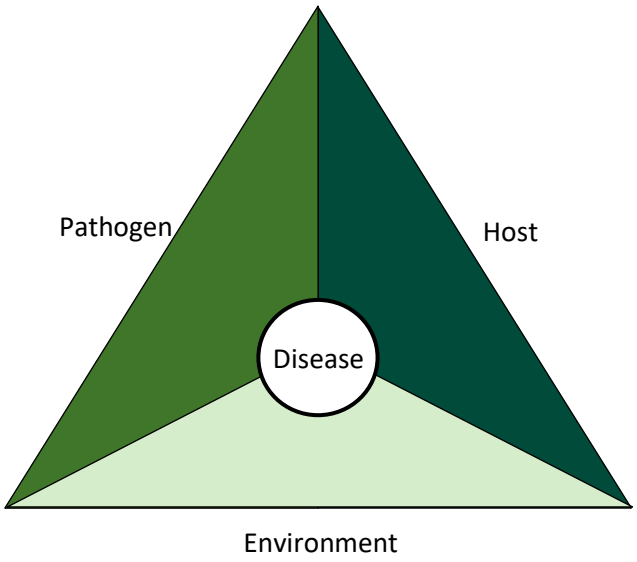

Figure 1.2. Basic model of the disease triangle, where the occurrence of a disease is dependent on interactions between the pathogen, the host organism and the environmental conditions (taken from Francl, 2001).

Factors affecting the development of a primary pathogen disease include the pathogen, the host organism, and the environment (Grulke, 2011). Disease development is dependent on these factors and the interactions between them. Figure 1.2 is indicative of the development of a disease where each factor (pathogen, host and environment) has an equal influence on the disease progression. However, this is not always the case.

The host has an influence on the development and progression of disease due to its intrinsic susceptibility and defence mechanisms. These can vary depending on the health of the host and the physical environment. Plants are exposed to microorganisms (pathogenic, or otherwise) on a daily

basis, however for a microorganism to have a detrimental effect on the host, it must access the interior of the plant (Chisholm et al., 2006). This could be easier for a pathogen if the host is already weakened due to an abiotic stressor (e.g. drought) or an existing genetic disposition (Jackson, 2003). Host organisms often become susceptible to disease after attack by defoliating insects, which weakens the host (although the disease development is not thought to be directly associated with this), as in the case of Horse Chestnut Bleeding Canker, which often affects trees already weakened by the Leaf Miner Ant (Koskella et al., 2017). The host microbiome, the collection of microbiota and their associated genes within the host organism (Vandenkoornhuyse et al., 2015), which varies depending on the species and particular habitat, also plays a key role in the protection of the individual against pathogen attack. Through the recruitment of protective microorganisms (particularly in the rhizosphere), pathogens are often excluded through colonisation resistance, and therefore suppressed, and disease is avoided (Beattie, 2006; Berendsen et al., 2012). If this is unsuccessful, however, and the pathogenic microorganisms reach the interior of the plant, the pathogen must subsequently survive the defence mechanisms developed by plants. These can include the release of various signalling molecules, and chemicals that deter or kill the invading pathogen (Kunkel and Brooks, 2002).

Aggressive and virulent plant pathogens employ a range of mechanisms to overcome host defences (Nomura et al., 2006), including effector molecules to gain access to the intercellular spaces of the host, whether this be through natural pores, or wounds (Jones and Dangl, 2006; Melotto et al., 2006). This could be through the disruption of plant cell structure, leading to higher pathogen dispersal, or nutrient loss. Effectors are also known to suppress plant defence mechanisms. Some pathogenic plant bacteria can release around 30 effectors into the cells of the host, which often increase virulence, by mimicking or disrupting plant cell functions (Jones and Dangl, 2006).

It is well known that environmental conditions play a key role in the development of plant diseases (Garrett et al., 2015). Phytopathogens operate under varied environmental conditions, including humid environments (West et al., 2012), and in freezing temperatures, often along with snow cover (Matsumoto and Hsiang, 2016). This indicates that although a host organism may have a very large and variable geographic range, the pathogens that affect these species may be limited to certain areas within this range (Garcia-Guzman et al., 2016). Environmental conditions that are unfavourable to the host can also lead to a more rapid development of a disease, as the host is weakened through stress and is therefore more susceptible to pathogen attack (Mittler, 2006). Temperature, rainfall, humidity and atmospheric CO_2 levels are all climatic factors that can affect the prevalence of plant diseases (Jeger and Pautasso, 2007). It is this interdependent relationship between the pathogen, the host, and the environment that determine how detrimental an epiphytotic incident (specifically relating to

plants, an event where the infection of susceptible host organisms increases rapidly at a dramatic rate (Encyclopaedia Britannica, 2018)) could be (Scholthof, 2007).

Primary Pathogen Case Study: Dothistroma Needle Blight

Dothistroma Needle Blight (DNB), also known as red band needle blight, is an example of a primary pathogen disease driven by a single species, *Dothistroma septosporum.* DNB affects several conifer species in the UK, pine trees in particular appear to be the most susceptible and are most frequently infected. In the UK, Lodgepole pine, Corsican pine and Scots pine are all affected by DNB (Fraser et al., 2015). DNB is caused by the fungus *Dothistroma septosporum,* a hemibiotrophic fungus that utilizes the biotrophic strategy of associating with the host through infection using specialised hyphae through which nutrients are absorbed, as well as the necrotrophic strategy of secreting enzymes and toxins causing cell death and absorbing nutrients that are then released (Koeck et al., 2011). After entering the needles through stomatal pores, *D. septosporum* grows extracellularly, close to the mesophyll (leaf, or in this particular case pine needle) cell tissues, which are killed by the fungus, allowing continued growth through the absorption of nutrients from the necrotic mesophyll tissues. The fungal stromata continues to develop under the surface of the needle, which eventually ruptures leading to defoliation of needles, and allowing the conidia to be released, thus spreading the disease (Barnes et al., 2016; Bulman et al., 2013; de Wit et al., 2012)

Symptoms of the disease include discolouration of the ends of infected needles, which can appear yellow and brown and then turn red, this can be seen in Figure 1.3. This discolouration is caused by the production of dothistromin, a broad-range mycotoxin produced by the fungus at a very early stage of development, indicating a role within initial plant invasion (Bradshaw and Zhang, 2006; Kabir et al., 2015). It is within the red parts of the needles that the fruiting bodies of the fungus the grow and develop (Forestry Commission, 2018). In June and July, the fruiting bodies of the fungus release spores that infect other needles, and the initially infected needles are shed, this leads to “lion’s tail” branching (Figure 1.3B). Defoliation of the needles continues to occur each year, weakening the host tree, which leads to loss of growth and timber production, and ultimately death (Fraser et al., 2016; Woods et al., 2005).

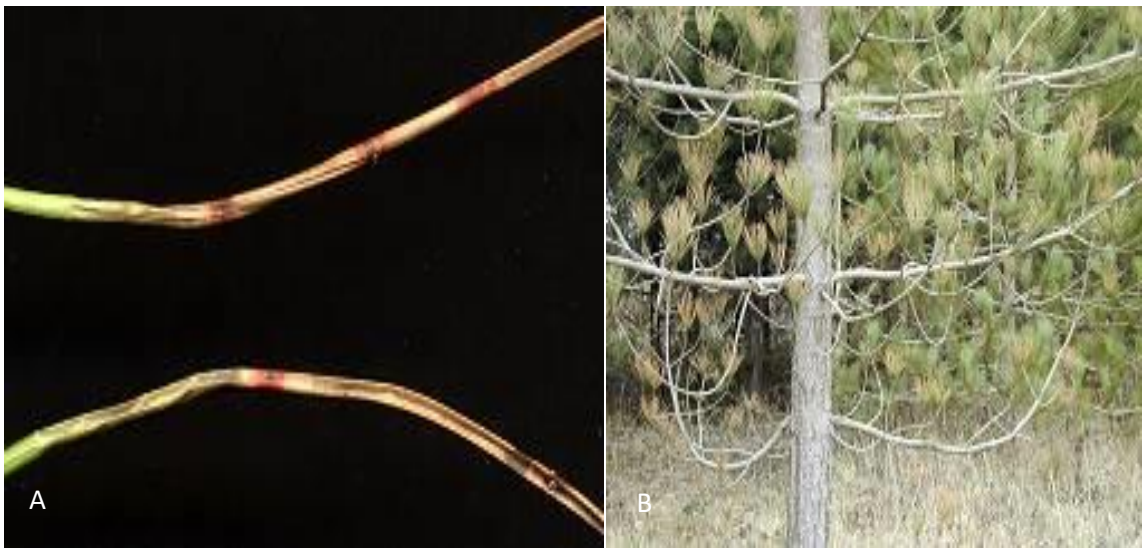

Figure 1.3. Symptoms of Dothistroma (red band) needle blight. A. Infected needles showing the yellow-brown colour with red bands, where the fruiting body of the fungi is present. B. An infected tree showing the typical "lions tail" branching where the infected needles have been shed leaving only the small collection of healthier needles at the end of the branch.

A temporal global distribution map of DNB shows that although DNB has been prevalent since 1899, it only became a major issue in the UK in around the 1950's (Fig. 1.4). However it took over half a century to be identified in Northern Ireland (Drenkhan et al., 2016). Although occurring across the globe, it is suggested that due to *D. septosporum* being fungal, the spread of the disease is dependent on moisture levels in the environment (Kabir et al., 2013). High moisture levels are required for the spread of the fungi, and it is suggested that spores travel long distances in humid winds and mists (Mullett et al., 2016). Spread of *D. septosporum* and therefore DNB is also aided by transfer of infected needles on clothing and footwear, as well as global trade of timber and derived products (Barnes et al., 2014), studies have also found that fungal spores can be transported via smoke created when biomass including diseased tree tissue is burned (Mims and Mims, 2004).

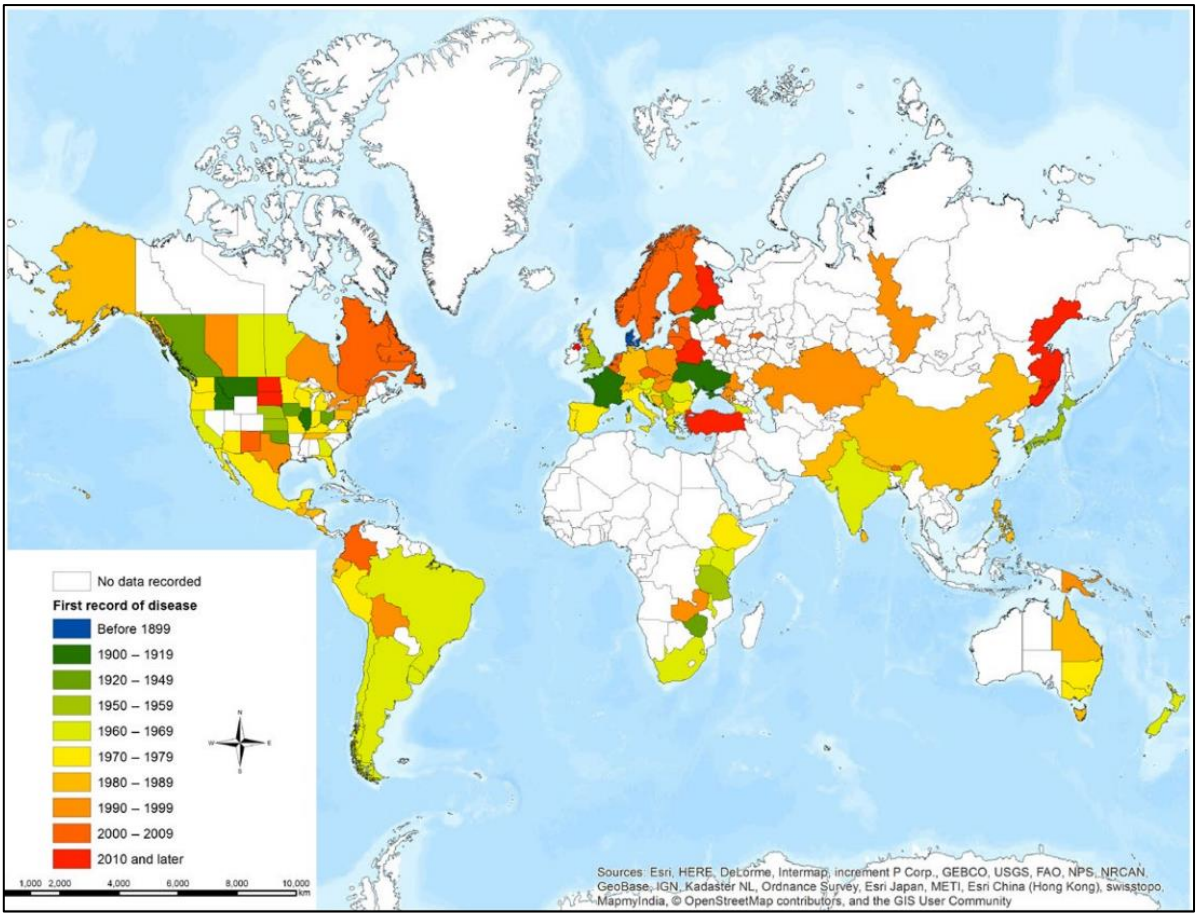

Figure 1.4. Global distribution of Dothistroma Needle Blight showing how the disease has spread almost worldwide in just over a century. Taken from Drenkhan et al. (2016).

As global warming and climate change become ever-increasing concerns within the field of phytopathology, it is thought that the spread of diseases such as DNB which are dependent on temperature and humidity will increase further (Lindner et al., 2010). Furthermore, increased global trading of timber and tree derived products (Henders et al., 2015) risks spreading DNB further across the globe. Consequently, it is critical that management strategies are developed to control and eradicate DNB.

1.5 Decline Diseases

Although a majority of diseases currently affecting the trees and woodlands in the UK are caused by single primary pathogens (although influenced by the environment, host and pathogen virulence), some tree diseases are not driven by a single pathogenic species. In decline diseases, trees are infected with secondary pathogens only after they have been predisposed by a combination of various biotic and abiotic factors, including genetic potential, climate change and insect attack (Amoroso et al., 2015; Ostry et al., 2011; Woo, 2009). Acute Oak Decline (AOD) and Chronic Oak Decline (COD), are decline diseases driven by numerous interacting factors that result in the decline of the host, and ultimately

death (Denman, 2017; Denman et al., 2018; Kolb et al., 2016). Decline diseases are more accurately explained using Manion's Decline Spiral (Manion, 1981 (Figure 1.5)).

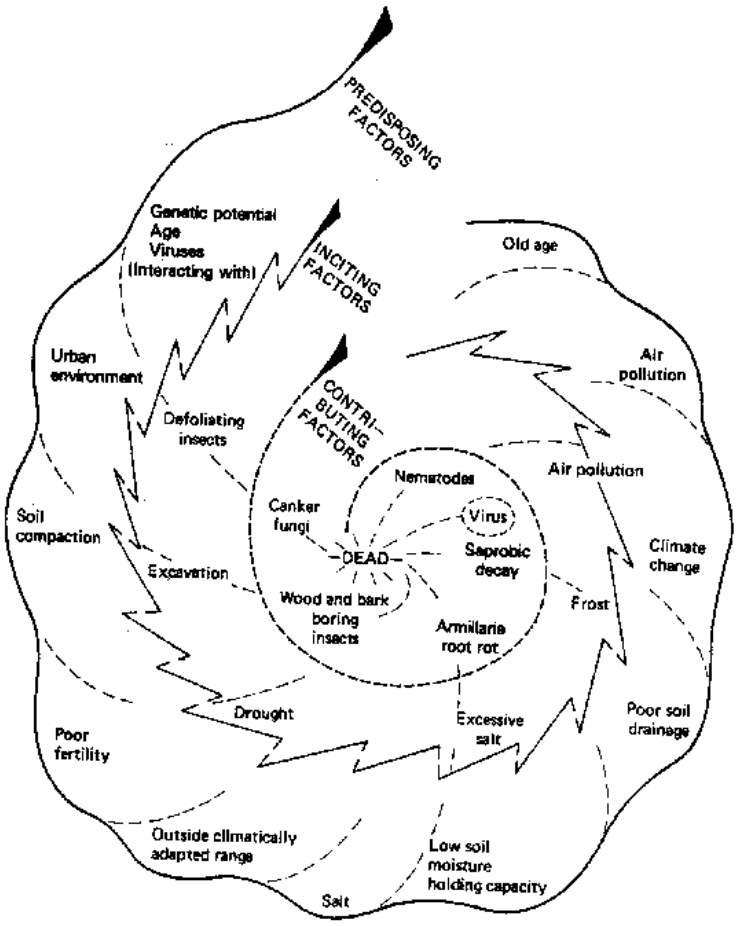

Figure 1.5. Manion's Decline Spiral, showing the three categories of factors that affect the development of a decline disease; predisposing factors, inciting factors, and contributing factors. Taken from Manion (1981).

Due to the complex, multi-factorial nature of diseases within the wider decline complex, the exact cause of these diseases is often unclear and difficult to determine. This is due to the sheer number of factors that can affect the host and the development of the disease (Brown et al., 2018). Manion's Decline Spiral conceptualises the gradual cumulative effect of both biotic and abiotic stressors in ecline diseases, categorising them as predisposing factors, inciting factors, and contributing factors, which accentuate the inward spiral of tree health status, ultimately leading to death (Manion, 1981).

Predisposing factors include stressors that cause initial weakening of the host tree. This could be the host's genetic susceptibility to certain pathogens, the general nature of the host's environment, and other generic environmental conditions that are relatively constant. Temperature and water levels are key aspects, which individually do not cause the development of the decline disease within the host organism, but do cause initial weakening meaning the organism is predisposed and more susceptible

to other stressors (Berg et al., 1994; McCarthy et al., 2001). Inciting factors include changes to the host's environment and therefore further stress and weakening of the host organism. This can include events such as flooding, drought and frost (Mattson, 1998; O'Brien et al., 2010). Contributing factors include issues that can cause further weakening of the host organism which often leads to its demise. This can include events such as insect attack (from wood or bark-boring insects), and infection by fungi, bacteria, or other microorganisms (Oliva et al., 2016). This final layer of the decline spiral, seen in Figure 1.5, is the one that ultimately causes the death of the host organism, as without this the organism would have more chance of recovering from the events of the inciting factors.

Decline Disease Case Study: Acute Oak Decline

Figure 1.6. A symptomatic oak with Acute Oak Decline demonstrating several necrotic lesions on the stem (Forestry Commission, 2017b).

Acute Oak Decline (AOD) is a decline disease affecting mature oak species in the UK (Denman et al., 2014; Sapp et al., 2016). AOD occurs mainly on English (or Pedunculated) oak, *Quercus robur*, and Sessile oak, *Quercus petraea* (Forest Research, 2018b). The disease can be characterised by the dramatic onset of four main symptoms. These include necrosis of the inner bark tissue, which seeps through cracks in the outer bark and gives the appearance of weeping lesions on the main trunk of the tree (Figure 1.6 and Figure 1.7A). The symptoms also include larval galleries of the *Agrilus biguttatus* beetle in close proximity to the lesion (Figure 1.7B), which are often (but not always) accompanied by the D-shaped exit holes (Figure1.7C) of the adult beetles (Brown et al., 2017a, 2015; Denman et al., 2014, 2010). These symptoms can appear rapidly and can cause mortality within 5 years of infection (Denman et al., 2010).

Figure 1.7. Symptoms of Acute Oak Decline associated with the Agrilus biguttatus beetle. A. A typical AOD lesion B. The larval galleries of the beetle underneath the outer bark of the tree. C. The typical D-shaped exit hole of the adult beetle. (all images taken from Forest Research, 2018b and Forest Research, 2018c).

AOD symptoms have been observed in the UK for around four decades, however it only became prevalent in 2006. Since this time, AOD has spread from its origin in the south-east of England, northwards along the English-Welsh border, most recently being discovered in the south of Wales (Broberg et al., 2018; Forest Research, 2018d). The most current distribution map (from 2016), can be seen in Figure 1.8A. Several abiotic factors have been found to correlate with the distribution and spread of AOD, including temperature, rainfall and elevation (Brown et al., 2018).It is known that as climate change becomes an ever-increasing concern, the conditions required for the disease to spread are becoming more widespread, for example, increasing temperature means that more northern latitudes will be tolerable for the disease-causing microorganisms (Holdenrieder et al., 2004). A map modelling the probability of AOD occurrence across the UK (Figure 1.8B) predicted using environmental predisposition factors (temperature, rainfall, elevation, etc.) and shows that at present the optimum environment for AOD to thrive is still the south east of England, however there are small areas across the UK that possess the optimum environmental predisposition factors to support the development of AOD. It is also suggested that as climate change becomes an increasing issue, the numbers of areas with these optimum conditions will increase, allowing for further spread of the disease (Brown et al., 2018).

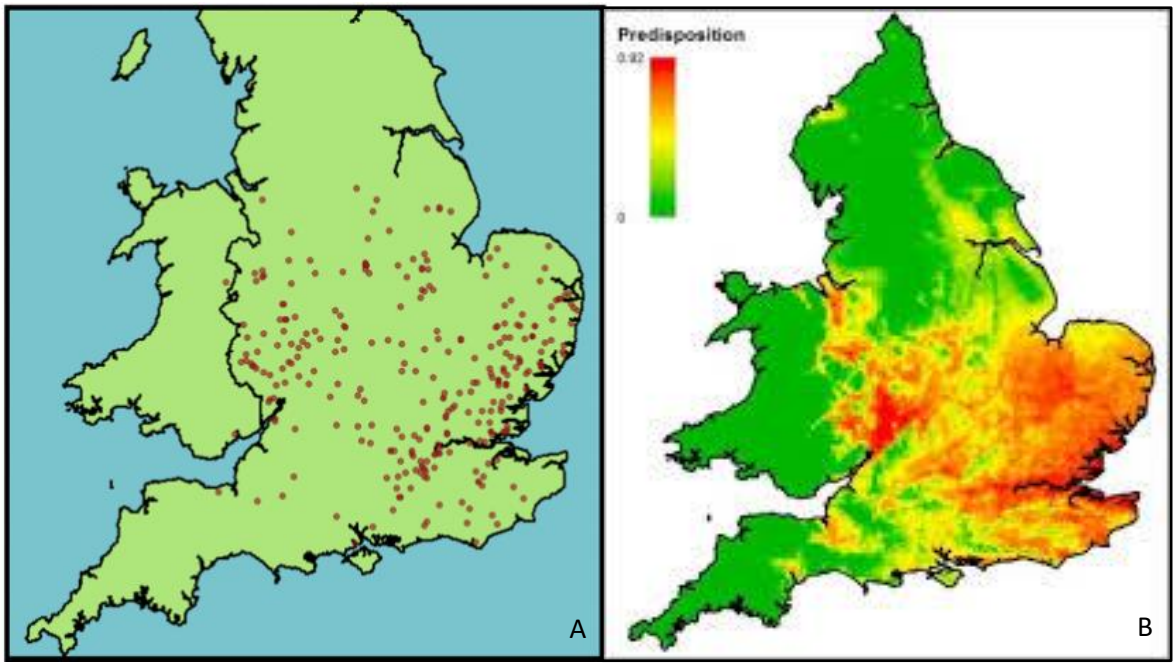

Figure 1.8. A. a distribution map of Acute Oak Decline (correct as of 2017) showing the locations of confirmed AOD sites across the UK (Forest Research, 2018d). *B. Map of the areas of the UK predisposed to AOD according to Brown et al. (2018). Red areas have the optimum conditions for AOD, yellow areas have some of these conditions, and green areas are unlikely to be susceptible to AOD due to various environmental factors, such as elevation, precipitation and temperature.*

It is the predicted spread of the disease that has caused an increased sense of urgency to determine an effective management strategy to control AOD. The difficulty with this decline (and other diseases that are a part of the wider decline complex) is that due to the multifactorial nature of the disease development, the exact cause and transmission of AOD is not definitively known (Denman et al., 2018). Knowledge on the transmission of AOD is basic, and the information regarding the reservoirs of the bacteria associated with the disease is underdeveloped.

1.6 Biotic Causal Agents of AOD

The current AOD outbreak was first documented in the 1980's and officially characterised in recent years (Denman, 2017). AOD can be explained through a modified decline spiral, which includes bacterial pathogens as well as insect attack as contributing factors (Denman et al., 2016a). The continuous isolation of two bacterial species, *B.* goodwinii and *G. quercinecans*, from trees affected with AOD indicated that there is a distinctive polymicrobial element to the development of AOD, along with the insect attack from the British *Agrilus biguttatus* beetle (Broberg et al., 2018; Denman et al., 2018, 2016a).

Agrilus biguttatus is a bark boring buprestid beetle native to the UK, which lays its eggs beneath the outer bark of a mature host tree (usually *Quercus robur* or *Quercus petraea*). Once hatched, the larvae then grow and develop, forming larval feeding galleries in the cambial interface, where the cambium

and inner bark meet (Reed et al., 2018; United States Department of Agriculture, 2018). Finally, the larvae pupate and emerge as adult beetles through distinctive D-shaped exit holes in the outer bark (Denman et al., 2010). Close associations have been suggested between *A. biguttatus* and AOD-associated bacteria, as often there are signs of both on declining trees. However, it is not fully understood whether the presence of one affects the presence of the other (both are attracted to already weakened trees), or whether they are both just attracted to the host trees at the same time (Brown et al., 2017a).

Isolation studies revealed multiple novel key bacterial phytopathogens associated with the disease, including *Brenneria goodwinii* and *Gibbsiella quercinecans* (Brady et al., 2010; Denman et al., 2012). These bacteria have been consistently isolated from the lesions found to be present on AOD-symptomatic oak trees (Kaczmarek et al., 2017). Other bacterial species have also been isolated, including *Rahnella victoriana,* however this bacterium has been isolated from both AOD-symptomatic trees and healthy trees (Denman, 2017; Denman et al., 2018, 2016a). An adaptation of Koch's postulates has indicated that these bacteria (along with the *A. biguttatus* beetle larvae) cause the lesions associated with AOD, and microbiome investigations have confirmed that the bacteria associated with the disease contain various plant pathogen virulence components, such as effector molecules and pathogenicity factors (Broberg et al., 2018; Denman et al., 2018). Continuous isolation of *B. goodwinii* and *G. quercinecans* from the necrotic lesions on AOD-symptomatic oaks across the UK, along with the microbiome and infectivity studies, have proven that these bacteria are the key microbial components involved in the development of the AOD disease (Brown et al., 2016; Denman et al., 2018).

The spread of the disease from tree to tree is extremely localised with healthy trees next to infected trees becoming infected. It is this that has led to the inference that the transmission of the disease is a result of interacting biotic factors (such as insect vectors, bacteria in the immediate vicinity, or endophytic microorganisms) as opposed to abiotic factors (such as wind, climate and pH), and research on the disease has been focused on this (Brown et al., 2016, Sapp, et al., 2016, Denman, et al., 2016).

1.7 The Genera *Brenneria* and *Gibbsiella*

Knowledge of *B. goodwinii* and *G. quercinecans,* two newly described bacterial species associated with AOD, including their behaviour and ecology in forest habitats is severely limited. Although research is currently being undertaken to bridge this knowledge gap, hypotheses on their ecology and activity can be established by studying the closest relatives of these bacteria and analysing the microbial ecology of other members of these genera.

The genus *Brenneria* comprises Gram-negative, rod shaped bacteria within the family *Pectobacteriaceae*. Known to cause soft rot and wilting in many plants, the genus *Brenneria* contains many phytopathogenic species, including *B. populi*, and *B. paradisiaca* (Biosca et al., 2003; Li et al., 2015; Naushad et al., 2014). Species from the genus affect a huge number of host plants, including (but not limited to) poplar (*B.* populi), alder (*B.* alni), banana (*B. paradisiaca*), willow (*B. salicis*) and walnut (*B. nigrifluens*) (Li et al., 2015; Maes et al., 2009a; Ménard et al., 2004; Poza-Carrión et al., 2008; Samson et al., 2005), although each species appears to be a host species-specific pathogen. Members of the genus *Brenneria*, family *Enterobacteriaceae*, cause primarily necrogenic infections, including maceration and vascular wilt (Kado, 2006). Infection mainly occurs in the xylem (the water transporting) vessels of the infected trees (Janda, 2006). Although isolated from diseased trees, some species of the *Brenneria* genus have also been isolated from healthy tree hosts, indicating that they may represent endophytic bacteria, and are not always pathogenic (Maes et al., 2009a).

As a member of the genus *Brenneria*, *B. goodwinii* may therefore be an oak-specific hemibiotroph, causing necrogenic infection with a similar lifestyle to *B. alni*, a pathogen of alder trees that causes bark canker, or *B. nigrifluens* and *B. rubrifaciens* that cause bark and deep bark canker in walnut, respectively (Li et al., 2015). Other members of the genus *Brenneria* are known to be endogenous members of the tree microbiome, lying dormant in the inner bark tissue until environmental changes trigger virulence as secondary pathogens. For example, *B. salicis,* an endogenous pathogen and the cause of watermark disease on willow trees, has been isolated from healthy and diseased trees, and the interactions between this bacteria and it's tree host are not fully understood (Maes et al., 2009a). The isolation of *B. goodwinii* from both healthy and diseased oak trees suggests that not only is *B. goodwinii* a pathogenic bacterium specific to oak trees, but that it appears to be an endophyte, and does not always cause disease.

The genus *Gibbsiella* is a group of Gram-negative bacteria, which lie within the family *Enterobacteriaceae*, and includes secondary phytopathogens, such as the species *G. acetica* and *G. greigii*. All of which have been associated with necrotic tissues on numerous tree species including various oak species as well as apple, pear and horse chestnut, as well as leaf litter (Brady et al., 2017, 2014a; Geider et al., 2015). Members of the genus *Gibbsiella* have also been isolated from the gut microbiome of wood and bark-boring insects, suggested to be a result of the lignocellulose degrading abilities of the genus members (Rizzi et al., 2013). *Gibbsiella quercinecans* appears to have a broad host range and may therefore represent a general secondary forest pathogen that is broadly distributed in forest ecosystems.

1.8 Environmental Reservoirs of Forest Pathogens

Tree diseases can be caused by a multitude of pathogens, including fungi, bacteria, viruses and oomycetes (Eyles et al., 2010; Shigo, 2003). Each of these pathogens requires a suitable host in order to survive and develop. Selecting an unsuitable host; a host that has not been previously weakened, is not susceptible to the pathogen, or a host with strongly developed defence mechanisms could be detrimental to the development and lifecycle of the microorganism, with the pathogenic microorganism being removed or killed by these defence mechanisms (Keeling and Bohlmann, 2006). Outside of the host organism, pathogenic microorganisms can often survive in what is known as the "environmental reservoir". These differ depending on the microorganism itself and the host organism (Bartoli et al., 2015; Vezzulli et al., 2010). Environmental reservoirs can therefore have a significant impact on the ecology and development of these diseases (Morris et al., 2010). Forest pathogens often utilize forest soils and bodies of rainwater such as natural ponds and puddles as non-host environmental reservoirs (Mordecai, 2011).

Globally, forest soils (of all types) are home to many microorganisms, both beneficial and pathogenic, as well as commensals, amensals and saprophytes (Lloyd, 1980; Pal and Gardener, 2006). These microorganisms are required for various ecosystem functions, such as nutrient cycling and promoting plant growth (van der Heijden et al., 2008). Pathogenic microorganisms that reside in these forest soils use it as an environmental reservoir and survive by using the nutrients within the soil. Once identified, they then affect their desired host organism either by entering via the roots and vascular systems, or by interacting with the host organism externally under the soil line (Chaparro et al., 2012), which affects the functioning of the host (McCann et al., 2013; Mendes et al., 2013; Samson et al., 1998). For example, *Pythium* is a genus of oomycetes that contains pathogenic species that utilise forest soil as an environmental reservoir. This group of phytopathogenic microorganisms are saprophytic, fungi-like water moulds (Parveen and Sharma, 2015) found universally in soil, which can interact with the roots of the host organism in the rhizosphere and cause diseases such as seed rot of alfalfa (Berg et al., 2017). Members of the *Pythium* genus have been occasionally isolated from larger bodies of water, although it is suggested that the water is utilised as a route to spread further across the forest environment (Naznin et al., 2017).

However, some species of pathogenic microorganisms are known to survive in the environment by primarily utilizing water as an environmental reservoir (Bartoli et al., 2015), such as the well-known bacterium, *Pseudomonas syringae* (Monteil et al., 2013), which has over 50 different pathovars, including pathogenic and non-pathogenic species, all of which are Gram-negative rod shaped bacteria, and can be differentiated by their host range (Green et al., 2010). These include many plant and tree

species, including various food producing species such as kiwi, leek and wild cherry trees (Ménard et al., 2003; Nowell et al., 2016). One pathovar, *P. syringae* pv. *aesculi*, is an airborne pathogen that falls to the ground in rainwater (Monteil et al., 2014), this particular species has been isolated from necrotic lesions on trees suffering from Horse Chestnut Bleeding Canker across the UK and Europe (Schmidt et al., 2008; Webber et al., 2008). After being isolated from lesions in the phloem tissue, it has been suggested that *P. syringae* pv. *aesculi* gains access to the host plant via natural entry points such as leaf scars and growth cracks after falling on the host in rainwater (Steele et al., 2010). Another pathovar of *P. syringae, P. syringae* pv. *tomato*, has been determined to gain access to plant tissues through the stomatal openings on leaves. Once through the stomata, *P. syringae* pv. *tomato* releases a virulence factor (coronatine) which prevents the closing of the stomata, allowing for further access of pathogenic microbes (Zhou et al., 2017).

1.9 Aims, Objectives and Hypotheses

Currently, the environmental reservoirs and modes of transmission of the two major bacterial causal agents of AOD, *Gibbsiella quercinecans* and *Brenneria* goodwinii, are unknown. In order to fully understand the main cause and transmission methods of the disease, we must first understand if the bacteria responsible for these drastic symptoms are able to survive without a host and then how they are able to move from one infected tree to infect the next.

The overarching aim of the study is to investigate whether rainwater and forest soil, both known bacterial reservoirs in a forest habitat, are suitable reservoirs for the persistence and transmission of *B. goodwinii* and *G. quercinecans*. The hypotheses to be tested are; *B. goodwinii* is a specific oak endosymbiont and will therefore decay quickly outside of the oak host in rainwater and forest soil, and *G. quercinecans* has a broad distribution in forest ecosystems and will therefore survive outside of the oak host in rainwater and forest soil.

This will be completed through the execution of the following objectives:

A. To develop antibiotic resistant strains of *B. goodwinii* and *G. quercinecans* for selective isolation and enumeration of these organisms in survival experiments.
B. To develop culture-based methodologies to detect viable *Brenneria goodwinii* and *Gibbsiella quercinecans* cells in rainwater and in forest soil.
C. To determine the survivability of *B. goodwinii* and *G. quercinecans* in rainwater and soil
D. To use these optimised methods to assess the ecology and survival of *B. goodwinii* and *G. quercinecans* in the forest environment.

These data will provide evidence for the question of survivability of *B. goodwinii* and *G. quercinecans,* two bacterial causal agents of necrotic lesions in oak trees suffering with Acute Oak Decline, in rainwater and forest soil. Understanding the ecology of tree pathogenic bacteria is an important endeavour in the field of microbial ecology and forest pathology, informing further research and management opportunities for the causal agents of AOD.

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CHAPTER 2

Characterising Growth Conditions and Development of Antibiotic Resistant strains of *Brenneria goodwinii* and *Gibbsiella quercinecans*

2.1 Introduction

Activities of bacterial and fungal cells were first observed in around 1665, when basic microscopes (developed by Robert Hooke and Antoni Van Leeuwenhoek) allowed microorganisms to be visible for the first time (Gest, 2004). The study of microorganisms and their behaviours has progressed greatly since this time, with the development of advanced culture-based methods (including various media developments and isolation techniques) and eventually molecular-based methods, such as qPCR and metagenomics (Canfora et al., 2016; Davis, 2014; Remenár et al., 2015). These highly developed processes have become crucial steps in the understanding of the ecology and activities of microorganisms and their communities across a range of species and environments (Anderson and Cairney, 2004; Ramette and Tiedje, 2007).

Microbial Communities

Rainwater microbial communities have been extensively studied worldwide due to the common use of rainwater as a source of drinking water (Daoud et al., 2011). These studies have found that, although initially rainwater is widely contaminant free, it can easily become contaminated with pathogenic and non-pathogenic species (through contact when falling through the atmosphere (Polymenakou et al., 2012), or ground contact) and rapidly develop into a complex community of microorganisms, which could have significant effects on the ecosystem (Schets et al., 2010). However, one species of phytopathogenic bacteria, *Pseudomonas syringae* (the cause of Horse Chestnut Bleeding Canker) is known to fall to the ground in rainwater and snow (Mertelik et al., 2013; Morris et al., 2010, 2008).

Forest soil communities are also extremely diverse, representing a significant percentage of the living biomass present on Earth (with 10^3-10^4 kg of biomass per hectare of surface soil). Soil microorganisms are key drivers in many ecological systems and cycles (Baldrian et al., 2012; Fierer et al., 2007b; Torsvik and Øvreås, 2002). It is well known that the microbial communities associated with plant root systems have a profound effect on plant health, with roles in nitrogen fixation and nutrient cycling, however forest soils can also contain phytopathogenic species (Azcón-Aguilar and Barea, 1997).

Exploring Decay Rates as a Method to Measure Survivability of Microorganisms in the Environment

Using measurement of decay rates, defined as a change in culture counts (colony forming units,

CFU/ml) over time (Anderson et al., 2005) is a well-established method of measuring survivability of microorganisms (Salem et al., 2006). Decay rate experiments typically use culture-based methods to identify the number of viable cells in a sample at regular time points throughout the experiment (Antoniou et al., 1990), and these experiments provide a clear visual representation of the growth and decay of target species over time.

Limits of Using Culture-Based Methods to Detect Viable Cells in Environmental Samples

Easily cultured microorganisms, consisting of species that develop quickly in synthetic environments with high nutrient levels and moderate temperatures, are considered to make up less than 1% of the number of species in an environmental sample (Hugenholtz, 2002). Secondary to the initial conditions required by the bacterium, such as temperature, pH and humidity (Kirk et al., 2004), there is also a need to understand the specific growth strategy. This could be oligotrophic, this usually occurs in environments where nutrients are scarce, or copiotrophic, which usually occurs in nutrient-rich environments (Koch, 2001).

Oligotrophic species are usually unable to grow in environments that are rich in nutrients (Hashimoto et al., 2006), and therefore do not grow well on the standard nutrient rich agars used in most laboratories. Microorganisms associated with host species, such as endophytes (where the species resides within the host organism), symbionts and pathogenic microorganisms, are also likely to require specific conditions in order to mimic those provided by the host organism (Chaparro et al., 2012).

This phenomenon has been called the “great plate-count anomaly”, and led an increase in the use of molecular based methods in microbial ecology studies (Remenár et al., 2015), however these are not without limitation, and a pure culture is still required in order to obtain DNA for analysis (Lagier et al., 2015). Many microbial species also appear visually very similar, again making isolation of pure cultures difficult, therefore, a combination of culture-dependent and independent techniques are used in order to obtain accurate results (Martin-Sanchez et al., 2018).

Using Selective Bacterial Culture to Detect Viable Cells in Environmental Samples

As indicated above, culture-based bacterial studies are not always efficient at isolating specific species. Interspecies competition in standard lab culturing (for example on standard nutrient agar plates) can provide biased results, with some species thriving in this environment and others being underrepresented (Rodríguez et al., 2018). A method to improve culturing of underrepresented microorganisms is to use selective media (Mcdonald et al., 2012).

There are numerous established methods for the production of selective media, which vary depending on the target species. Variations include; nutrient enriched or impoverished media

(oligotrophic/copiotrophic), chemical compounds (to inhibit the growth of certain bacterial groups, or indicate presence with a reaction such as visual colour change), antibiotic media can also be used (with resistant bacterial strains). These can also all be used in conjunction with temperature and oxygen availability manipulation to provide suitable conditions for a specific bacterial species (Lagier et al., 2015).

Manipulation of nutrient availability relies on the understanding that different species utilize nutrients in different ways, and this affect the speed at which they grow in a standard nutrient rich environment (Hashimoto et al., 2006). For example, copiotrophs, as stated above, are species that thrive quickly in nutrient rich environments, and can grow rapidly in short periods of time, however the nutrient availability must remain high for the species to remain viable and continue to grow. Oligotrophs, on the other hand, do not thrive in these seemingly optimal conditions, and take much longer to grow, although these species are often more resilient towards stress and can survive long periods with minimal nutrient availability (Fierer et al., 2007a; Koch, 2001).

Chemical compounds such as silver nanoparticles (Sondi and Salopek-Sondi, 2004), bile salts (Lagier et al., 2015), benzidine hydrochloride (Snyder and Atlas, 2015) and various acids (Baral and Shah, 2014) are known to inhibit the growth of various bacterial species or produce a visual change to indicate the presence of a species. Chemical compounds such as these can be used as rapid diagnostic tests, and are regularly used in the clinical microbiology industry (Snyder and Atlas, 2015).

Antibiotics can also be added to standard lab media to selectively culture bacteria (Lagier et al., 2015). Many bacterial species have naturally occurring antibiotic resistance genes (Nordmann et al., 2018a), through mutation or horizontal gene transfer, which can be beneficial in an environment with antibiotic producing competitors, and seems to have amplified with the increasing use of antibiotics in a clinical setting (Martínez, 2008). An example of a species with a high level of natural antibiotic resistance is *Pseudomonas aeruginosa,* which posesses mechanisms to defend against many antibiotic compounds, such as efflux systems and the ability to reduce pore expression (Bălăşoiu et al., 2014). Although resistance genes can be detrimental to human and plant health, they can also be beneficial to research by allowing researchers to exploit this function and utilize antibiotic containing media to selectively culture specific bacterial species (Gould et al., 1985).

The bacterial plasmid is the key factor in the development of antibiotic-resistant bacterial strains, as resistance genes are located on this circular piece of DNA. Various methods have been developed to generate antibiotic-resistant mutants, however all rely on either transformation of the resistant genes or mutations in the plasmid (Bennett, 2009).

Methods to Generate Antibiotic-Resistant Mutants

Spontaneous evolution of resistance is a natural occurrence that follows incorrect DNA replication, resulting in an error in the DNA code (Watford and Warrington, 2019). One of the possible mutations caused by the incorrect replication of DNA is genetic resistance to antibiotics, which due to the short generation times of many bacteria, can result in resistant bacterial strains quickly evolving (Woodford and Ellington, 2007). This process can be exploited in order to obtain antibiotic-resistant strains by exposing the bacterium to antibiotics, which stimulates an SOS response from the cell whereby the effects of the antibiotic are minimised. This results in stress-induced mutagenesis, which can lead to resistance (Foster, 2007).

Continual passage is a method that involves slowly increasing the strain's tolerance to increasing concentrations of an antimicrobial compound. This is done by passaging (re-plating) successful colonies onto the same concentration, followed by higher concentrations of the antimicrobial compound (Apolónio et al., 2014; Birosova and Mikulasova, 2009).

Using a gradient plate involves creating a gradient of antibiotic concentrations that the strains are then inoculated onto. Successful colonies will be visible higher up the concentration gradient, and can then be isolated and cultured to be used as resistant strains (Cohen et al., 1989; Szybalski and Bryson, 1952; Zanetti et al., 1980).

Electroporation of cells and subsequent transformation of a resistant plasmid is a well-established and efficient method for generating antibiotic-resistant bacterial strains. Transforming a resistant plasmid ensures that the resistance genes are present in the transformed cells, leading to more successful production of resistant cells (Dower et al., 1988; Guerche et al., 1987; Kraemer and Iandolo, 1990).

Aims and Objectives

The main aim of this chapter was to produce antibiotic-resistant strains of *B. goodwinii* and *G. quercinecans,* two phytopathogenic bacteria associated with lesion formation in acute oak decline, in order to selectively isolate these strains from samples of rainwater and forest soil. This will also includet the positive control of *Pseudomonas syringae* pv. *aesculi.*

The aim of the study will be met through the completion of the following objectives:

A. Investigate the growth rates of *B. goodwinii, G. quercinecans* and *P. syringae* pv. *aesculi*, and produce a growth curve.
B. Characterise the growth rates of *B. goodwinii*, *G. quercinecans* and *P. syringae* pv. *aesculi* for inoculation under controlled quantities.
C. Assess growth of naturally occurring microorganisms in rainwater and forest soil.

D. Utilise various methods to generate antibiotic-resistant strains of *B. goodwinii* and *G. quercinecans* that can be selectively cultured from rainwater and forest soil samples.

2.2 Methods

2.2.1 Bacterial Strains, Growth Conditions and Confirmation of Identity

The type strains of *B. goodwinii* (FRB141), *G. quercinecans* (FRB97) and *P. syringae* pv. *aesculi* (FRB130), were obtained from Forest Research and were grown initially from glycerol stocks (stored at -80°C). Strains were streak plated onto standard nutrient agar (for *P. syringae* pv. *aesculi* 5% sucrose was added (Green et al., 2009)) and were incubated for forty-eight hours at 28°C.

Prior to the experiment, the identity of the type strains was confirmed using PCR and sequencing of the bacterial DNA gyrase subunit B (gyrB) gene. DNA was extracted from the cells with the use of a "boil prep method"; a single colony was picked from an agar plate containing a pure culture of the isolate using a sterile inoculation loop, and suspended in 20 µl of PCR-grade water. Subsequently, the cell suspension was heated to 95°C for two and a half minutes, vortexed vigorously for 10 seconds, and heated again at 95°C for a further two and a half minutes. After being briefly vortexed, 1 µl of extracted DNA was used as the template for a PCR reaction using Bioline MyTaq™ Red Mix. Each 50 µl reaction consisted of 25 µl of 2x MyTaq™ Red Mix, 1 µl of 10 pmol each of GyrB gene specific oligonucleotide primers gyrB07F (5' – CMCCYTCCACCARGTAMAGT – 3') and gyrB02R (5' – CMCCYTCCACCARGTAMAGT – 3') as described by (Brady et al., 2010), 22 µl of nuclease-free H_2O, and 1 µl of the DNA template (from the "boil prep" extraction method above). PCR reaction conditions were: initial denaturation at 95°C for 1 minute, followed by 35 cycles of; denaturation at 95°C for 15 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 10 seconds, producing a PCR product which was approximately 750 base-pairs in length. Ten microliters of the PCR product was run in a 1% agarose gel at 100 V for 50 minutes, and 1 µl of the PCR product was quantified using the Qubit™ dsDNA HS Assay Kit. The unpurified PCR product was then sent, along with the accompanying gyrB07F sequencing primer, for sequencing to Macrogen Europe, Netherlands.

The identity of each strain was confirmed by searching the gyrB sequence of each strain against the NCBI BLAST database, using the "Nucleotide BLAST" feature (NCBI, 2017), for *B. goodwinii, G. quercinecans* and *P. syringae* pv. *aesculi,* using >97% sequence similarity as a cut-off for species delineation. After the identity of strains was confirmed, each strain was plated onto fresh nutrient agar approximately once per week, throughout the duration of the experiment. Fresh glycerol stocks of each strain were also made regularly. Stock plate cultures were wrapped in para-film, after an initial incubation at 28°C for 24-48 hours and stored at 4°C.

Strains grew as expected, with *B. goodwinii* producing small, white circular colonies, which grew slower than *G. quercinecans* which produced larger, circular cream colonies, and grew quickly (within 24 hours). *P. syringae* pv. *aesculi* grew as large irregular cream colonies which grew over a forty-eight hour period.

2.2.2 Growth curves of *B. goodwinii*, *G. quercinecans* and *P. syringae* pv. *aesculi*

Growth curves (to an OD_{600} value of 0.5) were produced for each of the strains, *B. goodwinii*, *G. quercinecans* and *Pseudomonas syringae* pv. *aesculi*; the latter is a well-characterised phytopathogen that can survive in soil and rainwater, and was used as a positive control for the survivability investigations in Chapter 3. Growth curves were used to give a reference timescale for incubation periods and also to provide an estimate number of colony forming units (CFU's) at an OD_{600} value of 0.5. The value of 0.5 was chosen to ensure the cells were still in "log phase" (an actively metabolising state) and had not yet reached the "stationary phase" (senescence).

Initial starter cultures were produced by picking colonies from agar plates containing pure cultures and inoculating into universal tubes containing 10 ml nutrient broth. *B. goodwinii* and *G. quercinecans* were inoculated into nutrient broth, and *P. syringae* pv. *aesculi* was inoculated into nutrient broth with the addition of 5% sucrose (Green et al., 2010). These were then incubated at 28°C, shaking at 150 RPM, overnight.

After overnight incubation, 250 ml conical flasks containing 100 ml nutrient broth (*B. goodwinii* and *G. quercinecans)* or nutrient broth with 5% sucrose (*P. syringae* pv. *aesculi*) were inoculated with 1 ml of the corresponding bacterial culture (1% v/v inoculum). Each growth curve was repeated in three replicate flasks. Each broth culture (three replicates each of *B. goodwinii* in nutrient broth, *G. quercinecans* in nutrient broth and *P. syringae* pv. *aesculi* in nutrient broth with 5% sucrose added) was incubated at 28°C, shaking at 150 RPM. The optical density (OD_{600}) of each was measured every 15 minutes until an OD_{600} of 0.5 was reached. At this point, the cultures were immediately held on ice and refrigerated at 4°C until all cultures had reached this value and the next step of the experiment could begin.

The next step of the experiment involved producing serial dilutions of each of the cultures (10^{-1} to 10^{-10}), and plating 100 µl of each dilution onto the corresponding solid media (nutrient agar or nutrient agar with 5% sucrose added). After a 24-hour incubation at 28°C, the number of colony forming units (CFU's) on each plate was counted, and the CFU/ml was determined. The mean CFU/ml of the three biological replicate cultures were calculated and a growth curve was produced. Optical density (OD)

values of each growth curve were utilized as a reference for all experiments in Section 2.2, and also for the investigations in Chapter 3.

2.2.3 Enumeration of Naturally Occuring Microbial Growth in Rainwater and Forest Soil

In order to determine the background level of microorganisms present in rainwater and forest soil, enumeration of soil and rainwater microorganisms was performed as described below.

Collection and Analysis of Rainwater Samples

A large bucket, lined with two large autoclave bags, was placed on the roof of the Memorial Building, Bangor University. The bucket was placed approximately two meters away from any walls or obstructions to avoid splashing, as this could cause microorganisms from the floor or other obstructions to contaminate the samples. Rainwater was collected over a twenty-four-hour period of heavy rainfall and immediately processed in the laboratory.

Processing of the rainwater sample included producing a ten-fold serial dilution from 10^{-1} to 10^{-8}, using Phosphate Buffered Saline (PBS) solution for dilution, and then plating 100 µl of each dilution (including 100 µl of the original undiluted sample) onto standard nutrient agar plates and spreading with sterile L-shaped spreaders. This process was repeated in triplicate, with three biological replicates (water samples), and three technical replicates of each (nine values for each dilution). The plates were then incubated at 28°C for forty-eight hours, after which the number and types of colony were observed.

Collection and Analysis of Soil Samples

Soil was collected from approximately the top 6 inches of soil beneath a mature oak tree at Treborth Botanic Garden, Bangor University, and was immediately processed in the laboratory. Processing involved suspending 1 g of the soil sample in 5 ml of PBS solution, three times to create three biological replicates, and using these to produce ten-fold serial dilutions from 10^{-1} to 10^{-8}. Each of the dilutions (including the initial soil suspension) were plated onto standard nutrient agar plates (100 µl of each), plated in triplicate, as above, and spread using sterile spreaders. After a forty-eight-hour incubation at 28°C, the colony numbers and types were observed.

2.2.4 Generating Antibiotic-resistant Mutants of *B. goodwinii* and *G. quercinecans*

Antibiotics were selected based on previous research on the antibiotic sensitivity of *B. goodwinii* and *G. quercinecans* by Dr James Doonan (Bangor University, personal communication, October 2018). The information provided from sensitivity testing, using agar containing antibiotics at the recommended working concentration (Table 2.1), was used to select Chloramphenicol for *B. goodwinii* resistance

experiments and Kanamycin for *G. quercinecans* resistance experiments, as neither type strain (FRB141 and FRB97) had natural resistance to either antibiotic.

Table 2.1. Results of antibiotic sensitivity tests performed by Dr James Doonan (Bangor University), indicating that B. goodwinii is sensitive to all antibiotics tested and G. quercinecans is resistant to two of the four antibiotics tested.

Species	**Sensitive**	**Resistant**
Brenneria goodwinii	Kanamycin Chloramphenicol Ampicillin Tetracycline	
Gibbsiella quercinecans	Kanamycin Chloramphenicol	Ampicillin Tetracycline

Detecting Resistant Mutants of B.goodwinii and G. quercinecans with Spontaneous Evolution Experiments

The test for the evolution of spontaneous resistance was based on the probability that at some point, a random mutation would occur, and lead to resistance to the antibiotic. The test also aimed to determine the minimum inhibitory concentration (MIC) of the antibiotics, which could then be used in later experiments.

Antibiotic-containing medium, both solid nutrient agar plates and 10 ml liquid nutrient broths, of a range of concentrations was produced. Chloramphenicol medium was produced with increasing concentrations of 3.5 µg/ml, from 3.5 µg/ml to 35 µg/ml (the recommended working concentration). Kanamycin media was produced with increasing increments of 5 µg/ml, from 5 µg/ml to 50 µg/ml (the recommended working concentration).

An overnight 10 ml broth culture of each of *B. goodwinii* and *G. quercinecans* was prepared, by inoculating a 10 ml nutrient broth with a colony picked from a pure plate culture of the bacterium, and incubating overnight at 28°C, shaking at 150 RPM. One hundred microliters of the overnight culture was inoculated into the respective antibiotic broths (*B. goodwinii* into the Chloramphenicol broths and *G. quercinecans* into the Kanamycin broths). These were then incubated for 48 hours at 28°C, shaking at 150 RPM. After the 48-hour incubation, 200 µl of each broth was plated onto the corresponding concentration of antibiotic in solid agar media. These plates were incubated at room temperature (approximately 20-22°C) for 48 hours, and successful colonies (*G. quercinecans* on 5 µg/ml and 10 µg/ml kanamycin agar) were re-plated onto nutrient agar containing the same concentration as before and incubated again at room temperature for 48 hours.

However, this method was determined to be inefficient, and therefore the following method was developed using the *G. quercinecans* strain that had grown at 10 µg/ml of Kanamycin.

Developing Antibiotic Resistance in B. goodwinii and G. quercinecans Through Continual / Serial Passage

The process of continual/serial passage (consecutive inoculation of bacteria into liquid media containing increasing concentrations of antibiotic, Figure 2.1), is a method regularly used in clinical microbiology for a number of reasons, including identifying resistant strains of bacteria (Gullberg et al., 2011; Khan et al., 2018). Here, the *G. quercinecans* strain that was found to be resistant up to 10µg/ml of Kanamycin (in both liquid and solid media) was used.

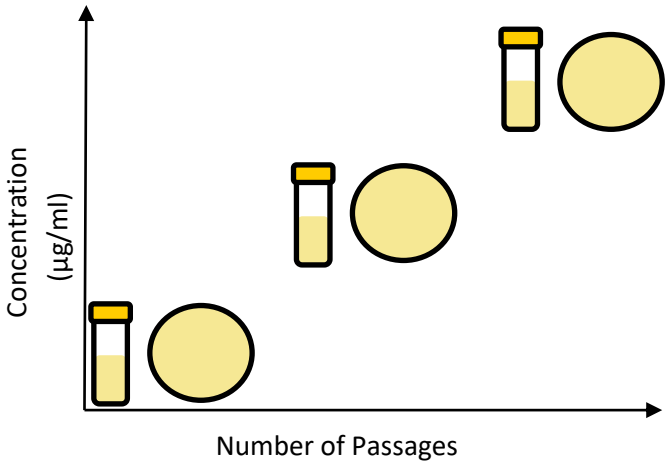

Figure 2.1. Diagram of the proposed continual serial passage method for producing antibiotic-resistant strains, through the steady increase in antibiotic concentration and subsequent passage to reinforce the resistance.

The above strain was inoculated into a 10 ml nutrient broth containing 10 µg/ml of Kanamycin. Following a 48-hour incubation at 28°C, shaking at 150 RPM, 100 µl of the broth culture was plated onto a Kanamycin agar plate with a concentration of 10 µg/ml, which was incubated at 28°C for 48 hours. A successful colony was isolated from this plate and used to inoculate 10 ml nutrient broth with a concentration of 15 µg/ml. After a 48-hour incubation at 28°C, shaking at 150 RPM, 100 µl of the broth culture was plated onto nutrient agar with a Kanamycin concentration of 15 µg/ml and incubated for forty-eight hours at 28°C.

The continual/serial passage (increasing by 5 µg/ml every passage into broth media) was continued until growth in the broth culture was unsuccessful. This occurred at a concentration of 25 µg/ml, which indicated that the method of continual/serial passage was unsuccessful, meaning the method would not be used to produce resistant bacterial strains for the survivability experiments in Chapter 3.

Using a Gradient of Antibiotic Concentration to Generate Antibiotic-Resistant Strains of B. goodwinii and G. quercinecans

The next method tested was a "gradient plate", which involved using a large square bioassay tray to produce a gradient of antibiotic concentrations. Standard nutrient agar was poured into the tray which was then left to set at an angle. Antibiotic agar (at the working concentration) was then poured over the antibiotic agar which was set on a flat surface. This resulted in a gradient effect, with a higher concentration of antibiotic agar at one end, and a lower concentration of antibiotic at the other (Figure 2.2A). One plate with Chloramphenicol and one plate with Kanamycin were produced.

An overnight broth culture of each bacterial species was produced (a colony from a stock plate was inoculated into a 10 ml nutrient broth and incubated at 28°C, shaking at 150 RPM, overnight), and 200 µl of the broth culture was added to one side of the gradient plate and was spread along the gradient in a straight line with a sterile spreader (Figure 2.2B), this was repeated five times on each plate, with the *B. goodwinii* broth being plated onto the Chloramphenicol gradient, and the *G. quercinecans* broth plated onto the Kanamycin gradient. The two plates were incubated at room temperature (approximately 20-22°C) for forty-eight hours. The gradient plate method was determined to be too inefficient to produce confidently resistant strains without further development of the method.

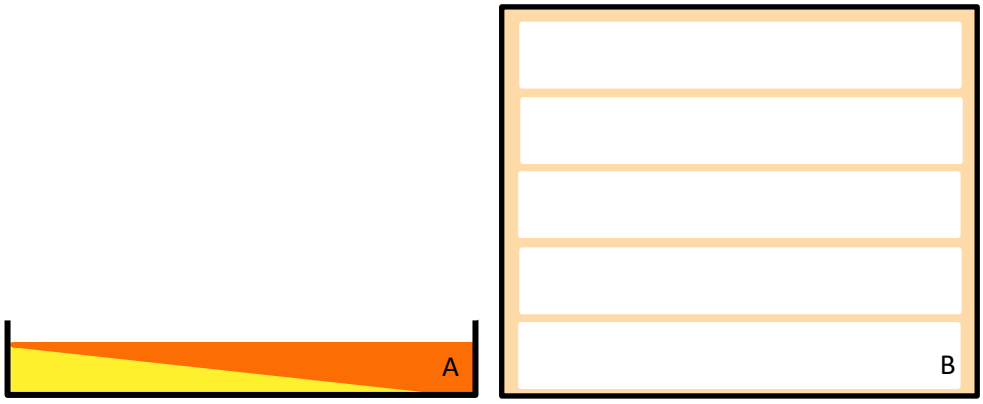

Figure 2.2. Diagram of the proposed gradient plate method for producing antibiotic resistant strains, (A) Diagram of the cross section of the antibiotic gradient plate with standard nutrient agar represented by yellow, and the antibiotic agar represented by orange. (B) Diagram whereby white indicates streaks of bacterial culture, in five replicates.

2.2.5 Producing Electrocompetent Cells and Transformation of a Resistant Plasmid

The relative success of *G. quercinecans* in comparison to *B. goodwinii* throughout the various resistance experiments lead to this strain being selected to go through the electroporation process first. Electroporation is a technique which uses an electrical pulse to increase cell membrane

permeability, allowing a resistant plasmid to be inserted. The following protocol was produced through the modification of a standard transformation protocol for *E. coli* (Drury, 1996).

Producing Electrocompetent Cells

An overnight broth culture was produced by selecting a colony from a pure agar plate culture and inoculating into a 10 ml nutrient broth, which was incubated at 28°C, shaking at 150 RPM overnight. This 10 ml broth culture was used as the inoculum for a 100 ml broth starter culture, which was also incubated at 28°C, shaking at 150 RPM. Once the OD_{600} value of the starter culture had reached between 0.3 and 0.4, the 100 ml broth was split into two 50 ml falcon tubes and kept on ice.

The two 50 ml falcon tubes were centrifuged at 2000 *x g* for twenty minutes at 4°C, and the supernatant was removed, ensuring the cell pellet was not disrupted. The cell pellets were then re-suspended, each in 40 ml of ice cold distilled water. The tubes were centrifuged again (2000 *x g* for twenty minutes at 4°C, and the supernatant). The cell pellets were re-suspended, each in 20 ml of ice cold distilled water, and these were centrifuged again at 2000 *x g* for twenty minutes at 4°C. The supernatant was removed from both tubes, and the cell pellets were re-suspended, each in 40 ml of ice cold 10% glycerol. At this point, numerous 1.5 ml Eppendorf tubes were put on ice. The cell suspensions were centrifuged a final time at 2000 *x g* for twenty minutes at 4°C. The supernatant was removed by aspiration with a pipette, to ensure that the cell pellets were not disrupted in any way. The cell pellets were then re-suspended, each in 1 ml of ice cold 10% glycerol by pipetting gently up and down. These 1 ml cell suspensions were then aliquoted (150 µl each) into the ice-cold Eppendorf tubes and immediately frozen at -80°C.

Transformation Through Electroporation

The first step of the electroporation procedure involved producing various media. This included nutrient agar plates containing 50 µg/ml of kanamycin, SOB and SOC media were produced using standard protocol (Hanahan and Harbor, 1983).

Four of the frozen aliquots of electrocompetent cells were thawed slowly over ice, and 40 µl of each was transferred to ice-cold Eppendorf tubes, along with 1 µl of the Kanamycin-resistance plasmid (pHSG298DNA) at a concentration of 1 ng/µl. The cells and plasmids were mixed by gentle pipetting up and down.

The cell and plasmid mixtures were transferred to ice cold 10 mm electroporation cuvettes, which were then placed into the electroporation chamber and pulsed once at 1.8 kV. One millilitre of SOC recovery media was immediately added to the cuvette and mixed by gentle pipetting up and down.

The cell suspension in SOC media was transferred to fresh Eppendorf tubes. This was repeated for all four aliquots.

The SOC medium cell suspensions were incubated at 28°C, shaking at 225 RPM for one hour. After this incubation, 100 µl of the cells from each tube were plated onto agar plated containing 50 µg/ml of Kanamycin. This was also repeated with 200 µl of cell suspension. All plates were incubated at 28°C, and the tubes were also returned to the incubator at 28°C for twelve hours. After this further incubation, another 100 µl and 200 µl of each cell suspension was plated onto 50 µg/ml Kanamycin agar plates and incubated at 28°C for twelve hours.

After the twelve-hour incubation period, colonies on each plate were counted and a range of colonies from each successful plate were picked and re-plated onto 50 µg/ml Kanamycin nutrient agar, in order to reinforce the resistance. After another twelve-hour incubation at 28°C, the plates were wrapped in para-film and stored at 4°C to slow bacterial growth.

The transformant colonies were inoculated into 50 µg/ml Kanamycin nutrient broth, and after a twelve-hour incubation at 28°C shaking at 150 RPM, 100 µl of each broth was plated onto 50 µg/ml Kanamycin agar, and 250 µl was glycerol stocked (at a final glycerol concentration of 40%) and stored at -80°C.

In order to confirm the successful transformation of the Kanamycin-resistant plasmid, the plasmid was extracted using the Bioline Isolate II Plasmid Mini Kit. The protocol for "Isolation of low-copy plasmid, P1 constructs or cosmid DNA from *E. coli*" (Bioline, 2018) was slightly modified to fit the growth conditions of *G. quercinecans,* by replacing LB broth with nutrient broth. However, agarose gel electrophoresis (1% gel, 100 V for 45 minutes) of these extractions was inconclusive.

2.3 Results and Discussion

2.3.1 Confirming Strain Identity Through PCR Amplification and Consequent Sequencing of the Bacterial Gyrase B Gene

Stock plates for all three species grew as expected, with *B. goodwinii* producing small, white, circular colonies and *G. quercinecans* as slightly bigger, cream, circular colonies. *P. syringae* pv. *aesculi* formed irregular cream colonies which were larger than *B. goodwinii* and *G. quercinecans* (Figure 2.3).

Figure 2.3. Stock plates of bacterial strains, (A) B. goodwinii stock plate with very small, circular, white colonies present. (B) G. quercinecans stock plate, with small, circular, cream colonies present. (C) P. syringae pv. aesculi stock plate, with medium sized, irregular cream colonies present.

DNA extraction of the above stock strains and subsequent PCR amplification of *B. goodwinii*, *G. quercinecans* and *P. syringae* pv. *Aesculi* using specific bacterial gyrB gene primers resulted in a product of approximately 750 base pairs. Agarose gel electrophoresis of the PCR product confirmed the presence of the bacterial gyrB gene in all three samples (Figure 2.4).

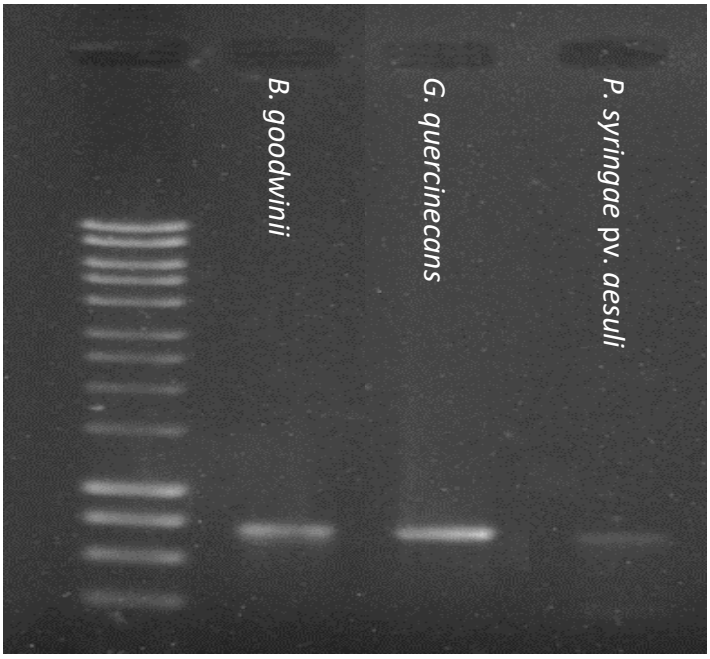

Figure 2.4. Agarose gel electrophoresis of the bacterial gyrB gene in B. goodwinii, G. quercinecans and P. syringae pv. aesculi. 1Kb hyperladder indicates the product to be approximately 750bp in length, confirming the presence of the bacterial gyrB gene.

Sequencing of the bacterial gyrB gene, followed by an NCBI BLAST search confirmed the identity of the three strains (Table 2.2).

Table 2.2. Results of the NCBI BLAST search of the bacterial gyrB gene from B. goodwinii, G. quercinecans and P.syringae pv. aesculi, including the result, similarity % and the GenBank accession number.

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

Understanding the growth conditions, for example, preferred media and expected visual appearance of the colony types, through exploring the literature (Brady et al., 2010; Denman et al., 2012; Green et al., 2009), lead to simple initial identification of the strains, which was followed by molecular DNA analysis to confirm the identity of the strains. This was imperative to be able to determine the inoculum size for decay experiments in Chapter 3. It also inproved the accuracy of the study, further increasing its validity, by ensuring that there were no contaminants throughout the experiment

2.3.2 Growth Curves of *B. goodwinii, G. quercinecans* and *P. syringae* pv. *aesculi*

The growth curve produced from the OD_{600} values obtained from *B. goodwinii*, *G. quercinecans* and *P. syringae* pv. *aesculi* (Figure 2.5) shows initial fluctuations over all three species, which settle into a steady increase as the time progresses. *G. quercinecans* was the first strain with all three biological replicates to reach the OD_{600} value of 0.5, followed by one replicate of *P. syringae* pv. *aesculi* (the other two biological replicates did not reach the required OD_{600} of 0.5). Two of the *B. goodwinii* biological replicates reached the 0.5 OD_{600} value within the 12 hour period, however one did not.

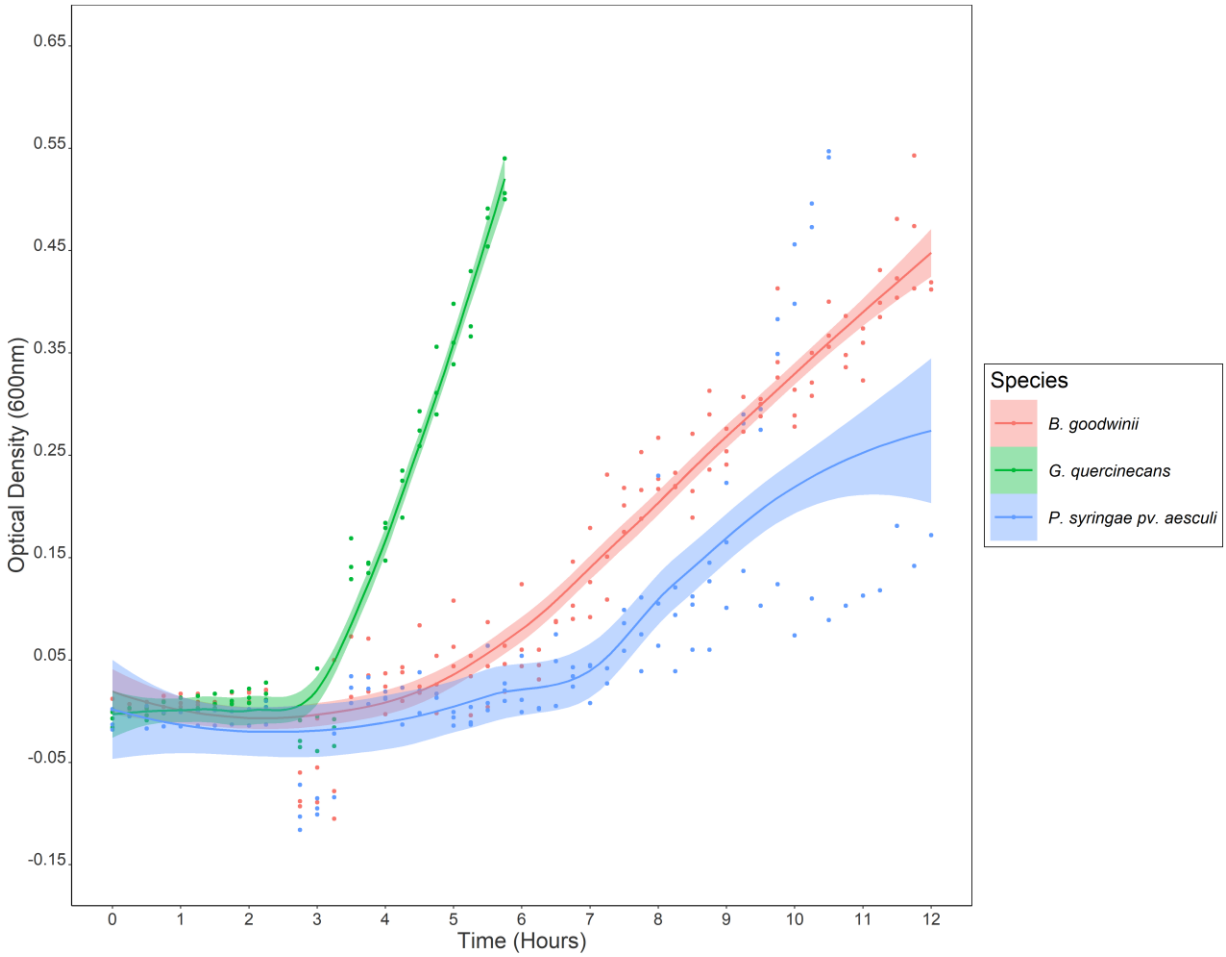

Figure 2.5. Growth curves for B. goodwinii, G. quercinecans and P. syringae pv. aesculi, produced by measuring the optical density value at 600nm(OD_{600}) of three biological replicates of each strain at 15 minute intervals for 12 hours, or until the OD_{600} reached a value of 0.5. Confidence intervals of 95% are indicated by the shaded region of the corresponding colour for each species

At an OD_{600} of 0.5, colony forming units (CFU's) per millilitre for each species were calculated through serial dilution and plate counts of colony forming units (CFU) per millilitre. This was calculated as the mean of the three biological replicates, or the number of replicates that reached an OD_{600} of 0.5.

Table 2.3. Approximate CFU/ml of B. goodwinii, G. quercinecans and P. syringae pv. aesculi at an OD_{600} of 0.5, estimated by calculating the mean CFU/ml of three biological replicates.

Species	**Approximate CFU/ml**
Brenneria goodwinii	1.13×10^{14}
Gibbsiella quercinecans	1.7×10^{8}
Pseudomonas syringae pv. *aesculi*	2.1×10^{7}

The results of the growth curve indicated that each of the bacteria required a different incubation time to reach an optical density of 0.5 (OD_{600}) at the recommended temperature of 28°C (Brady et al., 2010; Denman et al., 2012). The differences in time between the bacterial species is notable, as it

indicates a considerable difference in growth rates of the bacteria, even at an optimum temperature and nutrient availability. This could be indicative of differing ecological strategies, or could indicate how each of the species occurs in a different environment, with different conditions leading to the diversity in growth rates (Golovlev, 2001).

G. quercinecans took only a short amount of time to reach the OD_{600} value of 0.5, with a rapidly increasing log phase, which shows that with the provided conditions, *G. quercinecans* thrives. The short generation time indicates that *G. quercinecans* (in all three biological replicates, indicated by the narrow 95% confidence margins) has the ability to grow quickly and monopolise the available nutrients in a short amount of time.

B. goodwinii required almost the whole twelve-hour experimental period to reach the desired OD_{600} value of 0.5. *B. goodwinii* also had a more gradual log phase, even in these apparently optimum bacterial growth conditions. The seemingly slow growth rate observed in this species could be explained by *B. goodwinii* cells being much smaller than that of *G. quercinecans* with short rods approximately 0.8 x 1 – 1.3 µm (Denman et al., 2012), whereas *G. quercinecans* cells are short rods approximately 0.9 x 1 – 1.5 µm (Brady et al., 2010), meaning OD_{600} values may have not been the most suitable method for measuring cell frequency in this case. *B. goodwinii* produced a much higher number of CFUs, several order of magnitudes higher than that of *G. quercinecans* and *P. syringae* pv. *aesculi,* suggesting that *B. goodwinii* has a much higher growth rate in these conditions when the number of CFUs are measured as opposed to OD_{600}.

P. syringae pv. *aesculi* had an uneven growth rate, with a slightly longer lag phase but a rapidly increasing log phase, and not all of the biological replicates reaching the OD_{600} value of 0.5. This may be explained by the conditions used for the growth curve experiment not being the optimum reequirements for this strain to grow efficiently.

Although the experiment was considered a success, not all of the replicates for each of the species reached the OD_{600} value of 0.5 (*B. goodwinii* replicate two and *P. syringae* pv. *aesculi* replicate three), which highlights the critical importance of having multiple replicates in experiments. Without the triplicate results there would be no way to calculate the mean of the experiments, leading to biased and unreliable data. Increasing the number of biological replicates (using individual starter cultures), as well as increasing the number of technical replicates (replicates taken from each starter culture) for each species, would allow for a more accurate and representative experiment (Prosser, 2010). Having three or more replicates per condition in a study also allows for more robust statistical analysis (Dogan et al., 2006), and is therefore crucial. Alternative methods could also have been used to produce an

accurate growth curve in the experiment, including the use of an automated turbidimeter, which could have reduced the time taken to produce the growth curve by removing the need to plate out serial dilutions (Begot et al., 1996).

The results gained from this study can be used to aid the experimental development and design of other related studies. For this study, these data were crucial to have an approximate count of cells to add in to the microcosms in Chapter 3. This was imperative in order to be able to calculate an objective numerical estimate of cell decay, rather than a simple trend line.

2.3.3 Identification of Microbial Counts in Rainwater and Forest Soil

Numerous microorganisms were present in unsterilized rainwater, most of which were small, white or cream colonies (Figure 2.6A), similar in appearance to the *B. goodwinii, G. quercinecans* and *P. syringae* pv. *aesculi* colony types. Similarly, various microorganisms were present in unsterilised forest soil (Figure 2.6B), including filamentous fungi, making it difficult to identify other colony types on the plate. This meant that without a selective agent in the cell culture process, identification of *B. goodwinii, G. quercinecans* and *P. syringae* pv. *aesculi* species, amongst a backround of autochthonous microbiota would be difficult.

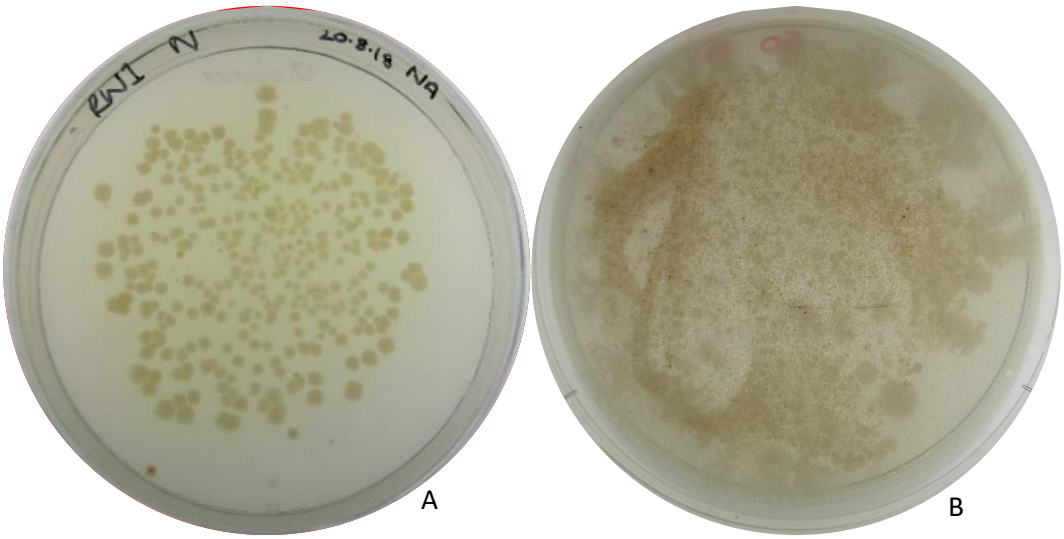

Figure 2.6. Microbial counts in rainwater and forest soil. (A) Undiluted rainwater on standard nutrient agar. (B) Undiluted soil suspension (1g of soil in 5ml PBS solution) on standard nutrient agar.

The testing for microbial growth of unsterilised rainwater and forest soil was a crucial step in the planning of following experiments in Chapter 3. The similar morphological appearance of the naturally occurring bacteria in unsterilised rainwater (with up to approximately 5000 CFU/ml of various bacteria estimated from serial dilutions repeated in triplicate) makes identifying *B. goodwinii* and *G.*

quercinecans colonies in the survivability experiments extremely difficult. The growth of various bacteria and fungi in unsterilised forest soil (with up to 40,000,000 CFU/ml of various bacteria and fungi estimated from serial dilutions repeated in triplicate), also makes the identification of *B. goodwinii* and *G. quercinecans* difficult and inaccurate. This means some form of selective medium needs to be developed in the cell culture process to allow for *B. goodwinii* and *G. quercinecans* to be identified.

2.3.4 Generating Antibiotic-resistant Strains of *B. goodwinii* and *G. quercinecans*

Detecting Resistant Mutants of B.goodwinii and G. quercinecans with Spontaneous Evolution Experiments

Generating antibiotic resistant strains of *B. goodwinii* and *G. quercinecans* was not successful at a concentration close to the recommended working concentration of the antibiotics (Table 2.5). Although *G. quercinecans* had successful growth at low concentrations of Kanamycin (5-10 µg/ml), this method was determined unsuccessful for producing resistant strains of *B goodwinii* and *G. quercinecans.*

Table 2.4. Spontaneous evolution of resistance results, where ✓ indicates successful growth with a turbid broth culture after the 48-hour incubation period, ✗ indicates unsuccessful growth with an apparently clear broth after incubation, and ? indicates an abnormal growth of small aggregates of cells in an apparently clear broth after incubation.

Species	Antibiotic	Concentration (µg/ml)	Growth
G. quercinecans	Kanamycin	5	✓
		10	✓
		15	?
		20	?
		25	?
		30	?
		35	?
		40	?
		45	?
		50 (Recommended)	?
B. goodwinii	Chloramphenicol	3.5	?
		7	?
		10.5	?
		14	?
		17.5	✗
		21	✗
		24.5	✗
		28	✗
		31.5	?
		35 (Recommended)	?

Spontaneous resistance testing was considered unsuccessful as the only *G. quercinecans* was considered to be potentially resistant. However this was still not resistant to a sufficient concentration of the antibiotic (only being resistant up to 10 µg/ml, when the recommended working concentration is 50 µg/ml of Kanamycin). As such, this method is not the most efficient method for producing antibiotic resistant strains, especially with Gram-negative bacteria (Marchese et al., 2000), resulting in atypical growth and varience in the phenotypes of the strains (as indicated by the aggregated growth in the above experiment). Further investigation and development of this method could have been undertaken, with the addition of media components and further study into resistance mechanisms of the species, however this further investigation may not have been successful, therefore leading to loss of time and resources (Silverman et al., 2001). Therefore other approaches were explored.

Building Antibiotic Resistance in G. quercinecans Through Continual / Serial Passage

The relative success of *G. quercinecans* compared to *B. goodwinii* throughout the previous spontaneous evolution experiment lead to the focus being transferred to *G. quercinecans* for the next stages of antibiotic resistance development. Continual/serial passage of *G. quercinecans* on slowly increasing concentrations of Kanamycin medium resulted in a successful growth in concentrations of 20 µg/ml (Table 2.5). However, abnormal aggregated growth was observed at 25 µg/ml, and so this method was considered unsuccessful.

Table 2.5. Continual/serial passage growth results of G. quercinecans in kanamycin broth, where ✓ indicates successful growth with a turbid broth culture after the 48-hour incubation period and ? indicates an abnormal growth of small aggregates of cells in an apparently clear broth after incubation.

Strain	Media	Concentration	Growth
G. quercinecans	Broth	10 µg/ml	✓
	Agar	10 µg/ml	✓
	Broth	15 µg/ml	✓
	Agar	15 µg/ml	✓
	Broth	20 µg/ml	✓
	Agar	20 µg/ml	✓
	Broth	25 µg/ml	?

The method of continual / serial passage, was more successful than the previous method tested, yielding a *G. quercinecans* strain resistant to up to 20 µg/ml Kanamycin. Other studies using this method have made it apparent that the minimum inhibitory concentration (MIC) of the antibiotic should be known beforehand (Drago et al., 2011).

Using a Gradient of Antibiotic Concentration to Generate Antibiotic-Resistant Strains of G. quercinecans

The results of the gradient plate experiment were determined to be inconclusive, as visible colony growth at a seemingly high concentration of antibiotic indicated a level of resistance in the strain (Figure 2.7), however with no objective method to determine the antibiotic concentration at a point on the gradient, this method could not be used without further investigation and development, using the approaches described above, which had proven unsucessful.

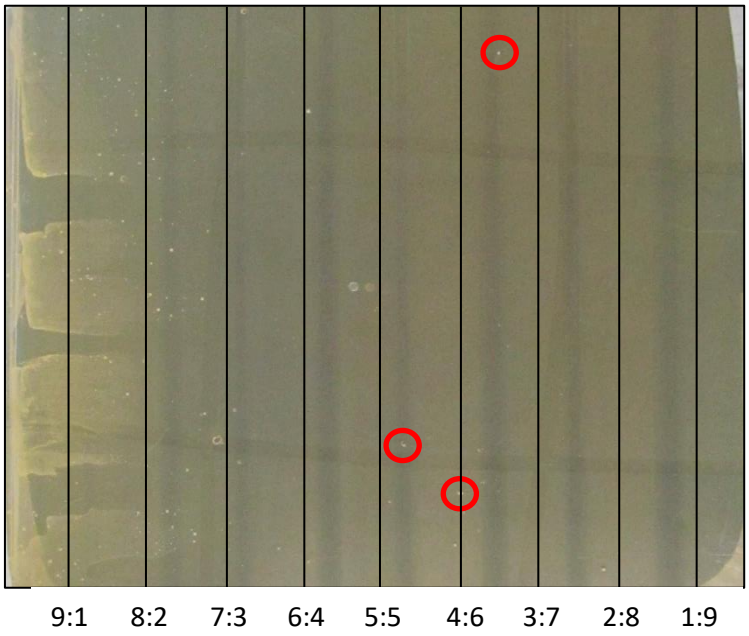

Figure 2.7 Gradient plate with approximate Nutrient agar : Antibiotic agar ratio displayed below. Individual successful G. quercinecans colonies are circled in red.

2.3.5 Producing Electrocompetent Cells and Transformation of a Resistant Plasmid into *G. quercinecans*

Producing electrocompetent cells and transforming a resistant plasmid through electroporation of the cell membrane was found to be the most successful of the methods tested to generate resistant bacterial strains. However, with multiple attempts and few successes, this method proved inefficient. After transformation, a small number of the Kanamycin plates had produced numerous colonies, with the most successful plate containing approximately 42 countable colonies (Figure 2.8). *G. quercinecans* colonies that had been seemingly successfully transformed, although still cream circular colonies, were much smaller than wild type colonies previously cultured on standard nutrient agar plates. The smaller colony types suggest a lower fitness of the strain at this concentration of kanamycin.

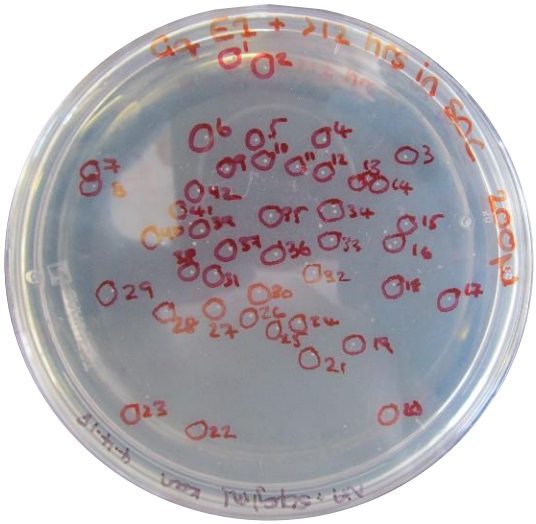

Figure 2.8. Agar plate with a concentration of 50µg/ml of kanamycin, and approximately forty-two small, but resistant G. quercinecans colonies.

Confirmation of presence of the Kanamycin-resistant plasmid using the Bioline Isolate II Plasmid Mini Kit, and subsequent agarose gel electrophoresis, was ambiguous, due to the apparent presence of genomic DNA in the extraction (Figure 2.9), indicating that the transformation of the plasmid into the electrocompetent cells was unsuccessful. This lead to the assumption that the colonies on the apparently successful plates did not contain the plasmid, but had managed to develop some natural resistance to kanamycin throughout the transformation process.

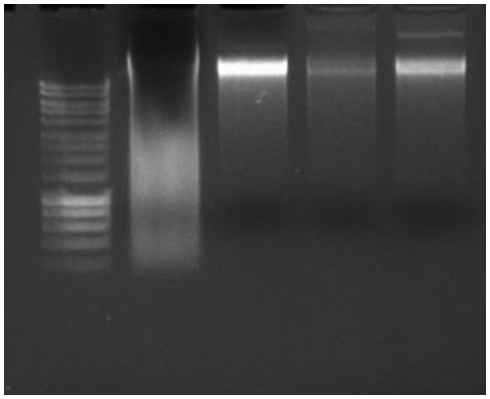

Figure 2.9. After a 1 kb hyperladder, the plasmid in its pure form is in the first lane, although is apparent as a smear with the distinct band being unclear. Three separate extractions from seemingly transformed G. quercinecans strains follow, although clear bands can be seen at >10 kb, indicating genomic DNA as opposed to plasmid DNA, with no band being obvious near the size of the pure plasmid smear.

The use of plasmid transformation was the best method tested to produce resistant strains of bacteria, although it is unclear whether the transformation was successful or whether the strains developed resistance naturally during the process. Kanamycin-resistant strains of *G. quercinecans,* resistant at the recommended concentration of 50 µg/ml were produced, however this method was highly

inefficient, with only three final strains that were resistant after further passage onto Kanamycin agar plates.

2.4 Conclusions

The main aim of this series of experiments was to develop antibiotic-resistant strains of *B. goodwinii* and *G. quercinecans,* two phytopathogenic bacteria associated with lesion formation in acute oak decline, in order to selectively isolate these strains from samples of rainwater and forest soil. The main objectives were to; investigate growth rates and OD_{600} values of *B. goodwinii* and *G. quercinecans* for mid-log growth phase, assess microbial growth in rainwater and forest soil, and to utilise various methods to generate antibiotic-resistant strains of *B. goodwinii* and *G. quercinecans,* which can be selectively cultured from rainwater and forest soil samples. Although some level of resistance to the antibiotic Kanamycin was developed in *G. quercinecans,* this appeared to have a large effect on the fitness of the strains, with colonies taking longer to form (based on the growth time from previous experiments), and also morphologically being much smaller than the wild type strain, meaning they could not confidently be used in the survivability experiments in Chapter 3. The failure to produce antibiotic-resistant strains of *B. goodwinii* and *G. quercinecans,* requires a different method of selective culture to be identified and explored in order to complete the survival experiments proposed in Chapter 3.

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CHAPTER 3

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

3.1 Introduction

It is well established that trees play an enormous role in the general functioning of the Earth, delivering key ecosystem services, supporting biodiverse ecosystems, and providing habitats for a huge range of plant and animal species (Andreote and Pereira E Silva, 2017; Boyd et al., 2013; Freer-Smith and Webber, 2017; Gamfeldt et al., 2013). Tree diseases, including diebacks, blights and bleeding cankers, have become more frequent in a range of economically important tree species, leading to the prioritised need to understand these diseases and how they affect the species individually, and forest ecosystems as a whole.

Acute Oak Decline

Acute oak decline (AOD) is an example of one such decline disease, affecting the UK's native Oak species. In recent years, AOD has spread at an alarming rate across the UK, from the south-east to the north of England, and as far west as south Wales (Broberg et al., 2018). As a part of the wider decline complex, influenced by both biotic and abiotic factors, AOD causes the rapid decline of tree health, with mortality recorded in as little as five years (Denman et al., 2014, 2012). Affecting the native UK oak species *Quercus robur* and *Quercus petraea,* AOD is defined by the presence of four major symptoms (Figure 3.1); cracks on the outer bark plates, "bleeding" patches on the stem and necrotic lesions in the inner bark, which are usually in close proximity to larval galleries of the bark-boring beetle, *Agrillus biguttatus* (Denman et al., 2014).

Figure 3.1 Four main symptoms of Acute oak decline, (A) Cracks in the outer bark plates, (B) Stem bleeds, (C) Necrotic lesions on the inner bark, (D) A. biguttatus larval feeding gallery (Forest Research, 2018).

Formation of necrotic lesions has been proven to be caused by *Brenneria goodwinii* and *Gibbsiella quercinecans* (Denman et al., 2018), two bacterial species that were described after being initially isolated from lesions of oak trees symptomatic of AOD (Brady et al., 2017, 2010; Denman et al., 2012; Sapp et al., 2016). This has been explored through a combination of culture-based and molecular methods. Isolation studies found *B. goodwinii* and *G. quercinecans* in AOD-symptomatic oak trees in various stages of decline, demonstrating that both species are clearly crucial in the development of AOD (Doonan, 2016). Koch's Postulates were tested through individual and combinations of bacterial inocula to recreate the polymicrobial cause of the lesions. It was found that the larvae of the *A. biguttatus* beetle increased lesion size, by apparently dragging the pathogenic bacteria through the inner bark. Finally, microbiome investigations identified *B. goodwinii* as the most abundant species in the AOD-symptomatic lesion margin, as well as being present in the AOD-asymptomatic metagenome. *G. quercinecans* was also present in AOD-symptomatic lesion metagenomes (Denman et al., 2018).

Although there is a strong correlation between the presence of the *A. biguttatus* beetle and AOD (Brown et al., 2015), initial hypotheses stating that the beetle acts as a vector for the bacteria associated with the disease (Denman and Webber, 2014) are lacking sufficient empirical evidence, therefore the exact role of the beetle in the development of the disease is currently unknown. It is suggested that the beetle larvae transport the AOD-associated bacteria throughout the inner bark when creating their larval galleries, as these are often located close to the necrotic lesions caused by the bacteria. Studies have investigated the spread and distribution of *A. biguttatus* (Brown et al., 2015), however there have been no such studies to explore the bacterial distribution in the forest ecosystem and the possible methods of entry utilized by these bacteria. Research on the role of bacteria in AOD has focussed mostly on characterising the individual bacteria associated with the disease (as well as how these bacteria behave in combination), and their gene expression, to determine their pathogenic traits which may aid in the understanding of how these bacteria are moving from host to host (Broberg et al., 2018). The ecology, transmission and survival of AOD bacteria beyond the tree host is therefore a key knowledge gap.

Environmental Reservoirs of Plant Pathogens

It is well known that rainwater and forest soils can act as environmental reservoirs for phytopathogens, such as the bacterium *Pseudomonas syringae* (Debener et al., 1991). One pathovar of this species, *P. syringae* pv. *aesculi,* is an airborne pathogen which falls onto trees in rainwater, and gains access through cracks and scars on the surface of leaves (Monteil et al., 2014; Morris et al., 2010). This species has been confirmed as the cause of horse chestnut bleeding canker (Mertelik et al., 2013; Webber et al., 2008), a disease affecting horse chestnut trees (*Aesculus hippocastanum*) across Europe (Bultreys et al., 2008). Symptoms of the disease include cracks in the bark plates (from

which a rust coloured liquid seeps), necrotic phloem tissues, and discolouration of the foliage. In some circumstances, mortality of a host can occur in less than five years (Green et al., 2009). Studies focusing on the survivability of *Pseudomonas syringae* pv. *aesculi* have demonstrated the prolonged viability and virulence of the species, which can still cause lesions on Horse Chestnut trees after being stored in rainwater for up to 6 years and soil for up to a year (Laue et al., 2014).

From the study of other *Gibbsiella* species, *G. quercinecans,* is thought to be a forest generalist, as the species have been isolated from various tree species (oak, pear, apple), as well as the gut of wood boring insects, the digestive system of a butterfly, and even the mouth cavity of a bear (Brady et al., 2014; Geider et al., 2015; Rizzi et al., 2013). *B. goodwinii* on the other hand, is thought to be a possible tree endophyte, as other species in the genus *Brenneria* are defined as being host-species specific pathogens, usually with no known environmental reservoirs. Members of the genus *Brenneria*, such as *B. silicis,* which causes watermark disease in Willow trees, and *B. nigrifluens* which affects walnut trees*,* are also present as endophytes in the vascular tissue of the host, before becoming virulent pathogens (Maes et al., 2009b; McClean et al., 2008).

As lesions formed on trees symptomatic of AOD occur in the phloem tissues (Brown et al., 2017b), the tissues of the plant that are involved in the transportation of sugar molecules and amino acids (nutrients vital for the growth and development of the plant) to the different components of the organism (Lalonde et al., 2003). Although the phloem tissues are not primarily involved in the transport of water (this is the function of the xylem tissues), the transportation of the nutrient molecules are driven by osmotic pressures and a hydrostatic gradient, meaning that water is controlled, and therefore heavily involved in these tissues (Hölttä et al., 2009). This could indicate that *B. goodwinii* lies within the tree tissues (in the phloem) and *G. quercinecans* infects the tree through the uptake of water, or by *Agrillus* transmission (after entering through bark cracks). When the two species accumulate in the phloem tissues, they then express pathogenic behaviours, causing the formation of necrotic lesions. These speculations have lead to the development of the following hypotheses, which will be tested in the study. The first is that *B. goodwinii* is a specific tree endosymbiont and will therefore decay quickly outside of the oak host in rainwater and forest soil, and the second is that *G. quercinecans* has a broad distribution in forest ecosystems and will therefore survive outside of the oak host in rainwater and forest soil.

Aims and Objectives

The overarching aim of the following experiments, used to test the hypotheses, are to determine whether rainwater and forest soil can provide the conditions required by *Brenneria goodwinii* and *Gibbsiella quercinecans* to survive outside of the oak host. This will test the hypotheses and

demonstrate whether rainwater or forest soil are environmental reservoirs for these phytopathogenic bacteria. The aim of the following experiments will be met through the completion of the following objectives:

A. Determine the survivability of *B. goodwinii* and *G. quercinecans* in sterile rainwater.
B. Develop a selective culture method to enumerate *B. goodwinii* and *G. quercinecans* survival in forest soil.
C. Determine the survivability of *B. goodwinii* and *G. quercinecans* in forest soil.

3.2 Methods

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

Strain identity and purity was confirmed as described in Section 2.2.1 Bacterial strains, growth condition and confirmation of identity.

Rainwater collection and microcosm processing

Rainwater survival experiments were to be carried out using sterile rainwater due to the failure to develp antibiotic resistant strains in Chapter 2. For the initial experiment, rainwater was collected over a two-week period in a large bucket lined with two large autoclave bags. The bucket was situated on the roof of the Memorial Building, Bangor University from the 11th May to the 14th May, and the surrounding area was cleared to reduce the risk of ground splash contaminating the sample. One litre of this was collected and frozen at -20°C until the commencement of the experiment. The frozen rainwater was thawed for six hours and then autoclaved twice, prior to 30 ml each being measured into twelve sterile 50 ml falcon tubes. These were to act as the rainwater microcosms for the initial experiment (Figure 3.2). The experiment was repeated, and rainwater was collected as before (from the 2nd June to the 3rd June 2018). Again, the collected rainwater was autoclaved (immediately after collection, and then a further two times on consecutive days). Once again, 30 ml of the sterilised rainwater was measured into twelve sterile 50 ml falcon tubes and these were used as the rainwater microcosms for the repeat experiment.

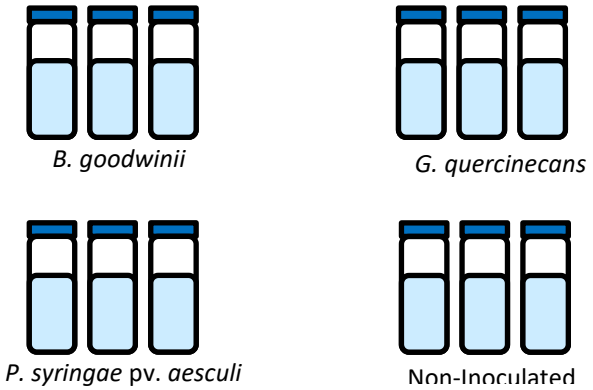

Figure 3.2. Diagram of the twelve falcon tubes to be used as rainwater microcosms for the experiment. Three biological replicates each, inoculated with either B. goodwinii, G. quercinecans, P. syringae pv. aesculi or a non-inoculated control.

Rainwater Microcosm Inoculation Procedure

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

Previous growth curve experiments in Chapter 2 found that at an OD_{600} of 0.5; a culture of *B. goodwinii* contained approximately 1.13 x 10^{14} CFU/ml, a culture of *G. quercinecans* contained approximately 1.7 x 10^{8} CFU/ml, and a culture of *P. syringae* pv. *aesculi* contained approximately 2.1 x 10^{7} CFU/ml. The varying CFU/ml of each species lead to the decision to add the volume of culture to give approximately 1 x 10^{6} CFU/ml in each 30 ml microcosm ((required no. of CFU / no. of CFU possessed) x Volume).

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

each of the non-inoculated control microcosms to adjust for added liquid volume and salts. After this initial inoculation, all twelve rainwater microcosms were vortexed vigorously for thirty seconds.

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

Rainwater Sampling Procedure

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

Two hundred millilitres of standard nutrient agar, and 200 ml of nutrient agar with 5% Sucrose were produced and poured into sterile square bioassay trays (sterilised through soaking in 5% bleach for a minimum of 30 minutes and then being UV treated before being wiped with 1% Distel prior to plating). These plates were then left to set for a minimum of three hours in the biosafety cabinet.

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

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

The colony forming unit (CFU) counts of *B. goodwinii, G. quercinecans* and *P. syringae* pv. *aesculi* were carried out on day T_0, T_1, T_7, T_{14}, T_{21}, T_{28}, T_{56}, and T_{84}.

The number of colony forming units per ml at each time points was calculated by multiplying by the dilution factor where countable colony numbers were present. A graph of strain viability over time was produced including 95% confidence intervals.

3.2.2 Validation of a Selective Medium for Identification of *B. goodwinii* and *G. quercinecans*

Due to the failure to develop an antibiotic resistant strain of *Brenneria goodwinii* or *Gibbsiella quercinecans,* a selective media method was validated for the ability to identify and differentiate *B. goodwinii* and *G. quercinecans* from resident microorganisms in forest soil. This was tested through a small-scale pilot study in which each aspect of the experimental methods was individually verified.

Eosin Methylene Blue (EMB) agar was used due to it being selective against Gram-positive bacteria and fungi. The three bacterial strains used in the study were tested for their growth capacity on this medium, and were successful. *B. goodwinii* and *G. quercinecans* were easily identifiable on the EMB agar due to the development of colonies with an iridescent green sheen. The growth of *P. syringae* pv. *aesculi* was similar to it's growth on standard nutrient agar. Small, off-white, glossy looking colonies grew and increased in size over a forty-eight-hour period when incubated at 28°C.

The next stage of method development was to test whether these bacteria would be identifiable when included in a soil sample containing standard soil microorganisms, including numerous bacteria and fungi. For this, a small amount of soil was collected (from beneath a mature oak tree at Treborth Botanic Gardens), and spiked with one colony (picked using a sterile inoculation loop) of *B. goodwinii* or *G. quercinecans* cells from a pure plate culture. Serial dilutions were then produced and plated onto EMB agar. The plates were incubated at 28°C for 48 hours and observed after this time.

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

Forest Soil Collection and Microcosm Processing

On the 4th July 2018, approximately 1 kg of soil was collected from beneath a mature oak tree at Treborth Botanic Garden, Bangor University. The soil was collected from the base of the tree between two buttress roots. After brushing away the topsoil and any forest debris (such as twigs and leaf litter), soil was taken from around four to six inches below the surface. The soil was collected in a clean plastic bag and the shovel used was disinfected with 1% Distel prior to digging. Once obtained, the remaining soil was used to cover the hole and the topsoil and debris was replaced to reduce further disturbance of the area. The collected soil was immediately taken back to the lab where it was further processed for the experiment.

Further processing of the soil was carried out in the biosafety cabinet to reduce the risk of the soil being contaminated. Larger aggregates of soil were broken down by hand and then sieved using a sterile (autoclaved and UV treated) 2 mm sieve to give even sized particles. Fifty grams of the sieved soil was weighed into a sterile (UV treated), resealable plastic bag. This was repeated twelve times (Figure 3.3), to give twelve soil microcosms (three replicates for *B. goodwinii* inoculation, three

replicates for *G. quercinecans* inoculation, three replicates for *P. syringae* pv. *aesculi* inoculation, and three replicates for the non-inoculated control).

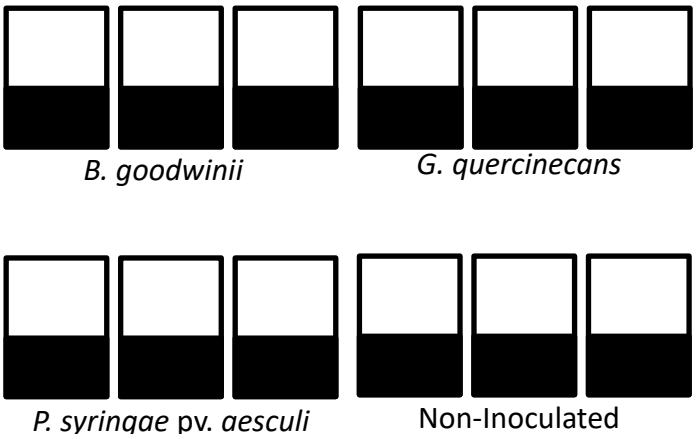

Figure 3.3 Diagram of the twelve bags to be used as soil microcosms for the experiment. Three biological replicates each, inoculated with either B. goodwinii, G. quercinecans, P. syringae pv. aesculi or a non-inoculated control.

Forest Soil Microcosm Inoculation Procedure

Ten millilitre starter cultures were produced for *B. goodwinii, G. quercinecans* and *P. syringae* pv. *aesculi* (in triplicate), by performing a 1% inoculation (100 µl) of an overnight broth culture (produced from stock agar plates, incubated overnight at approximately 25°C, shaking at 150 RPM) into 10 ml of nutrient broth (results in three replicate 10 ml cultures of each of the three bacteria). The cultures were then incubated at 28°C, shaking at 150 RPM, until they reached an OD_{600} value of 0.5. The cultures were then centrifuged at 3000 *x g* for five minutes to pellet the cells. The supernatant was removed and discarded, and the cells re-suspended in 500 µl of PBS solution, and vortexed briefly. The 500 µl cell suspension was then inoculated into the soil microcosm bags (Figure 3.3), 500 µl of PBS solution was added to the non-inoculated control microcosms to correct for moisture addition across the 12 microcosms. Each microcosm bag was shaken vigorously for 30 seconds to ensure even and thorough mixing. The microcosms were stored at 10°C throughout the experiment, as this is the average annual forest temperature in the UK.

Forest Soil Microcosm Sampling Procedure

The first sampling event took place within one hour of initial inoculation. This (and all subsequent sampling events) involved the following, which was carried out in the biosafety cabinet to minimise the risk of contamination:

Four hundred millilitres of Eosin Methylene Blue agar was autoclaved and 100 ml was poured into four petri dishes (150 mm x 15 mm). All equipment and consumables were UV treated for 20 minutes before starting the sampling process. Each microcosm bag was shaken vigorously for 30 seconds to ensure even and thorough homogenisation of the soil and bacteria within.

From each microcosm, 0.5 g of soil was weighed into a sterile Eppendorf tube four times, one of which was frozen at -20°C, for qPCR analysis. The three remaining soil samples (from the four taken) from each microcosm (technical replicates A, B and C), were used for the next stage of the sampling process. One millilitre of PBS solution was added to the three Eppendorf tubes, and the contents vortexed at a medium to high speed for 30 seconds. These undiluted samples were then used to create a serial dilution to 10^{-8}. This was performed in a 96-well micro plate (Figure 3.4) where each well contained 270 µl of PBS solution and 30 µl of the undiluted sample was added to the top row, and the contents of the well pipetted up and down five times to mix before moving on to the next dilution for each replicate on the next row.

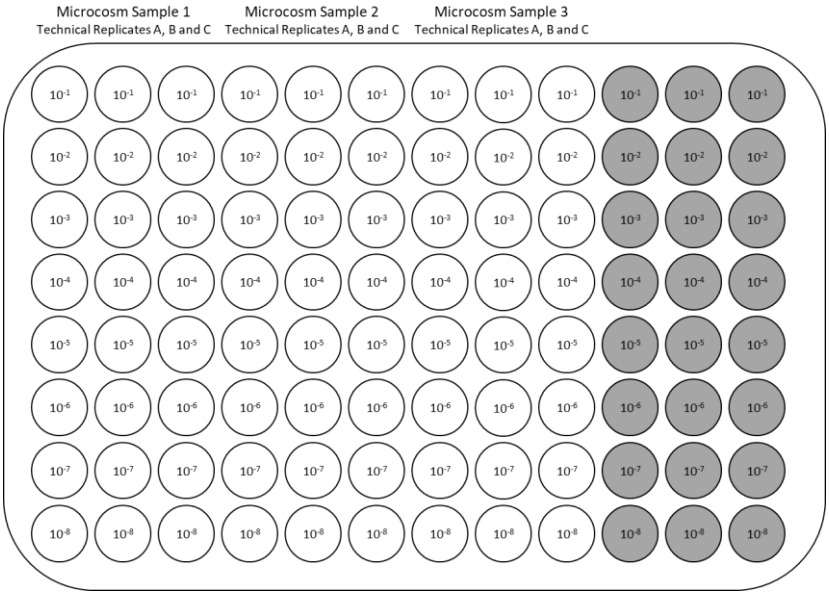

Figure 3.4. 96-well Microplate arrangement for soil microcosm serial dilutions

Twenty microliters of dilutions 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} were plated onto the EMB agar plates (1 plate per condition; *B. goodwinii, G. quercinecans, P. syringae* pv. *aesculi* and non-inoculated control). The four plates were incubated at 28°C for 48 hours and after this incubation period, the number of visible colonies was counted and recorded.

CFU counts were determined after T_0, T_1, T_3, T_7, T_{14}, T_{21}, T_{28} and T_{56} days. The number of colony forming units per ml at each time point was calculated by multiplying by the dilution factor.

qPCR Analysis of Soil Microcosms

In order to support the culture based methods of measuring the decay rates of the bacteria, molecular techniques were also used. Quantitative PCR (qPCR) analysis of the bacterial gyrase B (gyrB) gene, using the Bioline SensiFAST™ SYBR Lo-ROX kit (BioLine, 2018) was used to determine *B. goodwinii* and *G. quercinecans* gene copies in the micrososms.

Firstly, DNA extraction of each of the microcosm samples (three biological replicates of *B. goodwinii*, *G. quercinecans, P. syringae* pv. *aesculi* and the non-inoculated control) was completed using the MoBio PowerSoil® DNA Isolation Kit, following the manufacturers protocol (MO BIO Laboratories, 2016).

Primers were designed by Dr James Doonan, Bangor University, and were used for both producing the standards and also the qPCR reaction (Table 3.1).

Table 3.1. Primer names and sequences used for the qPCR analysis of B. goodwinii and G. quercinecans. All primers designed to target the bacterial GyraseB gene (GyrB).

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

qPCR standards for absolute quantification of gyrB gene copy number for *B. goodwinii* and *G. quercinecans* were produced. This involved amplification of the bacterial DNA gyrase subunit B (gyrB) gene using qPCR specific primer sets. DNA was extracted from cells grown from glycerol stocks, using a "boil prep method"; a single colony was picked from an agar plate containing a pure culture of the isolate using a sterile inoculation loop, and suspended in 20 µl of PCR-grade water. Subsequently, the cell suspension was heated to 95°C for two and a half minutes, vortexed vigorously for 10 seconds, and heated again at 95°C for a further two and a half minutes. After being briefly vortexed, 1 µl of extracted DNA was used as the template for the PCR reaction using Bioline MyTaq™ Red Mix. Each 50 µl reaction consisted of 25 µl of 2x MyTaq™ Red Mix, 1 µl of 10 pmol each of GyrB gene specific oligonucleotide primers for generation of qPCR standards (Table 3.1), 22 µl of nuclease-free H_2O, and 1 µl of the DNA template (from the "boil prep" extraction method above). PCR reaction conditions were: initial denaturation at 95°C for 1 minute, followed by 35 cycles of; denaturation at 95°C for 15 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 10 seconds, producing a PCR product which was approximately 750 base-pairs in length. The PCR product was run in a 1% agarose gel at 100 V for 50 minutes, and after viewing in a light box, the bands were cut out, and the bands

were extracted from the gel using the Qiagen QIAEX® II Gel Extraction kit (following the manufacturer's protocol (Qiagen, 2008)) the resulting DNA was then quantified using the Qubit™ dsDNA HS Assay Kit. The standards were calculated using the following equations:

$$\frac{X \text{ g of DNA (from the Qubit quantification)}}{\text{BP length (of the amplicon)} \times 660 \text{ (average weight of a base pair)}} = \text{Molecules/µl}$$

Where by the "BP length" of the resulting *B. goodwinii* amplicon was 959 bp, and the *G. quercinecans* amplicon was 1012 bp.

$$\frac{3 \times 10^8 \text{ (approximate number of gene copies required)}}{\text{Molecules/µl (from the above equation)}} \times 100 = \text{Volume to have in 100µl (for required gene copy number)}$$

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

3.3 Results

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

The results of the strain identity and purity confirmation are presented in section "2.3.1 Confirming Strain Identity Through Amplification and Consequent Sequencing of the Bacterial GyrB Gene".

The sterile rainwater survivability experiment (Figure 3.5) demonstrated that *B. goodwinii,* after being undetectable from T_0, had lost viability within hours of the experiment commencing. This occurred in both experimental repeats. *G. quercinecans* on the other hand produced countable colonies throughout the whole experimental period of 84 days, ranging from 227 CFU/ml (on Figure 3.5, day T_7, at the lowest point of the apparent acclimatisation period) to 14818 CFU/ml (on day T_{28}, the peak before the plateu and final decline occurred). This occurred in both repeats of the experiment. The presence of countable colonies in the NIC condition indicated that there had been a small scale contamination event, leading to microbial growth in the biological replicates of this condition.

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

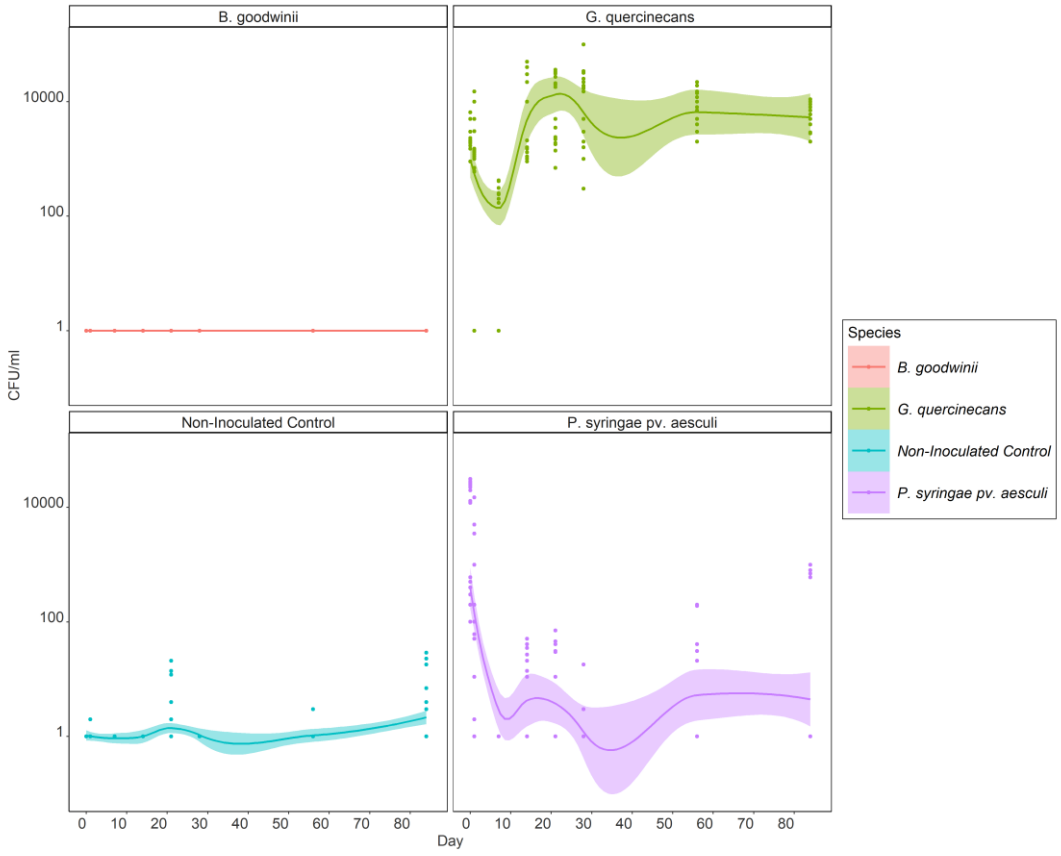

Figure 3.5. Colony Forming Units (CFU) per ml, of bacteria over time in a rainwater environment, showing that G. quercinecans, after an initial decline, recovered and maintained relatively high numbers of CFU/ml over the whole experimental period of 84 days.

3.3.2 Validation of EMB as a Selective Medium for the Isolation of *B. goodwinii* and *G. quercinecans*

Eosin Methylene Blue (EMB) agar was effective for detecton of viable *B. goodwinii* and *G. quercinecans* cells in soil samples. The inhibition of Gram-positive bacteria and fungi allowed the Gram-negative *B. goodwinii* and *G. quercinecans* to thrive, and the green iridescent sheen of the target bacteria (Figure 3.6) allowed for simple visual identification and colony counts.

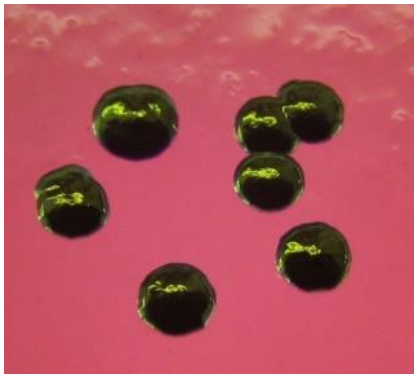

Figure 3.6. G. quercinecans colonies when cultured on Eosin Methylene Blue (EMB) agar. The obvious green colour of the bacterial colonies allows for simple visual identification.

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

The soil survival experiment (Figure 3.7) demonstrated that once again, *B. goodwinii* lost viability immediately after resuspension of a growing culture in PBS, causing cells to be undetectable from T_0. *G. quercinecans,* on the other hand had a small decline in CFU/ml at the start of the experiment (from 2.1 x 10^7 CFU/ml on day T_0 to 1.1 x 10^6 CFU/ml on day T_1), however (with the exception of T_{21}) there were countable colonies for 28 days (with approximately 22,222 CFU/ml at day T_{28}). At day T_{56}, there were no countable colonies of *G. quercinecans* demonstrating a 100% decline over the 56-day experimental period. *P. syringae* pv. *aesculi* (the positive control in the experiment) was countable up to T_{28}, with approximately 3.5 x 10^6 CFU/ml at this time point, but similar to *G. quercinecans, P. syringae* pv. *aesculi* was no longer detectable at T_{56}, signifying a 100% decline. As sampling was only carried out at T_{28} and then again at T_{56}, it is impossible to know at what point within this time *G. quercinecans* and *P. syringae* pv. *aesculi* declined to a point where they were no longer countable.

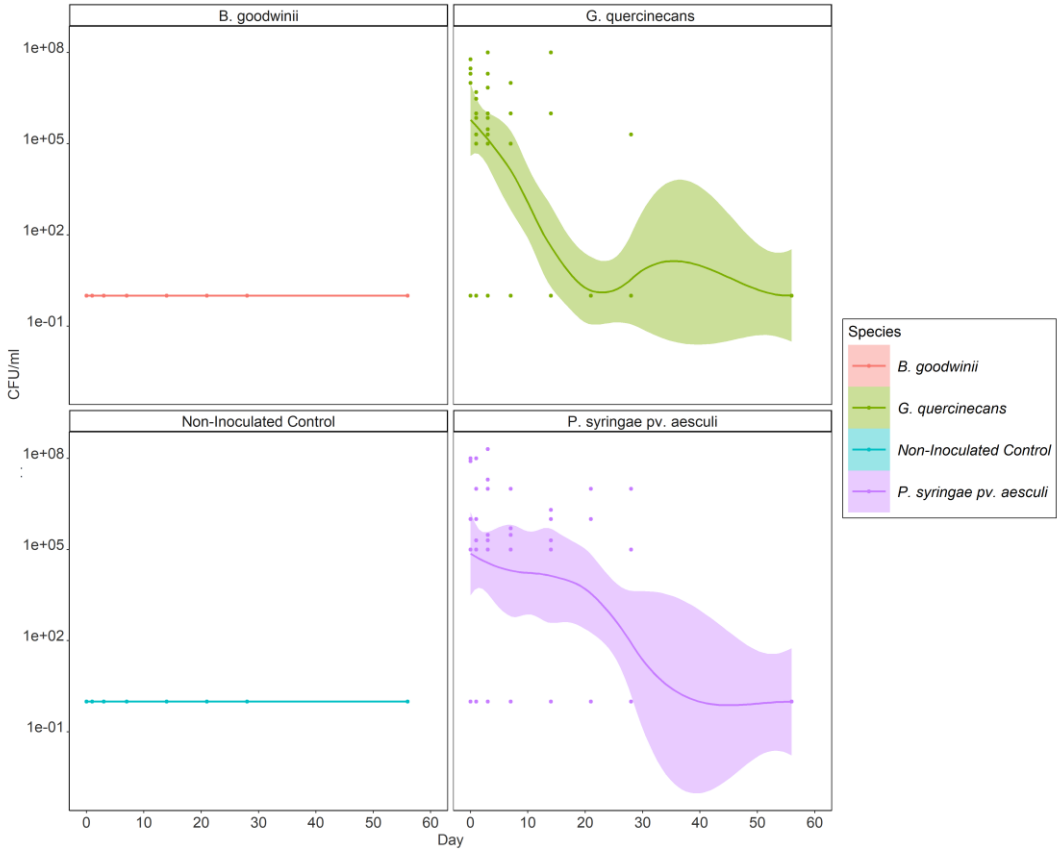

Figure 3.7. Colony Forming Units (CFU) per ml, of bacteria over time in a forest soil environment, showing that CFU/ml of G. quercinecans and P. syringae pv. aesculi fluctuated throughout the experiment, however did show an overall decline throughout the 56 day experimental period.

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

Results from the qPCR analysis using the *B. goodwinii* specific primers show that there are detectable levels of *B. goodwinii* in the *B. goodwinii* microcosms throughout the 28 day period (Figure 3.8). A rapid increase in detectable levels of *B. goodwinii* over the first 7 days of the experimental period peak (from approximately 12,373 gyrB gene copies/µl to approximately 16,189 gyrB gene copies/µl) and lead to a steady gradual decline, which still had detectable levels at day T_{28} (14824 gyrB gene copies/µl). Although gene ct values (gyrB gene copy count) values for *B. goodwinii* were obtained in the other three microcosm conditions (*G. quercinecans* microcosms, *P. syringae* pv. *aesculi* microcosms, and the Non-Inoculated Control (NIC) microcosms), it is assumed that this is due to the *B. goodwinii* specific primer also possibly partially matching with another species present in the soil. The primers used in this experiment were designed using pure cultures, and were not tested for matching in environmental samples.

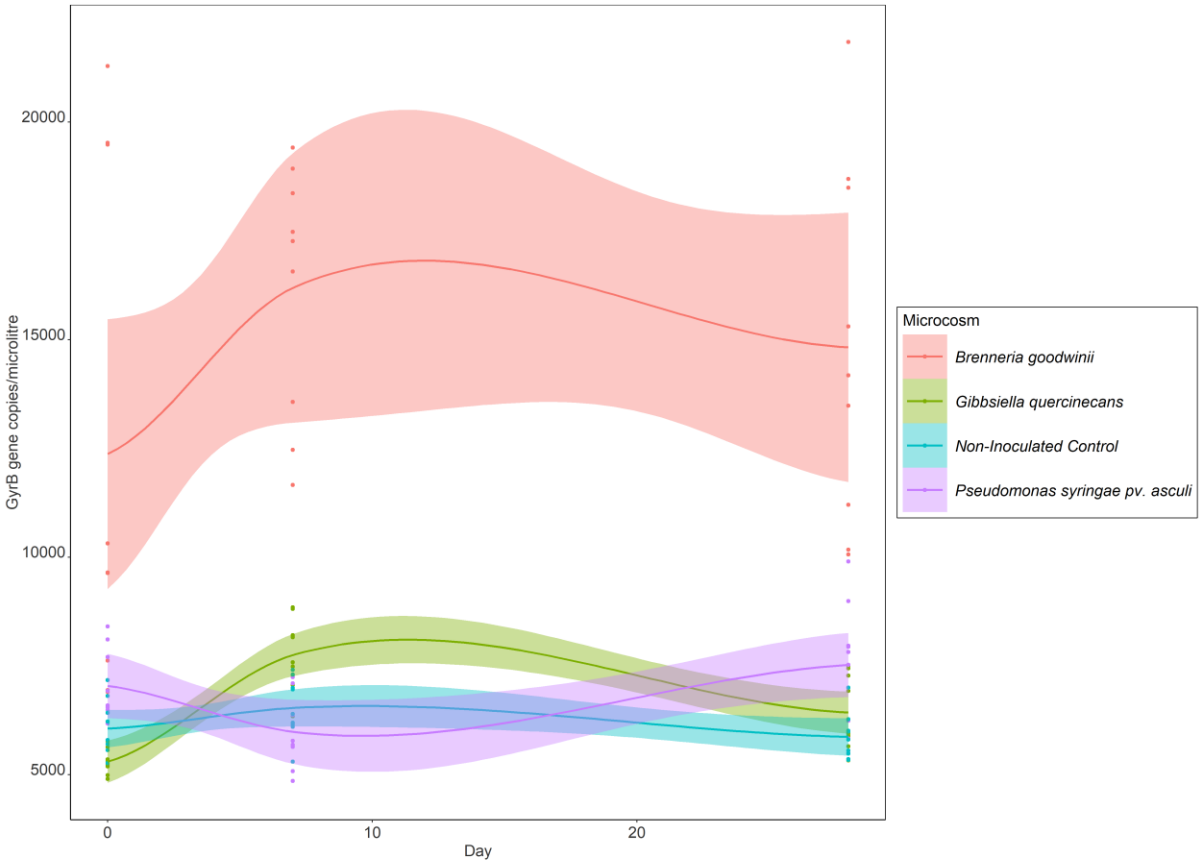

Figure 3.8. qPCR analysis of the each microcosm, detecting B. goodwinii DNA. A significantly higher number of B. goodwinii gyrB gene copies is present in the B. goodwinii microcosms (as expected). Although there is a reasonable level of detection of the gene in the other conditions (which was unexpected), it is seemingly consistant across all three conditions, leading to the suggestion that this is background contamination common to all microcosms, due to a partial match of the B. goodwinii primers with other environmental bacteria. Limit of Quantification: 250, Slope:-3.313, Y-Inter: 45.574, R2: 0.999, Amplification Efficiency: 100%, Error: 0.024

Nevertheless, the significantly higher detection of *B. goodwinii* gyrB gene copies for the *B. goodwinii* microcosms compared to the other conditions (which all had very similar numbers of gyrB gene copy detection throughout the experimental period, between 5,000 and 7,000 gyrB gene copies/µl), meant that the results were still valid.

Results from the qPCR analysis using the *G. quercinecans* specific primers show that there are detectable levels of *G. quercinecans* in the microcosms throughout the 28 day period (Figure 3.9). Following a similar trend to the culture based study, the initially high levels of *G. quercinecans* (approximately 33,415 gyrB gene copies/µl at T_0 where there were approximately 2.1 x10^7 CFU/ml) steadily declines over the course of the experiment (to 22,790 gyrB gene copies/µl at day T_7 where there were 1.4 x 10^7 CFU/ml, and just 9,445 gyrB gene copies/µl at day T_{56} where there were 1.1 x 10^7 CFU/ml). The other microcosm conditions (*B. goodwinii* microcosms, *P. syringae* pv. *aesculi*

microcosms, and the Non-Inoculated Control (NIC) microcosms), have negligible amounts of detection of *G. quercinecans* in them (all less than 100 gyrB gene copies/µl at ant time point), showing that the *G. quercinecans* specific qPCR primer is specific to *G. quercinecans*.

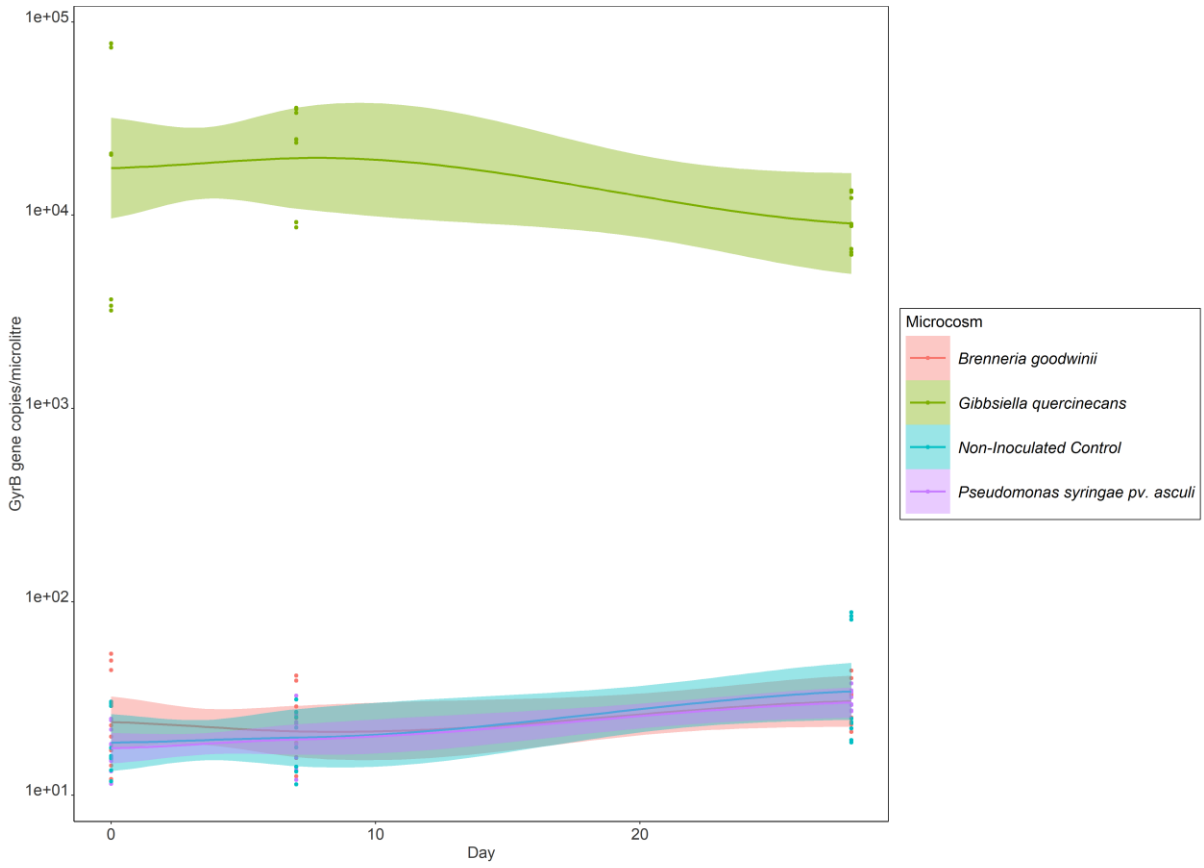

Figure 3.9. qPCR analysis of the each microcosm, detecting G. quercinecans DNA. A significantly higher number of G. quercinecans gyrB gene copies is present in the G. quercinecans microcosms, with extremely low levels of detection in the other conditions. Limit of Quantification: 250, Slope:-3.313, Y-Inter: 45.574, R2: 0.999, Amplification Efficiency: 100%, Error: 0.024

3.4 Discussion

The main aim of this study was to determine whether rainwater or forest soil could provide the conditions required to act as environmental reservoirs for *B. goodwinii* and *G. quercinecans*, bacteria associated with the rapidly spreading decline disease, acute oak decline (AOD).

Brenneria goodwinii

The results of the culture-based survival experiments, both in rainwater and forest soil, indicate that *B. goodwinii* does not have the ability to survive in a rainwater or soil based environment. This loss of viability suggests that natural bodies of rainwater and forest soils would not provide suitable conditions to act as environmental reservoirs for *B. goodwinii*. However, the molecular qPCR analysis

of the soil microcosms provided a contradictory result indicating that *B. goodwinii* DNA was detectable for up to 28 days in forest soil. Providing evidence to suggest that the *B. goodwinii* cells may have entered a "viable but non-culturable" (VBNC) state, as there was a rise in quantifiable *B. goodwinii* DNA between T_0 and T_7, and a slightly declining, but relatively consistent level of detection between T_7 and T_{28}. This change could also be explained by the degradation of cells leading to DNA from dead cells being present in the microcosms at a low level until T_{28}, therefore further investigation is required.

It is known that species in the *Brenneria* genus are plant pathogens, causing necrogenic infections of various tree species (Brady et al., 2012). Previous studies have confirmed that the development of AOD has a polymicrobial cause, and multi-omic data has confirmed that *B. goodwinii* is a key bacterial pathogen in this process (Broberg et al., 2018; Denman et al., 2016b). The results of this study raise the question proposed in the first experimental hypothesis, that *B. goodwinii* is a specific tree endosymbiont and will therefore decay quickly outside of the oak host in rainwater and forest soil. If the bacteria does not have the ability to survive in rainwater or forest soil (the two most common environmental reservoirs in a forest habitat), then is it possible that this phytopathogenic species is a specific tree endophyte, residing in the host tissues until predisposing factors and inciting factors (Manion, 1981) cause sufficient weakening of the host that *B. goodwinii* becomes virulent and attacks the tissues of the host. Some species in the *Brenneria* genus are known to have this endophytic lifestyle, such as *Brenneria salicis*, the cause of Willow watermark disease, which is spread through leaf to leaf contact (Maes et al., 2009b). *B. salicis* resides in willow tree tissue in a non-pathogenic form, with undescribed factors and stressors tipping the balance between endophyte and pathogen (Maes et al., 2002).

Gibbsiella quercinecans

The results of the rainwater survival experiment indicate that although there appeared to be an initial acclimatisation period, where a preliminary decline was followed by rapid multiplication of *G. quercinecans.* This strain has the ability to survive in rainwater for up to 84 days (approximately 3 months). This would provide sufficient time for the bacterium to transfer from one host to the next, suggesting that bodies of rainwater could provide sufficient conditions to act as an environmental reservoir for *G. quercinecans.* The results of the culture-based soil survival experiment indicate that after an initial decrease in CFU/ml, *G. quercinecans* slightly recovered around T_{28}, only to decline fully between T_{28} and T_{56}. The molecular qPCR analysis of the soil microcosms for detection of *G. quercinecans* were as expected from the initial culture-based results, following a similar pattern of decline. These results suggest that *G. quercinecans* has the ability to survive in rainwater and to a

lesser extent forest soil (although conditions in a sealed forest soil microcosm would no longer be considered natural after 28 days, due to the lack of nutrient and carbon cycling).

As well as the type species of the genus *Gibbsiella* being isolated from AOD-symptomatic oak trees in the UK (Brady et al., 2010), G*ibbsiella* species have also been isolated from a wide range of environments, including diseased apple and pear trees, and decaying wood from a compost pile (Geider et al., 2015), the intestinal tract of a butterfly in Korea (Kim et al., 2013) and the oral cavities of a bear, where acorns provide a high percentage of the autumnal diet of the bear (Saito et al., 2012). Molecular genetic analysis of various *Gibbsiella* species have also indicated divergence in a number of genes present across the genus. This has been linked to the requirement for these saprophytes to adapt to a range of conditions present in these differing environments, as well as the numerous microbial competitors present in these environments (Geider et al., 2015).

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

Validation of a Selective Medium for Isolation of B. goodwinii and G. quercinecans

The development of a selective media for the use in this particular experiment was a success. The use of the Eosin Methylene Blue (EMB) agar inhibited the majority of the other microorganisms present in the forest soil, which could have acted antagonistically, peventing the growth of the target bacteria. This along with the iridescent green colour produced by the bacterial colonies, made for easy identification of *B. goodwinii* and *G. quercinecans* in the samples, and PCR and subsequent sequencing of the strains confirmed this. The experimental design of this experiment allowed the use of EMB agar to be successful, predominantly the combined use of PCR sequencing and qPCR analysis for species confirmation, along with the EMB culture, which ensured that the iridescent green colonies present were in fact *B. goodwinii* and *G. quercinecans.*

Gram-positive bacteria, which are inhibited by EMB agar (Nordmann et al., 2018b), are often found to dominate the deeper layers of soils, however Gram-negative bacteria, not inhibited by EMB, are known to dominate soils with readily available substrates (Schindlbacher et al., 2011), such as the forest floor. This understanding must be present in the planning of any future experiments that may require other types of forest soils to be cultured in the search for *B. goodwinii* and *G. quercinecans.* Secondary to this, other bacterial species, such as *Escherichia coli* (*E. coli*) and some *Klebsiella* and

Serratia species, are also known to produce colonies with the same iridescent green sheen when grown on EMB agar (Leininger et al., 2001). In light of this, any culture-dependant study should be confirmed through the use of molecular tools such as PCR and qPCR.

Improving the Experimental Design and Future Research

Although the rainwater survival experiment indicated *G. quercinecans* had the ability to survive in rainwater, the experiment did not address whether these species would be able to survive in a natural body of rainwater with other microbial competitors. The decline of countable CFUs over the 84-day period in all of the conditions, could be due to a number of factors, not only the inability of the strains to survive in an aquatic environment. Other possible reasons for the decline could be due to the amount of biomass in the sterile rainwater being finite and becoming depleted over the 84-day period, leading to the lack of a food source for the bacterial strains. To address these issues, an improvement to the experimental design, and the focal point for future research, could include a repeat of the rainwater survival experiment with non-sterile rainwater, using EMB agar to add a selective component to allow for accurate colony counts. This would improve the validity of the rainwater survival experiment by determining whether the target bacteria of *B. goodwinii* and *G. quercinecans* could survive in rainwater with the presence of natural microbial competitors. This would provide results that are more representative of the normal activities expected to take place in the natural environments. It would also be of interest to investigate *Brenneria goodwinii* and *Gibbsiella quercinecans* in co-culture, as the presence of both species in AOD lesions indicates that they may facilitate each other.

An element of the study which holds a certain level of ambiguity is the contradictory CFU/ml counts (indicating almost immediate depletion) and the qPCR values (indicating a relatively constant level of cells) of *B. goodwinii* across the soil survival experiment. This could be addressed by a follow up experiment focusing on the length of time extracellular DNA (DNA that exists outside of the cell) persists in soils after cell death. The conditions regarding the death of a bacterial cell can affect the amount and quality of DNA that is released after death (Muela et al., 1999), which could affect detection of gene copies therefore this would need to be addressed in the experimental design. The future study would focus on the detectable quantity of specific bacterial DNA (*B. goodwinii* or *G. quercinecans*) over time to determine the length of time this DNA can persist and be detected. This would aid in determining whether the results in the qPCR analysis of this study are reliable, or whether DNA from dead cells was being detected, which would therefore support the hypothesis that *B. goodwinii* cannot survive in forest soil, but *G. quercinecans* does have this ability.

3.5 Conclusions

The aim of this study was to investigate whether rainwater and forest soil could provide the required conditions for AOD-associated bacteria, *B. goodwinii* and *G. quercinecans* to survive. This would address the question that these habitats could be acting as environmental reservoirs for these bacteria. The main objectives of the study were to use decay rates to determine the survivability of *B. goodwinii* and *G. quercinecans* in rainwater and forest soil, and to develop a selective culturing method for the isolation of these strains. The results of the study indicated that in culture-based experiments, *B. goodwinii* was unable to survive in rainwater (without natural microbial competitors) or forest soil, therefore supporting the hypothesis that *B. goodwinii* is a specific tree endosymbiont and will therefore decay quickly outside of the oak host in rainwater and forest soil, however molecular qPCR analysis indicated a measurable level of *B. goodwinii* DNA throughout the 56 day soil survival experimental period. This suggests that without further investigation, the hypothesis cannot be accepted. The results of the study revealed *G. quercinecans* to have the ability to survive in both rainwater (without natural microbial competitors) for up to 84 days, and forest soil for up to 28 days (with molecular qPCR data to corroborate this). This supports the hypothesis that *G. quercinecans* has the potential for a broad distribution in forest ecosystems and will therefore survive outside of the oak host in rainwater and forest soil. The information presented in this study will aid in the understanding of the microbial ecology of *B. goodwinii* and *G. quercinecans,* which is crucial to understanding the biology and spread of Acute Oak Decline.

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CHAPTER 4
Final Synthesis

4.1 Review of the Aims, Hypotheses and Objectives

This study set out to investigate whether rainwater and forest soil are potential environmental reservoirs for *B. goodwinii* and *G. quercinecans,* two bacteria associated with lesion formation (Denman et al., 2018) in acute oak decline (AOD). Therefore providing key knowledge on potential environmental reservoirs and ecological distribution with in a forest ecosystem for these important species. *B. goodwinii,* as part of the genus *Brenneria* has been suggested to be a host-species specific pathogen (as are all *Brenneria* species) and there has also been speculation that *B. goodwinii* could be a tree endophyte (similar to other members of the genus (Maes et al., 2009a)). This led to the first experimental hypothesis, that *B. goodwinii* is a specific tree endosymbiont and will therefore lose viability quickly outside of the oak host in rainwater and forest soil. *G. quercinecans*, as a member of the genus *Gibbsiella* was suspected to be a forest generalist with a broad host range. *G. quercinecans* has already been isolated from numerous environments and ecosystems (Kim et al., 2013; Saito et al., 2012), which led to the second experimental hypothesis, that *G. quercinecans* has a broad distribution in forest ecosystems and will therefore survive outside of the oak host in rainwater and forest soil. These hypotheses were to be tested through the four main objectives.

The first objective was to develop antibiotic-resistant strains of *B. goodwinii* and *G. quercinecans* (explored in Chapter 2). This was in order to use these strains for the survivability experiments in Chapter 3, where the strains were to be inoculated into rainwater and forest soil*,* and with the use of antibiotic containing media, to suppress the growth of autochthonous bacteria, the strains could be specifically re-isolated and identified with ease. Unfortunately, out of all of the methods tested for producing antibiotic resistant bacteria, none provided strains of *B. goodwinii* and *G. quercinecans* that were appropriate to use in the following experiments. *B. goodwinii* proved sensitive to very low concentrations of antibiotics, making progress difficult. *G. quercinecans* was relatively successful compared to *B. goodwinii,* as a strain resistant to up to 20µg/ml of kanamycin was developed. The recommended working concentration of kanamycin, however is 50µg/ml (OpenWetWare, 2010), therefore this strain was insufficient. A strain of *G. quercinecans* was obtained that was resistant to this concentration, however the electroporation and transformation process required to obtain this strain had a significant impact on the fitness of the strain, resulting in smaller colonies which required

a much longer incubation time to form. The failure to meet this objective led to need for an alternative method for selective culture of *B. goodwinii* and *G. quercinecans*.

The second objective was to develop methodologies to detect viable *B. goodwinii* and *G. quercinecans* cells in rainwater and forest soil. This was explored in Chapter 3, where the decision to use sterilised rainwater for the first stage of the survivability experiments was made. This allowed the investigation as to whether *B. goodwinii* and *G. quercinecans* had the physical ability to survive in rainwater without any naturally occurring microbial competitors. The rainwater was autoclaved, as opposed to filtered, to maintain biomass which the strains may have needed for nutrients. In Chapter 3, a new selective medium was validated. Eosin methylene blue (EMB) agar, selected for its ability to inhibit fungi and Gram-positive bacteria (Leininger et al., 2001), was used in a small scale pilot study where pure cultures of the strains were cultured on the medium, and also as a mixture of bacteria and soil were inoculated onto EMB. The vibrant, iridescent green colour produced by *B. goodwinii* and *G. quercinecans* colonies on this medium made identification of the strains in a soil mixture relatively simple. Green colonies were confirmed to be *B. goodwinii* and *G.quercinecans* through sequencing of the bacterial gyrB gene. The validated EMB agar medium was then used for the soil survivability experiment. Over the 56 day experiment, soil samples were taken and cultured on EMB agar, and green colonies were counted for analysis. The meeting of this objective allowed the survivability experiments to take place.

The third objective was to determine the survivability of *B. goodwinii* and *G. quercinecans* in rainwater and forest soil. Therefore, using the sterilised water, strains of *B. goodwinii* and *G. quercinecans* were inoculated and cell viability rates measures over the 84 day experimental period. The experiment demonstrated that *B. goodwinii* did not survive in rainwater. Leading to the suggestion that physiologically, the strain may have been unable to adjust to the change in osmotic pressure and mineral concentrations in rainwater. *G. quercinecans* on the other hand, appeared to thrive in rainwater with an overall increase (114% increase) in colony forming units over the 84 day experimental period. These data support the hypotheses, although the experiment would need to be validated by repeating with non-sterile rainwater to determine how *B. goodwinii* and *G. quercinecans* would behave in the presence of naturally occurring competitors. However, this was not possible in the timescale of this project. EMB agar was used for the forest soil survivability experiment, where again cell viability over time was measured. This was further validated with the use of qPCR analysis, quantifying the number of species specific gyrB gene copies throughout the experiment. The culture-based experiment again demonstrated that *B. goodwinii* did not survive and fully declined within hours of inoculation, leaving it undetectable from day 0. *G. quercinecans* however, survived and

remained viable in forest soil for approximately 28 days. The qPCR analysis of *G. quercinecans* presented a similar pattern of decline to the culture based experiment, which was to be expected. *B. goodwinii*, on the other hand, gave unexpected results. GyrB gene copies were quantifiable long after the strain had become unculturable, leading to the suggestion that the strain may have entered a viable but non culturable (VBNC) state. These data, with the exception of the ambiguous *B. goodwinii* qPCR analysis which requires further study, provide further evidence supporting the two experimental hypotheses.

The final objective was to use the data from the study to assess the ecology and activities of *B. goodwinii* and *G. quercinecans* in a forest ecosystem outside of the oak host. The majority of the data provided in this study support the two hypotheses. *B. goodwinii* did not remain viable in either of the potential environmental reservoirs of rainwater and forest soil, leading to the assumption that the species does not reside in these environments naturally. This along with the lack of environmental isolations of the species, further supports the suggestion that *B. goodwinii* is a tree endosymbiont. The potential of *B. goodwinii* to reside as a specific oak endophyte could be investigated further in future research. *G. quercinecans* remained viable in both potential environmental reservoirs of rainwater and forest soil for a minimum of 28 days this along with the knowledge that numerous studies have isolated *G. quercinecans* from various forest habitats (Geider et al., 2015) further supports that *G. quercinecans* has a broad distribution in a forest ecosystem and will survive in rainwater and forest soil.

4.2 Applications of the Knowledge

The data presented in this study has the potential for numerous applications, most importantly the aiding in development of management strategies. The impact of *G. quercinecans* being able to survive in both rainwater and forest soil is crucial in the understanding of how to help manage the disease. It means that as a general member of the forest ecosystem, *G. quercinecans* will never be completely eradicated, therefore limiting the contact of the bacteria with the tree may be the way forward in reducing the impact of the disease. This could be controlled through increasing drainage for trees likely to become waterlogged (another stressor for a declining tree), or by manipulating the soil microbiome to act as a defence for the tree, preventing *G. quercinecans* from entering the root system.

4.3 Future Research

Future research on this topic could include the repetition of the rainwater survival experiment using unsterilized rainwater and the selective media method (Eosin methylene blue agar). This would demonstrate whether *B. goodwinii* and *G. quercinecans* behave any differently in rainwater in the

presence of naturally occurring microbes. This would ideally confirm that *B. goodwinii* cannot survive in a water based environment, and also that *G. quercinecans* is not outcompeted by the naturally occurring microbiota in the rainwater, although the strain was not outcompeted by autochthonous soil microorganisms, therefore it is unlikely that it would be affected by naturally occurring rainwater microorganisms. This would be crucial to understanding the ecology of this forest pathogen.

An experiment that would be beneficial to the overall knowledge of microbial ecology, would be to investigate how long bacterial DNA persists in environmental reservoirs (rainwater and forest soil) after cell mortality. This would benefit molecular ecologists everywhere, and would answer the questions posed by the ambiguous *B. goodwinii* qPCR analysis in Chapter 3. If bacterial DNA can persist in the environment after cell mortality then it should be assumed that *B. goodwinii* cannot survive in forest soil, as expected. However, if it is found that bacterial DNA cannot persist without a living cell, and is broken down quickly, then it would be assumed that *B. goodwinii* can survive in forest soil, in a dormant, unculturable state. Testing this could potentially involve spiking soil with extracted *B. goodwinii* DNA and also *B. goodwinii* cells, to determine decay rates. This could also involve the use of Propidium Monoazide (PMA), which can be used in conjunction with qPCR to discriminate between viable and dead bacterial cells through covalent bonding to DNA of non-viable cells during the qPCR process, leading to the ability to determine between the two (Bae and Wuertz, 2009; Pan and Breidt, 2007).

Another way to test the potential theory that *B. goodwinii* may enter a viable but non culturable (VBNC) state, would be to induce cells into a VBNC state and then to attempt to resuscitate them using autoinducers (Ramamurthy et al., 2014). VBNC cells would be resuscitated and become viable and culturable again, proving that *B. goodwinii* becomes dormant when in non-favourable conditions, and may persist in environmental reservoirs between oak hosts, furthering the knowledge on the ecological distribution of the species.

Further research on *B. goodwinii* as a potential oak tree endophyte could include using molecular techniques such as qPCR to detect quantities of *B. goodwinii* in healthy oak trees on a large geographical and temporal scale as this would provide evidence as to whether *B. goodwinii* is a naturally occurring part of the oak microbiome. These investigations could also include studies into what potential factors lead to *B. goodwinii* becoming virulent and pathogenic. This future research would help determine whether *B. goodwinii* resides as a tree endophyte in the stem of healthy oak trees, similar to *B. salicis* (Maes et al., 2002) and *G. quercinecans* resides as a generalist in the wider environment, including rainwater. This could mean that *B. goodwinii* becomes virulent when there is an accumulation of *G. quercinecans* that has entered the tree through the uptake of water. This could

be easily tested through an experiment using *G. quercinecans* infected rainwater and *B. goodwinii* infected oak saplings, to determine whether they would show symptoms of AOD.

The information provided in this study enables the closure of key knowledge gaps surrounding the ecological distribution of *B. goodwinii* and *G. quercinecans* in the wider forest environment. This in turn will aid in the understanding the biology of AOD, and future management strategies to prevent the spread of this devastating disease.

4.4 References

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