University of Reading

Consideration of Pathogenicity of Bacterial species associated with Bleed canker on *Quercus sp.* and evaluation of potential methods of control

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Oliver Robert Booth

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Abstract

Since 2006 increasing numbers of mature and maturing Oak trees, both in forest and urban settings have suffered from bleed canker to the main stem. The event has been described as part of a syndrome named Acute Oak Decline in 2009. The syndrome involves an interaction between invasive beetle larvae of *Agrilus biguttatus* and pathogenic bacteria causing bleed cankers. This is reported to potentially result in the demise of an infected tree within a five -year period.

From an epidemiology study of 1 hectare of semi ancient natural woodland containing 192 maturing Oak trees it was seen that the bleed exudate occurred without the presence of the *Agrilus* beetle. The bleed exudate was seen to readily transfer through the Oak population over the 6year period. A significant proportion of the trees with the bleed exudate entered remission and that no connection was directly established between the death of the trees and the bleed canker alone. Tree ring analysis from a portion of the trees in the epidemiological study revealed that those trees that were symptomatic (with the bleed canker) did not exhibit a pre-disposition of reduced vitality and that those trees that showed recovery were predisposed to have smaller growth rings.

Soils analysis revealed that the soils within the woodland were acidic, with low levels of Calcium. Initial experimentation showed that Calcium amendment to the soils reduced the amount of bleed canker.

Three different bacterial species isolated from the bleed cankers were able to reproduce the bleed symptoms in pathogenicity tests, individually and in combination. However, the results were not consistent to satisfy Koch's postulates.

In consideration of potential biocontrol for the bleed canker a study of the culturable fungal endophytes within the main stems of symptomatic and non -symptomatic trees was carried out. This identified prevalent *Penicillium sp.* which *in vitro* were found to effectively suppress two of the three pathogenic bacteria identified as causal of the bleed exudate.

Given the independence of the Bleed Canker as a pathogenic agent exclusive of the general syndrome named Acute Oak Decline, it is proposed that the bleed canker is identified as Oak Bleed Canker.

Key words: Acute Oak Decline, Tree Ring Analysis, Calcium, Fungal endophyte, Penicillium, Oak Bleed Canker.

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Abbreviations

AOD Acute Oak Decline

BLAST Basic local alignment search tool, accessible at: www.blast.ncbi.nlm.nih.gov

Bp Base Pair

CE Controlled Environment

CFU Colony Forming Units

DBH Diameter at Breast Height (measured as of 1.5m from ground level unless otherwise stated)

DNA Deoxyribonucleic acid

KB Kings B medium

LB Luria Bertani medium

OBC Oak Bleed Canker

OD Optical Density

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

PDA Potato Dextrose Agar medium

Rpm Revolutions Per Minute

RNA Ribonucleic acid

Chapter 1: Contextual Background

1.1 Importance and Ecological significance of Oak trees

1.1.1 Global distribution of Oak species

The Oak is native to the northern hemisphere. There are approximately 600 species of Oaks in the genus *Quercus* (Mabberley, 1987). This includes deciduous and evergreen species that extend from the cool temperate latitudes to the tropical latitudes both in Asia and the Americas. *Quercus spp* appeared approximately 60 million years ago, spreading from south-east Asia into China, Europe and North America (Tyler, 2008). The modern species of Oak that are found through-out the UK probably emerged 14 million years ago (Tudge, 2006).

Pollen records and DNA data show that during the last Ice Age the refugia for *Quercus spp.* were Spain, Italy and the Balkans (Rackham 2003). After the retreat of the ice, *Quercus spp.* spread at approximately 1km a year (Tyler, 2008). It is notable that the UK population as with the whole Western European population came directly from the Spanish refuge (Oszako, 2004). The wide distribution, dominance and resilience of the genus is attributable to its adaptability to many environments (Logan, 2005; Tyler, 2008; Fig.1). More than twenty Oak species exist in Europe. These represent approximately 9% of all tree stock and approximately 27% of all broadleaved forests within Europe (Oszako, 2004).

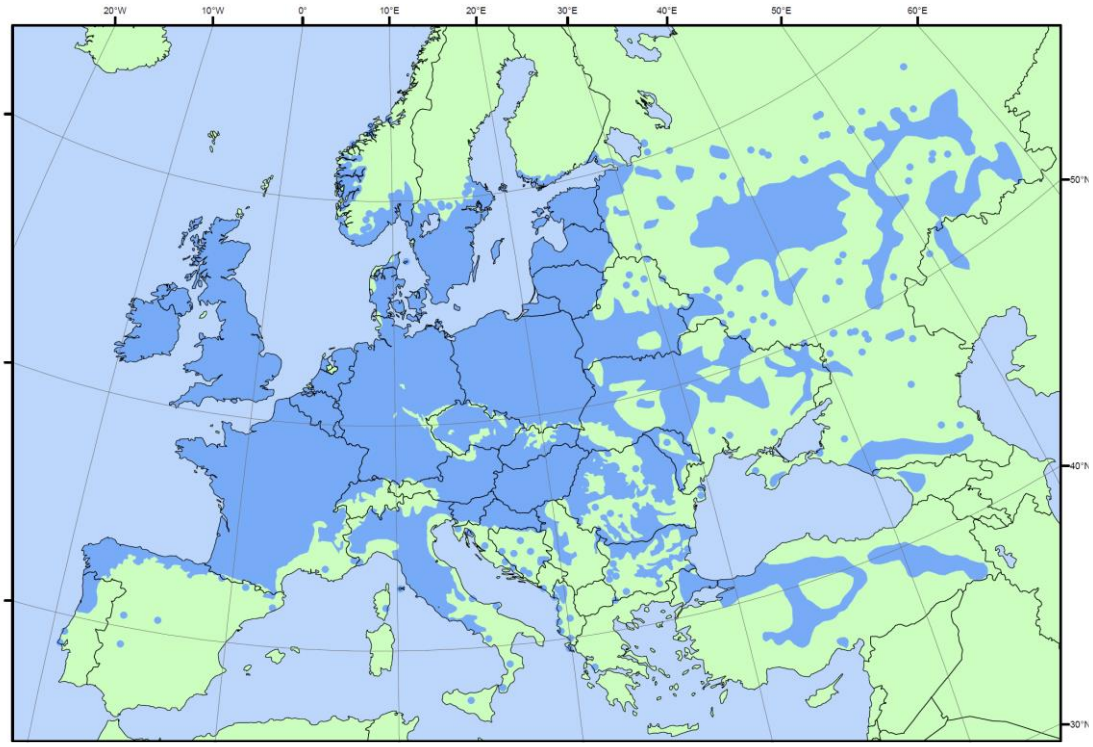

Fig. 1.1: Map showing The European distribution of *Quercus robur* as of dark blue (http://www.euforgen.org/).

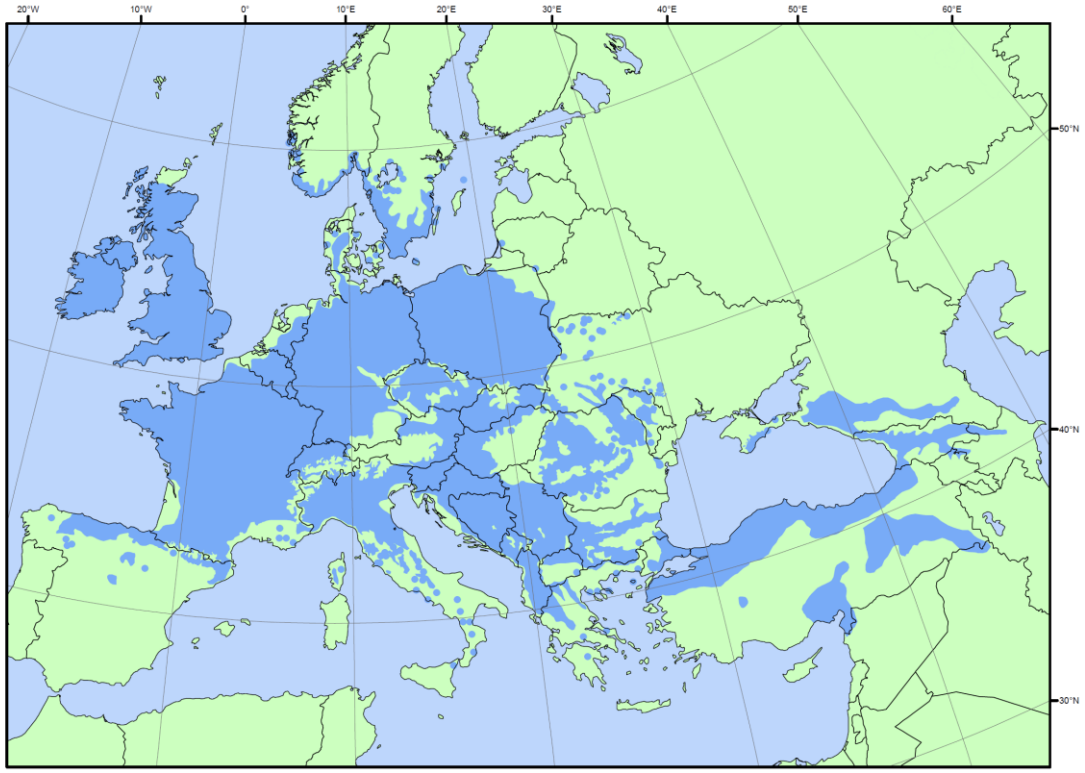

Fig. 1.2: Map showing The European distribution of *Quercus petraea* as of dark blue (http://www.euforgen.org/).

1.1.2 Ecological significance

The Oak tree predominates within the British landscape both as climax vegetation and through favourable management (Rackham, 2003). It is an essential component of our ecosystem. As with all trees Oaks act as carbon sinks (Liski et al, 2000; Schulze et al, 1999). They maintain watershed integrity and reduce soil erosion (Pimentel & Kounang, 1998; Zuazo, 2008). Planted in urban areas they reduce air pollution (Nowak, 2006), aid noise abatement (Fang et Ling, 2003; Ow & Ghosh, 2017) and air modulated temperature (Akbari et al, 2001).

Ecologically Oak trees are important. *Quercus spp.* generally support a greater range of biodiversity than any other tree in the UK. Oak trees support *circa* 500 species of insect within the mainland UK, that are either dependent or partially dependent upon the tree (Morris, 1974). It is commonly considered that the Oak is associated with more insects, birds and lichens than any other tree in Europe (Rackham, 2003 & Tyler, 2008).

Oak woodlands grow in a wide range of habitats. Rackham identifies up to seven of these habitats in the UK (Rackham, 2003). Of the eighteen woodlands identified in the National Vegetation Classification (NVC), four oak woodlands are described (Hall et al, 2004). These woodlands produce a wide association with many different plant communities as well as offering a wide variety of habitats to both invertebrates and general fauna (Tansley, 1939; Rackham, 2003). That Oak woodlands have been existent within the UK for the last 10,000 years means they have developed a unique relationship within the environment, contributing to the habitat and biodiversity of native species (Farjon, 2017). The consequent established provenance of Oak woodlands and trees plays a major role in the ecological resilience of landscapes (Holling, 1973; Johnson et al, 2009).

1.1.3 Historical and Cultural significance

Oak trees have been utilized throughout history by humans. Acorns have been used as a food source, the leaves and bark have been used for medicinal purposes, while the timber has been used for shelter, ship building and furniture making (Logan, 2005). Oak timber within the Forestry industry represents a large aspect of the total income generated, particularly in relation to broad leaved trees. The largest area of oak forest in Europe is an estimated 5.5 million hectares in France, followed by Russia with 4.3 million hectares (Oszako & Delatour, 2000). Oak timber is of relatively high value and an important economic factor in a number of European countries.

Oaks tend to live longer than most other tree species (Harris et al, 2003). Whilst most fully grown trees will reach 300 years before senescence and crown die back, an Oak pollard may exceed 700 years (Mitchell, 1966). Such long lived trees often become an historical and cultural landmark within the landscape. In the UK the Oak tree has gained numerous epithets through history reflecting its importance to the nation: 'King of the Forest' and 'Stay of the Nation' are but two, that show the cultural importance of the tree. Within the UK there are some 700 individually named Oak trees, often made historically famous within a local context (Harris et al, 2003).

1.2 History of Decline in Oak trees

1.2.1 History of Oak Decline in Europe

Issues relating to the early decline episodes of Oak trees in Europe have been documented for *circa* 250 years, in Germany in 1739 (Thomas, 2008), Switzerland in 1850 (Ragazzi et al, 1995) and France in 1875 and 1893 (Delatour, 1983). A wave of decline was reported through-out Europe in the early 1920s particularly in France and the UK. However, the severity of Oak decline (and record of this decline), have increased since the 1980s. The following European countries have all

recorded decline within their native forest Oak tree populations: Austria, Belgium, Czech Republic, Denmark, France, Germany, Greece, Hungary, Italy, Netherlands, Poland, Portugal, Romania, Russia, Slovakia, Spain, Sweden, Yugoslavia and the UK (Thomas et al, 2002). All have recorded significant incidence of oak decline, many of which are on-going (Oszako & Delatour, 2000).

Oak decline is associated with disease complexes in which pre-disposing, inciting and contributing factors combine and result in decline symptoms. Syndromes and associated pathogens are often similar, e.g. drought inducing stress and on- set of fungal infection or insect attack. Often A*rmillaria spp.* is reported or *Tortrix viridana*. However, in all incidence to date no single, universal, abiotic or biotic causal factor has been identified which could explain this widespread dieback of Oak (Oszako, 2004).

It is considered that the evolution of Oak decline phenomena in Europe is linked to environmental changes in the forest eco -systems that influence the health of the Oak forest and pest populations (Oszako, 2004). It would appear from a review of the literature, that *Quercus robur* is the most vulnerable to decline although *Q. petraea, Q. ilex* and *Q. suber* populations can also be impacted (Oszako, 2004).

1.2.2 History of Oak Decline in America

The United States of America suffered recently from an outbreak of what was termed Sudden Oak Death (SOD). This is directly related to the pathogen *Phytophthora ramorum*. The first report of SOD was in 1995 in California. SOD spread rapidly through-out the USA to become epidemic (Grünwald et al, 2012). Other diseases of Oak trees within the States include Oak wilt, a disease caused by the relatively aggressive fungal pathogen *Ceratocystis fagacearum*. Symptoms include wilting and discoloration of the foliage, premature leaf drop, and rapid death of the tree within weeks of the first appearance. Trees become infected with oak wilt in two ways: through

connections between root systems of adjacent trees, and through insects that carry the pathogen to other trees that have been wounded. Incidence of this disease has grown in significance since the 1980s in the Eastern USA (O'Brien et al, 2011).

Oak Decline is described as a slow-acting disease complex (Wargo et al, 1983). Trees are considered to be weakened by environmental stresses such as drought, waterlogging and frost or by pests such as defoliating or sucking insects. These weakened trees are then invaded and killed by insects and pathogens that are not normally successful in attacking healthy trees. Usually the progression of decline is slow, occurring over several years (Fan et al, 2008). The two major biotic factors associated with oak decline in 1983 were *Armillaria mellea* and *Agrilus bilineatus*, the two-lined chestnut borer (Wargo et al, 1983). Management solutions presented at the time by the U.S. Department of Agriculture Forest Service included:

1) Thinning to reduce competition for moisture and nutrients and promote better physiological condition of the remaining trees.
2) Silvicultural practices designed to encourage species best adapted to the site to reduce the effects of drought or frost.
3) Removal of weak and dying trees to reduce or delay population build-up of the two-lined chestnut borer.
4) Insecticide spray to reduce stress from insect defoliation in high-value forest stands.

1.2.3 History of Oak Decline in Britain

In 1997 the UKs Forestry Commission, Forest Research division, drew attention to the Dieback of Pedunculate Oak (Gibbs & Greig, 1997). The term 'dieback' was used in the context of tree diseases to describe a condition due to a complex cause, where a series of damaging agents progressively weaken a tree so that it eventually becomes vulnerable to organisms of secondary action (Gibbs, 1999). This term had been used in the 1920s for an episode of decline of

Pedunculate Oak in the UK (Gibbs, 1999). The damage then was associated with defoliation of trees in early summer by caterpillars of the oak Tortrix moth (*Tortrix viridana*). The issue was compounded by a powdery mildew fungus (*Erysiphe alphitoides*). The effects of the decline were particularly severe on the second flush of leaves that was formed after the first caterpillar defoliation (Gibbs & Greig, 1997).

A feature of many of the affected trees was the presence of *Armillaria*. It was then considered that this fungus was a secondary pathogen, killing trees that were already 'irretrievably damaged'. By 1925 Oak populations showed improvement as the decline event abated. This was attributed at the time to a reduction in the abundance of the Tortrix moth (Gibbs, 1999).

Gibbs (1999) research note was in response to problems between 1989 and 1994 when Forest Research received large numbers of reports of damage to oak. Symptoms began in the first year of the decline with overall deterioration of the foliage. The following year the death of fine twigs was followed by the death of small branches. The process continued through to the death of large branches and ultimately to the demise of the tree. It was concluded that drought was the main factor placing the trees under stress, since the summers of 1989 and 1990 were both classified as extremely dry. At the time an increasing presence of the beetle *Agrilus biguttatus* was noted on declining trees. The early stage of attack by the beetle on trees that still possessed some degree of vitality was often marked by the presence of a dark exudate on the bark surface. Gibbs concluded that different damaging biotic agents such as insect and fungal attack can result in the same final symptoms that may be influenced by abiotic factors such as drought or an imbalance in soil nutrition (Gibbs, 1999).

1.3 Symptoms of Oak Trees in Decline

Symptoms attributed to declining oaks may include:

1) Discoloration or yellowing of leaf. (Furher, 1998)
2) Tip dieback of smaller buds and branches to the upper and outer canopy of the tree. (Thomas et al, 2002).
3) Excessive twig abscission throughout the canopy. (Furher, 1998)
4) Crown transparency or thinning, where there is a visible thinning of the crown relative to the light penetration into the tree canopy. As well as relating to increased dieback of smaller laterals throughout canopy, this may also relate to dieback of larger branches throughout the crown. (Gibbs, 1999).
5) Development of epicormic shoots on branches and trunk relating to triggered dormant bud growth. (Gibbs, 1999; Thomas et al, 2002).

Less obvious signs may relate to:

1) Insect infestation. (Furher, 1998; Thomas et al, 2002; Thomas, 2008).
2) Fungal infection of varying areas and tissues of the tree. (Furher, 1998).
3) Reduced leaf size and shoot development. (Furher, 1998).
4) Bacterial slime flux on the trunks. (Furher, 1998; Thomas et al, 2002; Thomas, 2008).
5) Progressive necrosis of the bark and cambium effectively reducing the incremental growth of the stem, by affecting wood production. (Furher, 1998; Thomas et al, 2002; Thomas, 2008).

Whilst the visual indications of decline are many and varied, generally only a few of these symptoms are observable at any one time. Visual symptoms can also vary according to the environment and time of year. Symptoms maybe inter-related or relate to sequential phases of physiological decline. Yet there are no set of symptoms that are uniquely attributable to decline. (Furher, 1998).

1.4 Consideration of Factors affecting Oak Tree Decline

Existing literature generally shows that decline in Oak is not attributable to a single factor but rather a combination (Thomas, et al, 2002 and Thomas, 1998). Generally, such factors (of stress) occur within the same or consecutive years. Factors that have been recorded as contributing to Oak decline within the last twenty years include both biotic and abiotic factors:

1) Extremes of weather including summer drought, winter and spring frosts. Drought is believed to be a significant contributing factor in an oak decline syndrome. Trees may survive drought episodes but consequentially have depleted carbon assimilation due, primarily, to reduction in stomatal conductance (Thomas, 2008). Extreme frosts can damage the vascular tissue of the tree, damage fresh leaves, or induce embolism in early wood conducting vessels. (Hanninen, 2006).
2) Other abiotic factors affecting decline relate to soil imbalances, such as nitrogen deposition or nutrient imbalances. Excess Nitrogen from farm fertilizer run off has been shown to reduce tannin concentration and protein precipitation capacity in Oak trees (Thomas & Schafellner, 1999).
3) Soil nutrient imbalances, including decreased concentrations of Calcium are considered causal factors of decline (Thomas, 2002).
4) Hydromorphic soils (characterized by a reduction of iron, due to waterlogging and consequent lack of oxygen (Duchaufour, 1982)) and sites with fluctuating water tables were found to have a higher susceptibility to decline (Thomas & Hartmann,1998).
5) Biotic factors include severe (and repeated) insect defoliation, generally related to insect larvae. (Gibbs,1999).
6) Insect borers *Agrilus biguttatus* or *Platypus cylindrus* can contribute to damage to Oak trees in the later stages of decline. (Thomas, 2008; Brown et al, 2014).

7) Further biotic factors relating to fungal pathogens are *Collybia fusipes, Armillaria spp*. (Halmschlager, 1998), *Hypoxylon mediterraneum* (Thomas, 2008), as well as *Discula quercina* (Ragazzi and Moricca, 2008). Some of these species are viewed as opportunistic pathogens taking advantage of the trees weakened state, whilst others are considered to be latent, opportunistic, residing within the tree and triggered by the environment and the trees response to change.
8) Similarly, Oomycetes, in particular *Phytophthora spp.,* has been associated with Oak decline. *Phytophthora ramorum* is directly associated with Sudden Oak Death (Garbelotto & Hayden, 2012), while *Phytophthora quercina* is associated with Oak decline (Jung et al, 2000).
9) Bacteria, particularly related to stem bleeds has also been considered as contributing to Oak decline. *Brenneria quercina*, has been identified in Spain as the cause of bleed canker on *Quercus sp*. (Biosca et al, 2003) Novel bacterial pathogens have more recently been associated with Oak decline, specifically renamed as Acute Oak Decline (Denman & Weber, 2009). *Brenneria goodwinii, Gibbsiella quercinecans* and *Rahnella victoriana* have been associated with the bleed cankers symptomatic of this specified decline (Brady et al, 2010; Brady et al, 2011; Denman et al, 2011; Brady et al, 2014).

1.5 Concepts of Epidemiology of Tree Disease

Disease may essentially be viewed as a condition that is variant to the normal physiological functioning of an individual (Manion, 1981. Holliday, 1998). Disease may be divided into infectious agents, i.e. caused by a pathogen, and non- infectious causes by other factors. The former may be referred to as disease, the latter as a disorder (Holliday, 1998). In this way a disorder is a deviation of normal biological functioning of physiological processes arising from causes other than a pathogen, i.e. mechanical damage, mineral deficiency, pollution etc. Manion (1981) defines disease as any deviation in the normal functioning of a plant caused by a persistent agent. Manion' s definition is dependent upon time. A short disruptive interaction to the tree such as a hatchet blow differs from damage caused to a tree from, for example, a chemical that is slowly released into the atmosphere that causes damage to the tree. In this way Manion's definition of disease is that it is generally caused by a persistent biotic or abiotic agent. Tainter & Baker (1996) expound that it is not that the pathogen alone that is the disease. But rather that disease is the result of a dynamic interaction between a causal agent and the affected host causes.

There are many differing explanations and definitions of what disease is (Tainter & Baker, 1996). These are primarily focused on how the interaction between the host and pathogen may be viewed in a spatial-temporal relationship and, therefore, how we may interpret and manage disease.

1.5.1 Definitions

Aetiology (Etiology) is the study of the causes of disease (Tainter & Baker, 1996). It deals with the nature of the causal factor and its interaction or relationship with the host (Holliday, 1998). The application of Koch's 'Rules of Proof of Pathogenicity' concludes whether suspected pathogens are a cause of the disease (Holliday, 1998).

While Aetiology attempts to expound on the determinants, Epidemiology is rather conceived as the ecology of the disease and a forerunner of disease management (Tainter & Baker, 1996). It is

essentially the study of infectious diseases, their origin and their distribution in space and time within a population (Schumann, 1991).

1.5.2 Koch's Postulates

In 1884 Robert Koch expounded essential procedures for establishing pathogenicity of an organism, to distinguish whether the cultured microbe was the cause of the disease or co- existing with the diseased tissue – a saprophytic microorganism (Agrios, 2005; Holliday, 1998). The four criteria necessary to satisfy the premise of pathogenicity are as follows:

1. The suspected causal agent must be present in every diseased organism.
2. The suspected causal agent must be isolated from the diseased organism and grown in pure culture.
3. When a pure culture of the suspected causal agent is inoculated into a non- symptomatic host the host must exhibit the same symptoms.
4. The same causal agent must be re-isolated from the infected host.

1.5.3 Primary disease infection event

Disease results from the interaction between the host, the pathogen and the environment (Fig 1.3; Moore et al., 2011). This can be used to understand how epidemics develop and how they can be predicted and limited. Factors that will influence the effectiveness of the pathogen will include:

1) Virulence, how aggressive the pathogen is and how effectively it causes disease.
2) The reproductive cycle of the inoculum, how often and how much inoculum are produced.
3) The dispersal mode of the inoculum, e.g. whether it is airborne, waterborne or dependent on insect vectors.
4) The specificity of the inoculum in relation to the host.
5) The ability to adapt to different host species.

Factors that will affect the susceptibility of the host will include:

1) The Ontogenic resistance of the host where the susceptibility of the host is related to its age.
2) The Constitutive resistance of the host, i.e. those factors present within the host prior to infection. This may include physical barriers within the host as well as activation of the host's defence mechanism - Induced Systemic Resistance (ISR) (Choudhary, 2007).
3) Similarly, innate immunity maybe conferred by Systemic Acquired Resistance (SAR), where a resistance response occurs following a previous localised exposure to the pathogen (Durrant and Dong, 2004).
4) Seasonal development of the host and whether this favourable to the disease cycle of the pathogen.
5) The abundance or availability of the host for the pathogen to prevail over time.

Disease may be viewed as a sychronisation of different biological events affected by environmental factors. Such factors will include:

1) Humidity and temperature, drought and rainfall. Sudden changes to these conditions can exhibit within the host. Sometimes leading to stress conditions of the host (but also of the pathogen).
2) Soil conditions and nutrient availability again can affect the host condition. Lack of nutrient may lead to a weakened defence system within the host.
3) Climatic and weather conditions can affect the cyclical biological and ecological functioning of both the host and pathogen.
4) Surrounding management of other vegetation can affect dispersal and distribution of the pathogen as well as vitality of potentially competing host plants.

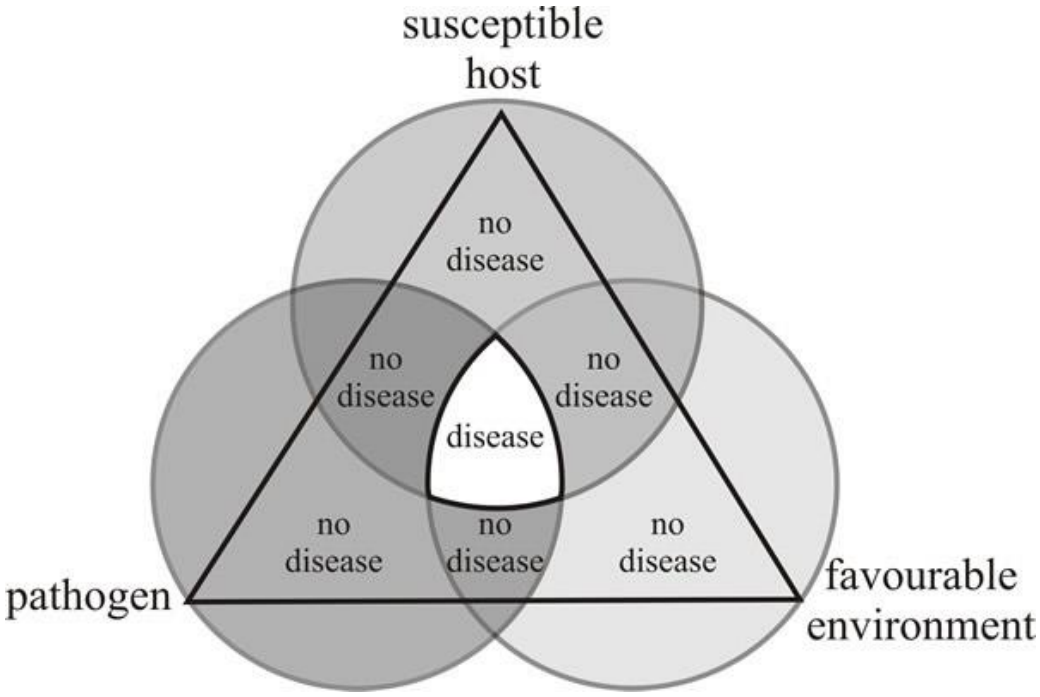

Fig. 1.3: The traditional disease triangle paradigm. (Moore et al, 2011).

1.5.4 Disease infection event and Time

A primary disease infection event may be established when the three aspects - host, pathogen and environment - align with each other to produce a disease event. Tainter and Baker (1998), consider that a disease square is more appropriate, distinguishing between biological and physical environmental conditions. Both such models focus on the development as a spatial relationship, rather than a temporal - spatial relationship, accounting for the further variable of time. Time will be relevant in relation to the specific point in time that the disease event occurs and its duration. This gives notion to a disease pyramid, (Agrios, 2005). Disease lifecycles are generally dependent on linear time. This is reflected in seasonal interaction between host and pathogen as well as the duration of the disease event, again often affected by climatic conditions.

1.5.5 The Host- Pathogen Complex

The traditional disease paradigm can be expanded to include a further consideration, the host - pathogen complex (Lucas, 1998). It is considered that a plant is healthy when it can carry out its

physiological functions to its optimal genetic potential (Agrios, 2005). Disease is the result of a dynamic interaction between a causal agent and the affected host (Tainter & Baker, 1996). This may be divided into infectious agents, i.e. caused by a pathogen, and non- infectious causes by other factors. The former may be referred to as disease, the latter as a disorder (Holliday, 1998). It is the successful establishment of a pathogen in its host that gives rise to the host-pathogen complex.

1.5.6 Disease Cycles

The disease cycle refers not simply to the life cycle of the pathogen, but to the disease in the host as function of the pathogen. There are four aspects to this cycle, pre-entry, entry, colonization and dissemination. Hence, a disease cycle can be viewed as either a continuous event - the pathogen is found to cause symptoms on the host throughout the entire disease cycle. Alternatively, it can be considered as a discontinuous event if the cycle is broken either by the pathogen entering a resting or non-pathogenic stage within the cycle (Gonthier & Nicolotti 2013).

1.5.7 The Symbiosis Concept of Disease

Symbiosis is the (comparatively) long term interaction between two or more different biological species. This persistent biological interaction can be mutualistic, but the term is used to encompass all variants, including commensalistic and parasitic relationships. Infectious disease can be viewed as an antagonistic symbiosis (Tainter & Baker, 1996). An organism that lives on or in another organism, by way of utilising it as a food source, is known as a parasite. In this sense parasitism is closely associated with pathogenicity (Agrios, 2005).

1.5.8 Manion's Spiral Theory of Decline

Plant disease is often more complex than single biotic or abiotic primary-causal-agent diseases.

Tree declines can be considered as a category of disease which is the result of a number of interacting factors.

The patterns of decline can often be mapped in relation to Paul Manion's Spiral Theory of Decline (1981; Fig. 4). This identifies 3 factors:

1) Predisposing factors. These are generally static or non- changing factors. Examples include climate, soil type, genetic potential of the tree. This places a permanent stress on the tree, pre-disposing it to the actions of other factors.
2) Inciting factors. These are classified as short in duration, of physical or biological nature, that tend to produce a drastic injury. Examples include insect defoliation, mechanical damage, frost or drought events. The plant attempts to recover but has difficulty because of the predisposing stress of the environment.
3) Contributing factors. These are those factors that produce noticeable symptoms on the host. Examples of this include bark beetles and decay fungi. These organisms tend to be persistent. They are often blamed for the condition but tend rather to be indicators of a weakened host.

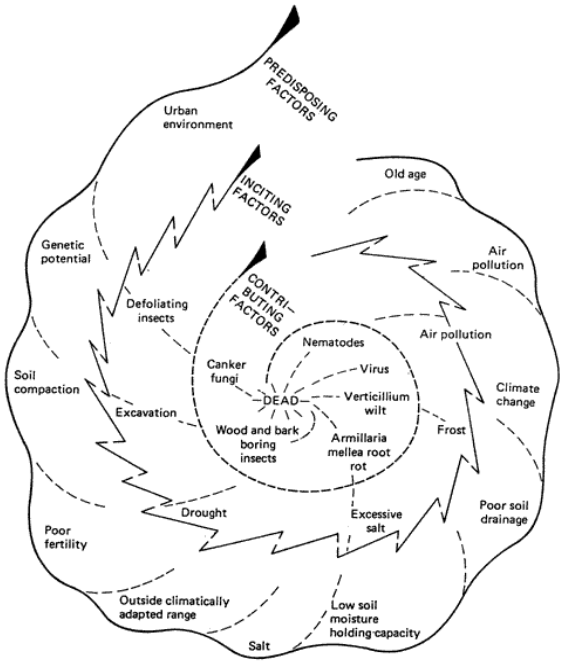

Fig. 1.4: Manion's Spiral Theory of Decline. A variety of factors that fit within one or more of the categories described lead to a spiral of decline resulting in the death of the tree (Manion, 1981)

1.5.9 Synergistic or Complex Disease Concept

It is worth noting that not all Forest Pathologists accept the concept of decline as a distinct category of disease. They believe it may better be defined as a collection of diseases with an incomplete understanding of the etiology of the disease event (Manion, 1981), involving synergistic action of multiple organisms.

Synergism is the association of two or more organisms acting at the same time to bring about a change that individually could not have occurred (Holliday, 1998). The host-pathogen interaction may result in a synergism that results in a pathogenic event (Woo et al, 2002). Equally such synergism may be as a result of a number of factors beyond the host-pathogen complex. Such factors that may not individually cause problems, but in the same spatial- temporal relation bring about a pathogenic event (Ragazzi et al, 1995).

1.5.10 Argument against Decline as a disease category

The main concern levelled at the decline concept is that it provides no practical insights to forest managers looking to find effective management options. (Tainter & Barker, 1996). Traditional Forestry Commission recommended management practices relating to trees defined as diseased, have generally been to implement sanitation felling. (e.g. Watermark disease of Willow, Norway spruce bark beetle, *Phytophthora ramorum* on Larch.). However, trees in decline are not necessarily diseased, with an identifiable pathogen that is the cause. This would be the case in relation to Chronic Oak Decline (Gibbs, 1999. Denman & Webber, 2009). More recent management practices in relation to Ash Dieback are less reactive. Sanitation felling has been resisted to allow potential revelations of disease resistance trees. (Mitchell et al, 2014). The

Forestry Commission practice note issued in April 2010 on managing Acute Oak Decline gives general advice to leave infected trees and monitor unless they pose a risk. Sanitation felling is recommended where there are a limited number of infected trees. The aim being to reduce infection levels in healthy trees. Biosecurity is urged and the recommended practice is to disinfect all tools and vehicles after use in areas of known infection.

The description of 'decline' does not identify potential issues that may then be addressed, i.e. it is a definitive description but leaves the cause or pathogen/s unidentified. Decline can occur as a natural process in a forest situation and as part of the natural process in the growth and development of a tree. (Sinclair & Lyon, 2005). The categorization of the tree with decline does not provide a suitable picture as to the future of the tree (and hence its management). (Ostry et al, 2011). It should be established whether the decline is a reversible effect. This will require a minimal understanding of the aetiology by which the traditional disease triangle should adequately describe the expression of decline symptoms without the need to ascribe multiple factors.

1.6 Recent emergence of Bacterial Canker of Oak

1.6.1 The naming of Acute Oak Decline

Denman and Webber (2009) at Forest Research first differentiated between different types of Oak Decline. This was formalised in 2010 as Forestry Practice note 15 issued by Forestry Research in response to the increasing number of native Oak trees (*Q. robur* and *Q. petraea*) that were in severe decline and exhibiting symptoms of bleeding canker on the main stem.

Oak decline is described as a number of different factors combining to create a syndrome. (Denman and Webber, 2009) Syndrome is not synonymous with disease. A disease is a condition where the normal functions of the tree are disturbed and harmed (Holliday, 1998). A Syndrome

maybe defined as a collection of symptoms that characterise or suggest a particular disease (Holliday, 1998).

The factors associated with Acute Oak Decline may be abiotic or biotic and vary in their intensity or involvement within the syndrome. There is little distinction between what is termed ‘traditional oak decline’ and ‘chronic oak decline’ only that the latter has been more recently associated with a root disorder. In this sense Oak declines maybe characterised as either Chronic or Acute (Denman & Webber, 2009).

The aetiological pattern that has emerged to Forest Research is related to the emergence time of the disease, the duration of the event and tree mortality. Acute Oak decline is classified as an event of relatively sudden manifestation, that is within a 5 to 10year period. It is considered to be an event that stabilises over time whilst leading to high mortality rate within affected populations.

Chronic Oak decline conversely is seen as developing over a longer time period (decades is suggested) with a low tree mortality rate, where some trees may recover (Denman & Webber, 2009).

An example of a previous episode of Acute Oak decline is given as the 1920s episode, where an increase in defoliating moths and mildew resulted in a high number of Oak mortalities over a short period of time.

The primary causal factor for the current example of Acute Oak Decline (AOD), is considered most likely to be a bacterial pathogen. This affects the main stem of the tree and disrupts the transportation of water, minerals and sugars. It is reported by the Forestry Commission as leading to the demise of the tree within 4 -5 years (Denman & Webber, 2010).

1.6.2 Description of Symptoms of Acute Oak Decline

The native Oaks of the UK are *Quercus robur* also known as Pedunculate Oak or English Oak and *Quercus petraea* known also as Sessile Oak. *Quercus robur* tends to pre-dominate in the South

and *Quercus petraea* in the North of the UK. The two species can interbreed, producing intermediate forms which can be difficult to differentiate.

Both species are affected by Acute Oak Decline. To date, *Quercus robur* is affected more than *Quercus petraea*. However, this may have more to do with the geography of the syndrome currently being reported in the Midlands and East Anglia where *Quercus robur* predominates (Denman & Webber, 2010).

AOD tends to occur more often in trees over 50 years old (Denman & Webber, 2010). The main symptom is extensive bleeding of a dark, sticky fluid from between fissures in the bark. On excising the diseased tissue necrotic patches are revealed developing under the bark plates. It is suggested that the discoverable bleeds may be associated either with splits in the bark or weeping patches on the stem. (Denman et al, 2014). However, all bleeds generally tend to have their origin from in between bark plates.

The first cankers will often predominate on the sun side of the stem. The cankers are generally recorded on the main stem up to a height of up to 12m (see Fig.1.5). The bleeding usually appears in spring to summer. Excising the cankers (for collection of samples), reveals that there is pressure behind the area of exudate. This pressure is believed to be a build up of sap as vascular tissue behind the stained area (canker) is blocked due to damaged tissue. It is considered that this pressure causes the weeping exudate from the area on the stem where the outer bark tissue is thinnest, i.e. bark fissures. When this first occurs it is seen as light coloured, aerated liquid issuing under pressure from between the bark fissures (see Fig.1.6).

Within two to three days the exudate becomes dark and viscous that then dries to leave a dark stain on the trunk. The stains may be washed off during heavy rain, making it difficult to identify affected trees.

Fig. 1.5: Typical symptomatic tree showing multiple cankers with recent exudation

Fig. 1.6: White arrow indicates fresh exudation from canker showing the preliminary light, aerated liquid.

Excavation of the canker at the bleed point underneath the outer bark reveals that the inner bark has decomposed. This leaves a fluid-filled cavity between the outer bark and the inner wood. The wood tissue becomes a soft dark pulp (see Fig.1.7), with a distinctive decaying odour. The remaining wood shows lesions that produce a dark brown necrosis of xylem and phloem tissue. The radial and transverse staining extends well beyond the perimeter of the bleed site. The depth of the affected wood maybe up to 5 centimetres into the tree (Fig. 1.8). Generally, this will have deteriorated both the primary and secondary phloem and vascular cambium – the inner bark, as well as the water conducting secondary xylem tissue – the sapwood.

Dieback within the canopy does not always correlate to the number of cankers on the stem. Cankers appear to develop and spread beneath the bark . If multiple lesions occur they tend to coalesce on the lower stem, in some instances causing girdling and consequent death of the tree.

Fig. 1.7: Initial excise of canker. White arrow indicates flow of clear liquid exudation.

Fig. 1.8: Partially excised canker. White arrow indicates spread of multiple lesions at a depth of approximately 5cm beneath the bark

1.6.3 Associated vector

It is not yet established how the suspected pathogenic bacterium/bacteria are spread. This may be through weather (e.g. wind and rain), or by animal dispersal (e.g. birds, squirrels). One potential primary vector associated with the disease is *Agrilus biguttatus* (Two spotted Oak Buprestid) (Fig1.9). Formerly known as *Agrilus pannonicus*, this beetle was listed on the Red data list in 1987 as a 'vulnerable' species, populations of which have grown in abundance in recent years (Gibbs & Greig, 1997).

The wood borer deposits its eggs in summer in the deep fissures of the oak bark. The eggs hatch and the larvae tunnel through the inner bark and cambium creating a lattice work of feeding galleries (see Fig. 1.10). The larvae remain in the tree for between 1 and 2 years, emerging as beetles in late spring.

They leave a characteristic ‘D’ shaped exit hole in the bark (see Fig. 1.11). The beetle then feeds on foliage of the tree (Vansteenkistea et al, 2005).

Fig. 1.9: Agrilus biguttatus (Lieutier et al, 2004)

Fig. 1.10: White arrow indicates feeding gallery at area of excised canker

Fig. 1.11: White arrow indicates Typical ‘D’ shaped *Agrilus* exit hole

Trees suffering from the bleed canker are reportedly more susceptible to *Agrilus biguttatus* attack. (Vuts et al, 2016). The relationship between the beetle and the bacteria is not yet fully understood and the order of cause and effect remains unclear. The beetle may be attracted to the Oak trees that are weakened/ pre- disposed to decline and already have the bleed symptoms or the beetle may be the vector, carrying the bacteria between trees. (Brown et al, 2014). More recent studies have shown that a greater percentage of Oak trees exhibiting bleed symptoms were more likely to die if *Agrilus* exit holes were also present, rather than if only the bleed symptoms were evident. (Brown et al, 2016). It is probable that the *Agrilus* larvae encourage the spread of the bacterial disease within the tree via the feeding galleries that are created (Vansteenkistea et al, 2005).

1.6.4 Types of Bleed / Exudate on Oak stems

Bleeds on Oak stems can originate from currently known disease symptoms. *Armillaria* infection can often cause small areas of bleed. The fungus is essentially a root decay organism. It establishes mycelia sheets beneath the bark of the main stem to a height of approx. 2m from ground level (Strouts & Winter,1994). This results in small dark black weeping patches on the stem. Generally, they do not bleed as profusely as the bleeds associated with the Acute Oak Decline. Whilst the bleed associated with *Armillaria* will only extend to a height of 2 metres, the bleed associated with Acute Oak Decline can extend up to a height of 12 metres.

Bleeds can also result from *Phytophthora sp.* infections. Again, these tend to be limited to the basal area of the trunk and will generally only spread up to 1 metre up the main stem. The exudate in this instance is blue/ black, as of ink. With AOD there tends to be an area of decay behind the area of exudate, this is not necessarily so with *Phytophthora*. Bleed canker associated with AOD are focused within the bark fissures and so presents as a vertical aspect of bleed sites over the stem. This is variant to *Phytophthora* which does not have a pattern of bleed, although often the bleeds are clustered (Strouts & Winter, 1994; Denman et al 2014).

Other declines of Oak trees are associated with Fungi resulting in similar bleed cankers. This canker can also produce bleed symptoms on Oak stems (Lynch et al, 2013). *Botryosphaeria stevensii* (anamorph *Diplodia mutila*) is reported to be the most frequently associated disease with Oak decline in Italy (Raggazi et al, 1999; Raggazi et al, 2000). It has also been found in relation to bleed cankers in Spain (Sanchez et al, 2003).

1.6.5 Historic descriptions of Bleeding on Oak trees relating to Bacterial infection

In 1967, a new bacterial disease of oak caused by *Erwinia quercina* was reported in California (USA), called drippy nut, due to bacterial ooze observed on acorns from species *Quercus agrifolia* and *Quercus wislizenni*. (Hildebrand & Schroth, 1967). This was later reclassified as *Brenneria quercina* (Hauben et al, 1998).

Brenneria quercina was identified on Spanish Oak trees, *Quercus ilex* and *Quercus pyrenaica* (Boisca et al, 2003). It was shown in this instance to be pathogenic, not only on acorns but also to cause bark cankers. In 2008 further work showed that *Serratia sp* bacteria were also isolated from samples taken from symptomatic cankers. Pathogenicity tests suggested that the *Brenneria quercina* and *Serratia* isolates are able to survive and grow on oak trees, and to produce bark bleeding symptoms. Both satisfied Koch's postulates and support the hypothesis that both bacteria are causal agents of oak disease. However, it has been postulated that *Serratia sp.* was a saprophytic bacterium, displacing the *Brenneria quercina* on older cankers. (Poza-Carrion et al, 2008).

It is believed that the more recent reports of the bleed cankers, primarily in English woodlands are unique (Denman & Webber, 2009. Denman et al, 2014).

1.6.6 Recent isolation and classification of bacteria from Bleeding Oak Cankers

Within the last seven years a number of different bacteria have been closely associated with the bleed symptoms characterizing Acute Oak Decline.

Phylogenetic analysis and phenotypic assays qualified and re-classified the *Serratia spp* (as of the Spanish research works identification of a pathogenic bacteria (Biosca, 2003)), as *Gibbsiella quercinecans* (Brady et al, 2010). Similarly, *Brenneria quercina* isolates were further examined using a polyphasic approach incorporating multi-locus sequence analysis (MLSA) of housekeeping gene sequences, DNA–DNA hybridizations and phenotypic characterization (Brady et al., 2011). This led to a further re-classification of the bacterium as *Lonsdalea quercina* with a further three new sub species.

A further bacterium also associated with canker bleed isolates was closely related to *Brenneria rubrifaciens* (Denman et al., 2011). This bacterium had previously been associated with deep bark canker on walnut (Wilson et al., 1957; Hauben et al., 1998). MLSA based on partial gene sequencing of four housekeeping genes (*gyrB, rpoB, infB and atpD*) confirmed that the isolates were within the genus *Brenneria*. DNA-DNA hybridization indicated that the isolates belong to a single taxon that was named *Brenneria goodwinii* (Denman et al, 2011). Further work has shown that *Rahnella victoriana* (Brady et al., 2014) and an unnamed *Pseudomonas* (Denman et al., 2016) are also consistently associated with the bleed canker.

The main bacteria primarily associated with the bleed canker and considered to be pathogenic are *Brenneria goodwinii*, *Gibbsiella quercinecans* and *Rahnella victoriana* (Denman et al., 2017). It has been proposed that the symptoms are causative of this polymicrobial complex with associated insect activity (Denman et al., 2017).

1.7 Knowledge Gaps relating to ‘Acute Oak Decline’

‘Acute Oak Decline’ was named circa 2009 by Forest Research, to distinguish it from Oak Decline described nearly 20 years previously (Gibbs, 1990). It is differentiated in relation to the speed of the decline and consequent mortality of the tree (Denman & Webber, 2009). It is identified by the presence of Bleed Cankers (possibly due to a bacterial pathogen) as well as the activity of *Agrilus biguttatus* beetle. However, since the initial description in 2009 many aspects of the disease had not been established as of 2012 and the commencement of this research.

1. It has not been established whether the disease event, described as Acute Oak Decline leading to fatality of Oak trees in 5 years, is caused only by the two associated indicators, Bleed cankers and *Agrilus* beetle, whether just one of these factors is required or indeed there are as yet unaccounted factors.
2. If the disease event is a decline as of the primary model for of decline in trees - Manion’s Spiral Theory of Decline, it would require that a pre-disposing factor for the decline is present, but not yet identified.
3. The principal vectors of the spread of the bleed canker are not established.
4. The causal agent/s relating to the bleed canker are not identified.
5. The severity of the decline was identified within the naming of the disease, yet the rate of consequent mortality directly related to the identified factors is not established.
6. It is not established if the decline, or disease is reversible.
7. It has not been considered what methods could be used to reverse the decline or halt the pathogen.

Management guidelines were produced within the Forestry Commission Practice note 15 (Denman, Kirk & Webber, 2010). However, the management advice provided is generally generic and indeterminate, primarily due to knowledge gaps of the condition or disease event.

1.7.1 Targets of research

This thesis considers aspects of these knowledge gaps as part of a specific consideration of the bleed canker. The primary focus of the study is to consider the Bleed canker in relation to the environment and the host over a period of time. However, it is not a study of the disease or decline event named Acute Oak Decline, which by its primary definition requires the presence of the Agrilus beetle as well as the bleed canker.

This thesis does not attempt to present a complete understanding of the disease event within the forest complex. The targets of this research are to address knowledge gaps related to the phenomenon of the bleed exudate on Oak trees and which I have termed ‘Oak Bleed Canker’.

The primary focus is to as of the following:

1. To establish whether there are any environmental factors that pre-dispose the trees to bleed cankers.
2. To identify the causal agents relating to the bleed canker
3. To identify possible methods by which to disrupt, halt or reverse the bleed canker.

Chapter 2: Materials and Methods

2.1 Writtle Forest Woodland Complex – the study site

2.1.1 History of Writtle Forest

The primary woodland site from which symptomatic and non-symptomatic trees have been sampled is Writtle Forest, Chelmsford, Essex. The Forest has a long history with records dating back to the Doomsday Book. Oliver Rackham has written about the woodland's history and its flora (Rackham, 1990).

Writtle Forest is defined as Ancient Semi Natural Woodland, covering an area of approximately 600acres. It was most likely once far more extensive but now exists as a mosaic woodland, within an area of approximately 15 square kilometres of predominant agricultural arable landscape.

It is typical of historic woodland management throughout Essex as being 'Coppice with Standards'. The coppice species tends to be either Hornbeam or Sweet Chestnut with Oak Standards and occasional Sweet Chestnut. Birch also dominates as a typical pioneer species. It takes advantage of changes in woodland structure and predominates as the natural regenerative species in the woodland. There are diminished enclaves of plantation planting dating from the early 1900s, typically European Larch and some Douglas Fir.

The woodland lies 4 miles South-West of Chelmsford, Essex and approximately 25 miles from the centre of London. It is the largest woodland in private ownership in Essex, it is second in size within the county of Essex to Epping Forest, 9 miles away. Epping is within the custodianship of the Corporation of London. It is particularly noted for its veteran Beech and Hornbeam pollards. Hatfield Forest, also within Essex, lies 12 miles to the north of Writtle Forest. It is owned by The National Trust and is renowned as the woodland with the longest continuous recorded history of

management - possibly throughout the world (Rackham, 1990). Hatfield Forest and Writtle Forest were within the same ownership up until the 1500s. The first Lord Petre took ownership in the mid -1500s since which time the woodlands have remained with the family up until the present day.

The woodland is typical of a W10 *Quercus robur – Pteridium aquilinum – Rubus fruticosus* woodland with variance of the sub-groups within differing compartments, as of the National Vegetation Classification for the UK (Hall et al, 2004). The Oak species that predominates is *Quercus robur*, typical for the south east area of the UK. However, *Quercus petraea* is well represented within the woodland, more so than in other Essex woodlands (Rackham, 1986).

2.1.2 First Documented Incidence of Bleed Cankers

In 2006 bleeding cankers were first noted on a group of Mature Oaks within an area of recent coppice to the north of the woodland. The episode was recorded and samples and photographs sent to Forest Research believing it then to be the newly reported *Phytophthora ramorum* (Sudden Oak Death). It was confirmed not to be *P. ramorum* or any other *Phytophthora sp.*. No other diagnosis was suggested at the time. The trees were felled as a matter of precaution, but with little success in containing the spread of the bleed cankers. Within four years several other areas throughout the woodland had Oak trees that displayed similar symptoms to the primary event, symptoms which now contribute to the disease/ decline event named as Acute Oak Decline.

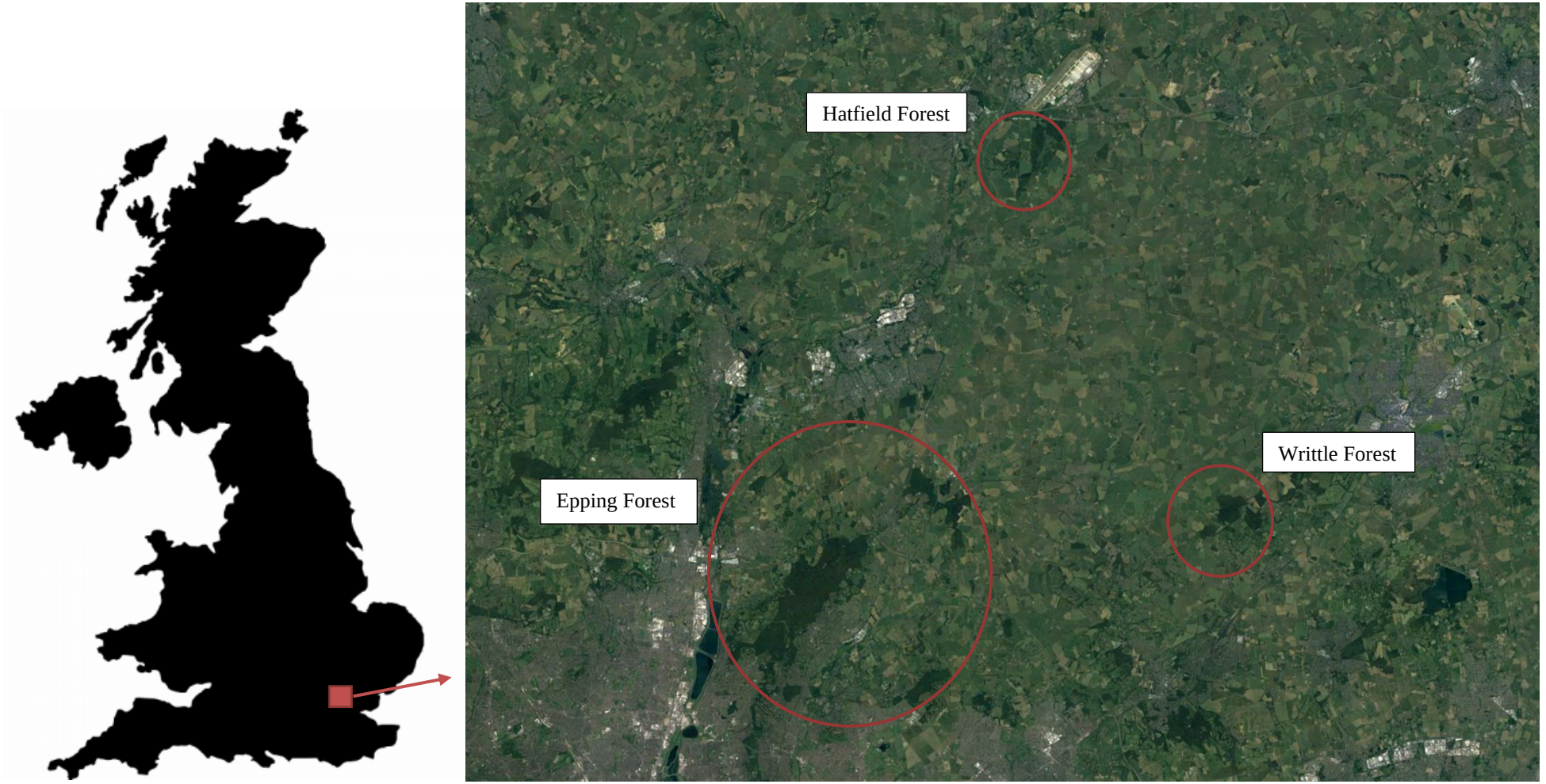

Fig.2.1: Location of Writtle Forest within the UK and contextualisation within the landscape (including nearby woodland)

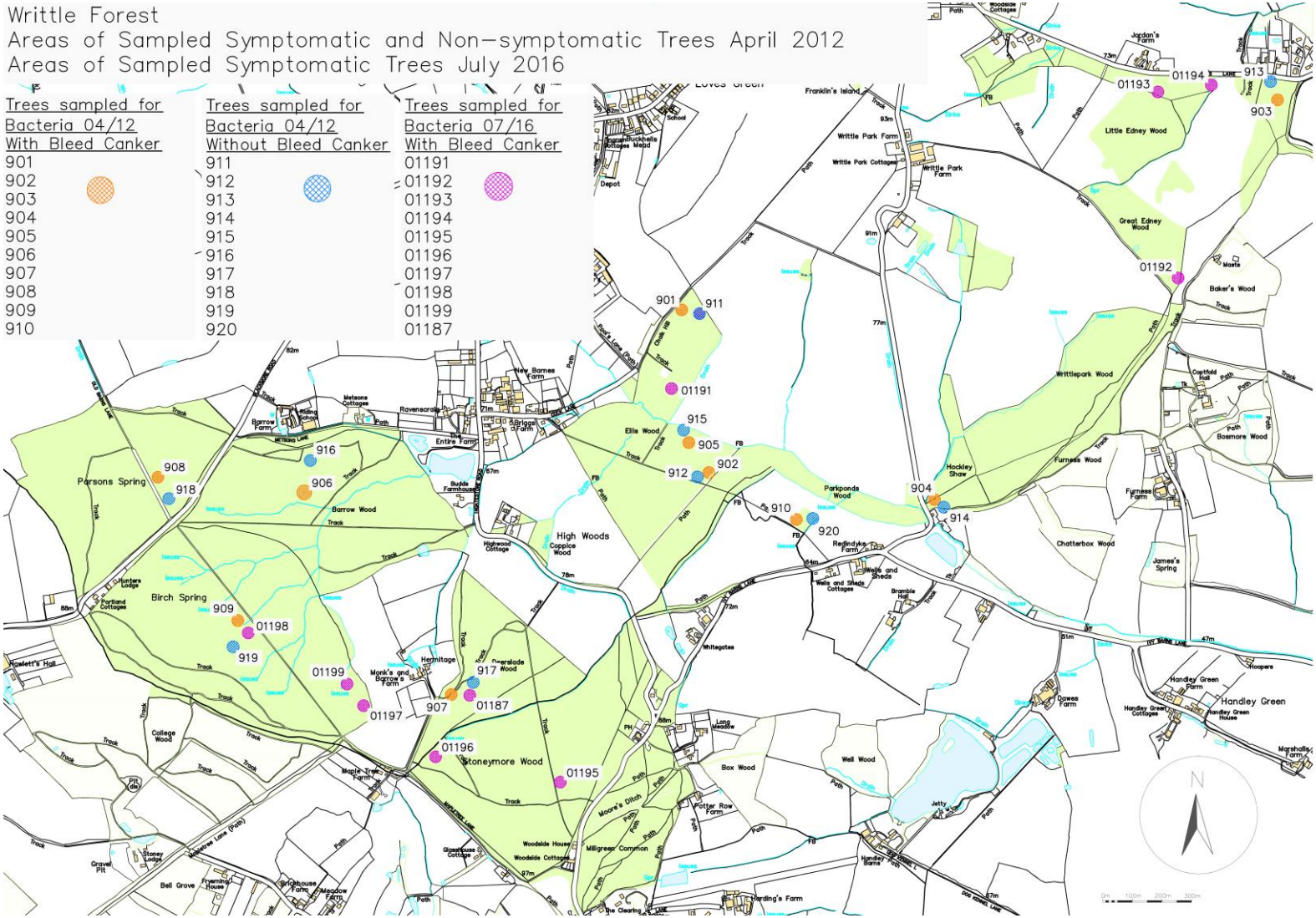

Fig. 2.2: Writtle Forest, Areas of trees sampled for Bacteria

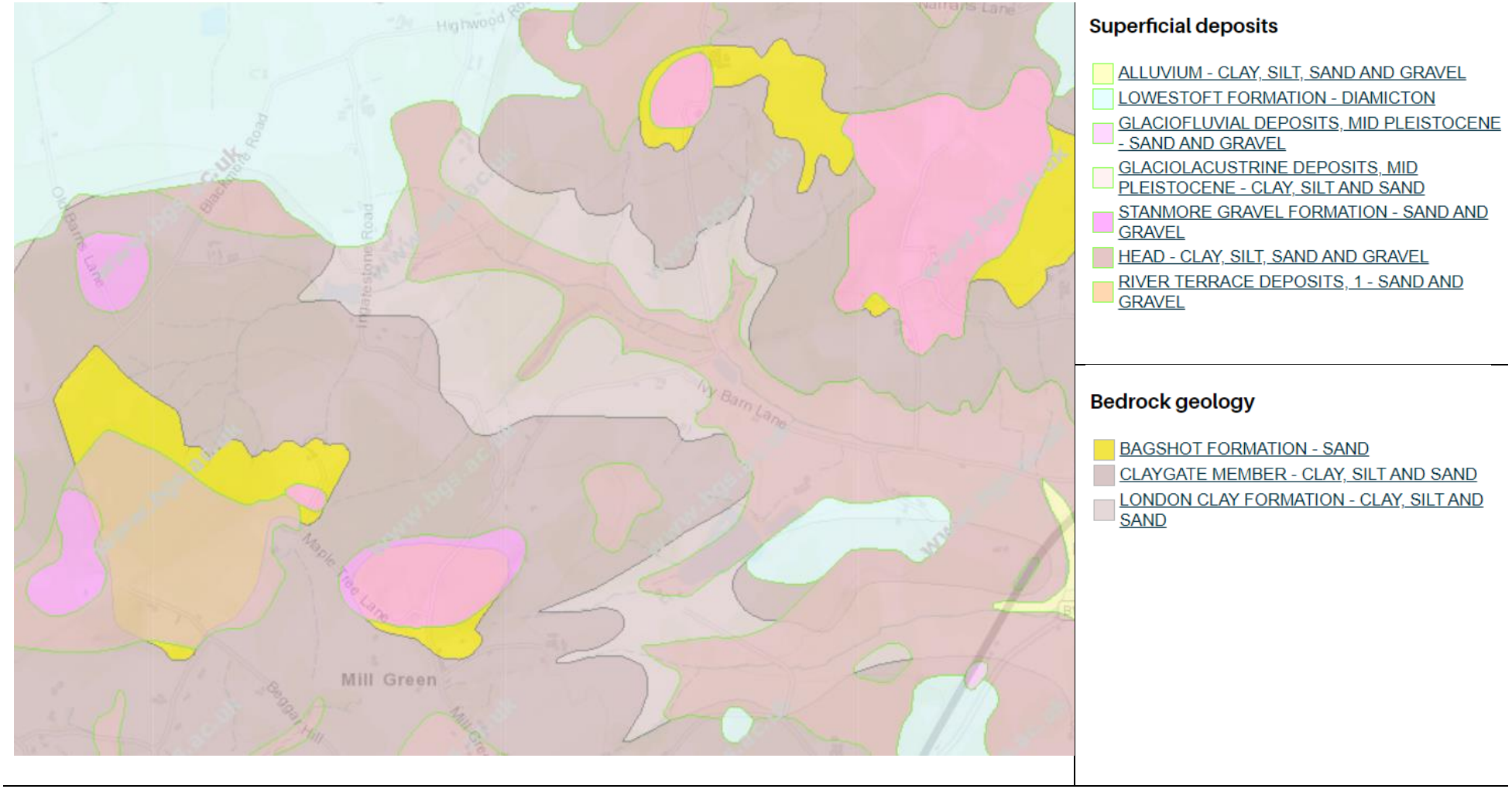

Fig. 2.3: Writtle Forest, Bedrock and Superficial geology (BGS Geology of Britain online viewer accessed November 2019)

2.2 Epidemiology study – methodology

The epidemiological studies were undertaken to understand the movement and virulence of Oak Bleed Canker within Writtle Forest. Data was gathered to establish the persistence and spread of the signs of exudate (cankers) on the Oak trees throughout the woodland. (These methodologies are further detailed in Chapter 3).

2.2.1 Gamma Scale

This approach was based upon adapted, simplified landscape models of scale (Holdenrieder et al, 2004). In this instance a Gamma scale extent is defined as of the entire Writtle Forest woodland complex (as of Fig. 2.2). A transect of the woodland was used by which to evaluate the extent and spread of the incidence of exudate on the Oak trees through the area over a period of six years. (as of Fig 3.1 Chapter 3).

This was evolved from an initial general sweep of the woodland to source all trees with Bleed Canker. Ten trees were selected based on distribution throughout the woodland and avoiding other symptomatic trees in close proximity. The aim was to select those trees that were found across the range of topography, soil type, management systems and companion tree species found throughout the woodland complex (and as of Fig. 2.3). This then formed the basis of a transect of the woodland which encapsulated an area of between 5% and 10% within each woodland with established Oak populations. The transect was reviewed biannually over the 6year period. Information pertaining to the number of Oak trees with bleed canker recorded with each tree that had canker being tagged to monitor progression of bleed.

Information pertaining to the ten selected trees was also gathered this related to:

1) Bleed, health and condition of the trees over a 6year period. Data was collected on the 10 symptomatic and 10 neighbouring non symptomatic trees (within 25m of the symptomatic tree) to track the progress of the bleed canker.
2) Collection of soil samples from the ten trees (and ten control trees) to compare possible pre-disposing factors relating to soil types (Chapter 4).
3) Samples gathered to identify the bacterial causal agent of the bleed canker (alpha scale diversity), (Chapter 5)

(Aspects pertaining to the general environment of the woodland of each of the ten symptomatic and non-symptomatic trees is noted within Appendix 4: 2012 Data relating to Oak trees throughout Writtle Forest).

2.2.1 Beta Scale

A Beta scale defined as one area of the woodland with a predominance of the disease incidence was also studied. (Barton et al, 2013). This was a section of 1 hectare woodland predominantly planted with Oak and exhibiting extensive signs of the Bleed Canker. The aim of the beta scale study was to consider the host pathogen interaction, the localized spread of the pathogen as well as the mortality rate of symptomatic trees. Within the beta scale these considerations could be made without such distinct variables such as differing soil types, general topography and localized microclimate.

2.3 Soil sampling – methodology

Data was gathered on each of the ten selected symptomatic and non-symptomatic trees. This information detailed a brief description of the woodland and the trees immediate position, the age of the tree, the diameter at breast height (1.5m), the height of the tree, the crown spread, percentage of deadwood within the crown, the number of cankers present on the main stem, the orientation

and height of canker, whether recent exudate was observable, the number of callused cankers, past history of wounding, evidence of beetle activity, existence of other pathogens present, the number of similarly symptomatic and dead oaks within a 25m radius of the tree. The soils within the woodland complex are relatively varied and samples were taken from areas of gravel, clay and silts and sands. (Fig. 2.3).

2.3.1 Soils information gathered adjacent to the sampled trees

Soils were collected at approximately 2.5m from the stem to the north of each symptomatic and non- symptomatic tree. Soils were collected from the topsoil immediately below the leaf litter, generally at a depth of approximately 150mm and at a depth of 300mm below ground level. At a depth of 300mm this was beyond the humus soils and generally constituted a sample of the sub soils.

Soils were collected and dried to approx. 1.5kg of sample, as of the soil sampling methodology provided by the soils laboratory (A&L Analytical Laboratories, Inc. 2790 Whitten Rd Memphis, USA). The samples were sent to Bartlett Tree Expert Laboratories for analysis to consider soil acidity, Nitrogen, Phosphorous, Potassium, Magnesium, Calcium, Sodium, Iron, Manganese, Copper, Zinc and Boron. (The methodology is further detailed in Chapter 4).

2.3.2 Soils information gathered Beta study area

Soils were similarly analysed by the same process from adjacent to symptomatic trees within the Beta scale area of woodland. This was done to 1) ascertain if there were any distinct differences between soils gathered over the woodland complex and 2) to establish basic field tests of soil ameliorants.

2.4 Tree Ring Analysis – Materials and Methods

2.4.1 Measurement of Tree Rings

Twenty trees were selected from the 1 Hectare Oak cohort within Stoneymore Wood. A non-destructive wood density assessment of trees was made using a Resistograph R650. This is an electronic, high-resolution, needle drill-resistance measurement device. The thrust and mechanical drive of the drilling device is measured. This provides high correlation between the measured values and the density of the penetrated wood (Fundova et al., 2018).

Drill readings were taken at a height of approximately 1.5m on the main stem, at a 90degree angle to the growth rings, at positions of drilling from north to south and south to north on each tree. These 2 readings for each tree were cross correlated to find the best tree ring measurement reading for each tree. All measurement readings were analysed using TSAP software (Cufar et al, 2008; Koprowski et al., 2010). This enables identification of the tree rings and cross referencing of data readings from the drill readings. It further allows for the incremental area of the tree rings to be estimated. Comparative readings and graphs were derived from the TSAP software.

2.4.2 Cross correlation of weather and tree ring data

The link between tree ring growth and climatic conditions is well established. Climatic events can be used as points of reference when interrogating tree ring data; (Fritts, 1973, Nabeshima, 2010). Rainfall and temperature data were provided on request from the National Meteorological Library & Archive. The information provided was for each day since 1959 from the area of Writtle, Essex. This data was cross correlated with the tree ring data to ensure best match of annual tree ring data to years. (These methodologies are further detailed in Chapter 5).

2.5 Bacterial Sampling– Materials and Methods

2.5.1 Collection of Bacterial samples from Writtle Forest April 2012

Samples were collected from ten symptomatic and ten non- symptomatic trees in April 2012. An area was chosen on symptomatic trees where the exudate was recent, prominent and accessible. These tended to be on the side of the stem exposed to most sunlight, at approximately 1.5m from ground level. An area of approximately 40mm x 30mm was cut using a chisel to a depth of approximately 20mm. This was sufficient to gather a single sample that included the outer bark, inner bark and sap wood from the bleed area. Each sample taken was placed in a sterile collection bag. The chisel was sterilised in 100% ethanol and flamed after collection of each sample. Samples were stored at 2 -5°C before preparation in the laboratory. When collecting samples from non-symptomatic trees the same sampling criteria was applied.

2.5.2 Collection of Bacterial samples from Writtle Forest July 2016

Over the course of the study it became apparent that exudate from the bleed areas was more extensive within the summer months. It was considered that the diversity of the bacterial species was often variable according to seasonality (Shen & Fulthorpe, 2015). Consequently, further samples were gathered in July 2016 from ten symptomatic trees only. This was to consider the combination of varying facultative anaerobic bacteria within the different vascular conductive tissues of the tree and to ascertain if there were further bacterial species involved in the Oak bleed process than those already isolated.

Trees were chosen on the basis of the biannual transect, the proximity to symptomatic trees discoverable in 2012 and the extent of the bleed at the time of the sampling. Sampling again tended to be on the side of the main stem exposed to sunlight at approximately 1.5m from ground level. In this instance a swab was taken of the exudate using sterilized cotton wool. This was placed in

Phosphate Buffer Solution (PBS) kept at 2 -5°C. Then an area of approximately 40mm x 30mm was cut using a chisel and samples taken as described in 2.5.1.

2.6 Bacterial profiling –Materials and Methods

2.6.1 Wood material selected for isolation of Bacteria

Lesions were sought on the peripheral area of infected tissue at the margin of necrosis. Lelliot and Stead (1987) recommend that specimens of diseased material taken for laboratory diagnosis, taken for Canker (or shoot) dieback, should include the edge of the lesion and several centimetres of healthy material. It is considered that the pathogen will be most numerous at the edge of the lesion but absent or in lower numbers in older tissue (Lelliot and Stead, 1987). From the Oak samples taken this area consists of two distinct areas, the non- affected, non-stained white sap wood tissue and the affected tissue, stained dark brown and forming a distinct zone. This was generally taken toward the uppermost aspect of the cankerous area.

2.6.2 Preparation of samples and Growth media - 2012 sampling

The sample was placed in a 2 ml bead-beating tube with 1mL of PBS. The tissue was homogenized at high speed (4m/s) for a total of 40 seconds, 1mL of the resultant liquid was aspirated. For those samples gathered in 2012 a dilution series was carried out using Phosphate buffered saline (PBS) as the diluent. 100µl of the pure sample was added to 900µl of PBS and mixed. 100µl of this solution (at 10^{-1}) was aspirated and diluted as before by adding to 900µl of PBS and mixed. This series was continued until a 10^{-6} solution was prepared. 100µl of each dilution was then added to a plate of Luria-Bertani agar (LB) and spread to create an even lawn. The plates were incubated for between 3 and 5 days at 20°C.

2.6.3 Preparation of samples and Growth media - 2016 sampling

Preparation of samples taken in July 2016 varied. It was apparent that the predominant bacterial species of interest were gram negative. Preparation of the samples was therefore based on protocols favourable to isolation of these bacteria and that would enhance morphological differentiation of bacteria on the growth plate. The sample was homogenized at high speed as of previous preparation. However, no dilution series was undertaken. The resultant liquid was streak spread on MacConkey Agar (with Crystal violet, Sodium chloride and 0.15% Bile salts). This media was used to select for gram- negative bacteria which could then be morphologically distinguished (generally according to the bacterial species ability to ferment lactose).

The plates were incubated for 24 hours at 27°C. The growth rate of the bacterial colonies was improved with the temperature increase. That the dilution series was not undertaken meant that discernable colony growth was quickly achieved within 24 hours.

2.6.4 Isolation and Storage of Colonies

Individual colonies as of the 2012 samples were purified from the sampled plates using a sterile loop and re-plated using the three- point streak method on to LB agar. The cultures were incubated for 2 days at 27°C. Individual colonies were picked from the 2016 samples and re-plated using the three- point streak method onto MacConkeys agar. The cultures were similarly incubated for 2 days at 27°C.

The morphology of colonies on the LB agar from the 2012 sampling, was then recorded using a magnification of 40 X, with illumination from above and below the plate being viewed. The colony morphology was recorded using standard descriptive terms (Prescott et al, 2005). These included; size, form, elevation, margin, colour and surface. An individual colony was then selected from the streaked colony culture and placed in 10mL of LB Broth. This culture was then agitated in a shake incubator (2,000 rpm) for 12 and 18 hours at 27°C. 600µl of the bacterial culture was aspirated

and mixed with 400µl of 40% glycerol. This was placed in a cryo- tube vial and frozen at -80˚C. Approximately 800 samples were isolated from both symptomatic and non-symptomatic trees and approximately 600 of these strains were frozen to form a library. Colonies isolated from the 2016 sampling on MacConkeys agar were similarly cryogenically frozen and stored.

2.6.5 DNA extraction

Bacterial isolates from 2012 sampling to be identified through Polymerase Chain reaction of 16SrRNA genes were first Gram stained to ascertain whether the isolates were gram–negative or positive. DNA was then extracted from the isolate according to manufacturer's instructions for Gram -negative or Gram -positive bacteria using a Quiagen 'DNeasy® Blood & Tissue kit.

A cheaper and simpler DNA extraction method was employed for the isolates taken from the 2016 sampling (knowing that the bacteria were gram negative). An individual colony was selected from a streaked colony culture and placed in 10mL of LB Broth. This culture was then agitated in a shake incubator (2,000 rpm) for 12 and 18 hours at 27°C. 1mL was aspirated and centrifuged for 1min at 12,000 rpm. The resultant supernatant was removed and the pellet resuspended in 200 µl Tris buffer solution (vortexed for 1min). This was then heated to 100°C for 20minutes and placed on ice for 15minutes. The product was then centrifuged for 1min at 12,000 rpm. The resultant supernatant is aspirated and constitutes extracted DNA, suitable for PCR.

2.6.6 Polymerase Chain Reaction (PCR) and sequencing of DNA

PCR was conducted using Gotaq™ with a Taq DNA polymerase enzyme and 16s rRNA universal primers Forward primer 8F AGAGTTTGATCCTGGCTCAGAGAGTTTGATCMTGGCTCAG, Reverse primer 1492R (1) GGTTACCTTGTTACGACTT (Turner et al 1999). The temperature cycle applied was an initial denaturing temperature of 95°C for 5mins. Then 30 cycles of the following: 95°C for 30 seconds, 54.5°C for 30 seconds (to anneal the primers), and 72°C for 90

seconds. At the end of these 30 cycles the DNA was heated for 1 cycle at 72°C for 5 minutes then left at a holding temperature of 10°C. Samples were purified and concentrated prior to submitting for sequencing using a Zymo Research DNA Clean & Concentrator™ kit.

2.7 Pathogenicity Testing– Materials and Methods

The aim of this work was to establish whether the typical bleed symptoms observable on symptomatic trees could be reproduced by infecting the tree with prepared inoculum of those bacteria isolated. This involved fulfilling criteria as of Koch's postulates. The first and second of these criteria are that the suspected causal agent must be present in every diseased organism and that the suspected causal agent must be isolated from the diseased organism and grown in pure culture. This was established through sampling, culturing the microbial population and identifying the discoverable bacteria. The third and fourth criteria are that when a pure culture of the suspected causal agent is inoculated into a non- symptomatic host the host must exhibit the same symptoms and that the same causal agent must be re-isolated from the infected host.

2.7.1 Inoculated trees

The Oak trees *Quercus robur*, approximately 5 years old, were purchased in bulk from a wholesale nursery. The origin of the stock was not known, although it was believed they were all of the same provenance. The trees were planted on a cultivated trial site within the grounds of University of Reading in 2013.

Whilst the bleed canker had most commonly been associated with mature trees it had also been seen on young trees (although far less frequently). Young Oak trees were more readily available, more cost effective to work with and easier to grow in controlled environments than to work with immovable, semi mature trees of economic value. It was considered that if the young trees could

reliably be infected then this would be an effective model by which to test the efficacy of methods of controlling the canker within minimum environmental variables. It was further hypothesised that living trees that were functional as a natural tree system, i.e. drawing water from the soil through the vascular system, would more readily produce the bleed symptoms witnessed on the diseased trees.

The first inoculations were carried out in January 2013 to establish whether the bleed symptoms could be replicated on the younger trees from the isolated bacteria from the symptomatic trees. This was successful and so inoculations were then carried out to trees in the field, Field Inoculations 2013. These were not successful. The original methodology of inoculation was thus re-employed, and trees were lifted and placed in glass house conditions, Glass House Inoculations 2014-15. This proved more successful although climatic conditions were highly variable. In 2016 climate control rooms became available at the University. The final inoculations were carried out within a controlled environment room, Controlled Room Inoculations 2016-18.

2.7.2 Field Inoculations 2013

The first inoculation experiments were made to trees within the Field. Initially trees were inoculated in April, as the young trees first came into leaf. The experiment was also carried out to trees in the middle of the growing season at the end of June. These Field Test Inoculations produced no bleed symptoms.

2.7.3 Glass House Inoculations 2014-15

Non- inoculated trees were then lifted from the field sites in winter. The trees were placed in 20 litre pots with an inert potting compost. The trees were then placed in semi -controlled glass house conditions at a temperature of approximately 18°C with 16 hours of light. Such conditions produced an early flush of the trees and once the trees were in leaf they were inoculated. These

inoculations brought some success and bleed symptoms were observable. Variations were made to the mode and amount of delivery of the inoculum to the tree to establish which dosages and delivery systems were most effective. An optimum system of delivery of the inoculum was achieved. However, the Glass house conditions were not consistent. The temperature and the sunlight hours within the glass house were only controllable in colder conditions.

2.7.4 Controlled Room Inoculations 2016-18

Controlled Environment rooms became available that would accommodate the trees. These control humidity, temperature and sunlight hours. Conditions were chosen that were considered to best represent growing conditions within a broad leaf woodland (Peterken, 2001). The humidity was set at 60%. 12 hours of daylight and 12 hours of dark were provided. The daylight hours temperature was set at 20°C, the non-light temperature was set at 16°C.

Trees were lifted from the field and placed in 20 litre pots with an inert potting compost in dormancy. The controlled environment rooms have a height restriction of approx. 2m and are limited in size. When the trees came into leaf they were pruned using natural target pruning techniques to a height of approximately 1.8m, with a crown diameter of approximately 1 metre. This represented approximately 50- 75% canopy loss for the tree.

2.7.5 Preparation of inoculum

Bacterial strains were utilized from the cryogenic library. Frozen stock was streaked onto Luria-Bertani agar (LB). Colonies were allowed to develop for 2 days at 24 -27°C. A single colony was then selected and placed in 10mL of Nutrient Broth. The culture was then agitated in a shake incubator (225 rpm) for 18 hours at 24- 27°C. The inoculum was then prepared at a density of 10^{9} CFU/mL in a Phosphate Buffer Solution (PBS). Where combinations of bacteria were used

as inoculum each bacterial suspension was prepared separately and added together at appropriate ratios to equate to the same amount of delivered inoculum for each test.

2.7.6 Delivery of inoculum - Field and Glass house Inoculations

Field Inoculations were made by creating a wound with a sterile blade into the stem of the tree at a 45degree angle. This was carried out within three areas of the stem at 150mm spacings. The inoculum was delivered 0.3mL by pipette downwards into the incision. The wound was then covered with parafilm.

This method was varied with the Glass house inoculations. It was considered that the reactive wound tissue that was created as a consequence of the blade cutting through the plant tissue, was physically substantially different, such as to resist the spread/ development of bacterial infection (Shigo, 1991). A different method of delivery was employed using a system called BITE - Blade for Infusion in Trees. This is a low impact tool for xylematic injections. It works by hammering a stainless steel, lenticular blade into the stem of the tree. This has the effect of separating the wood fibres of the tree reducing damage to surrounding tissue (Dal Maso et al., 2014. Acimovic et al, 2016). Given the size of the stem diameter of the trees to be inoculated (diameters ranged from 45mm to 90mm at area of wounding), the blade was inserted between 5mm and 10mm into the stem of the tree. The system delivers the product/inoculum via a syringe inserted behind the hollow blade. The syringe may then be used to push the product/ inoculum through the hollow blade into the area of divided fibres. The first inoculations made with this system used 1.5mL of inoculum pushed through the system with the syringe. However, the delivery system is designed to introduce large volumes of liquid, (generally as means of introducing chemicals to reduce disease in trees). Hence the delivery system did not allow for sufficient accuracy as to the amount of inoculum delivered.

2.7.7 Delivery of inoculum – CE rooms inoculation

The system of separating the tree fibres was used for the introduction of the inoculum to the Controlled Environment inoculations. However, the delivery was achieved using a hypodermic syringe inserted into the area of the divided fibres. The resultant wound area was then wrapped with parafilm and 1mL of the inoculum was then introduced with the hypodermic syringe through the parafilm into the wound pocket. The inoculum was introduced gradually into the area, within up to an 8hour period, to ensure suitable absorption into the tree's vascular system.

2.7.8 Monitoring of symptoms

Trees were checked every 7 days over a 90 to 100day period. Information was recorded relating to the appearance or otherwise of bleed symptoms. The amount of bleed was quantified with a scoring system. Other information that was recorded related to the development of callous or wound tissue at the area of inoculation. Each inoculation, at each recorded time point was photographed with Sony ILCE5000L Compact System Camera with SEL-1650 Zoom Lens, mounted on a tripod, set on full zoom - 50mm, at a distance of 450mm from the area of inoculation.

2.7.9 Re-isolation of Bacteria from infected plant

Woody tissue was isolated from the area of the inoculation where bled symptoms were prevalent. The material was macerated in a 2 ml bead-beating tube with 1mL of PBS at high speed (4m/s) for a total of 40 seconds. The resultant liquid was streak spread on MacConkey Agar (with Crystal violet, Sodium chloride and 0.15% Bile salts). The plates were incubated for 24 hours at 27°C. Individual colonies were selected for re-isolation based on variation in visible morphology. The isolated colonies were streaked on LB agar and incubated for 24hrs at 27°C

An individual colony was then selected from the streaked colony culture and placed in 10mL of LB Broth. This culture was then agitated in a shake incubator (2,000 rpm) for 12 and 18 hours at

27°C. 1mL was aspirated and centrifuged for 1min at 12,000 rpm. The resultant supernatant was removed and the pellet resuspended in 200 µl Tris buffer solution (vortexed for 1min). This was then heated to 100°C for 20minutes and placed on ice for 15minutes. The product was then centrifuged for 1min at 12,000 rpm. The resultant supernatant with extracted DNA was aspirated. PCR was conducted using Gotaq™ with a Taq DNA polymerase enzyme and 16s rRNA universal primers Forward primer 8F AGAGTTTGATCCTGGCTCAGAGAGTTTGATCMTGGCTCAG, Reverse primer 1492R (1) GGTTACCTTGTTACGACTT (Turner et al 1999). The temperature cycle applied was an initial denaturing temperature of 95°C for 5mins. Then 30 cycles of the following: 95°C for 30 seconds, 54.5°C for 30 seconds (to anneal the primers), and 72°C for 90 seconds. At the end of these 30 cycles the DNA was heated for 1 cycle at 72°C for 5 minutes then left at a holding temperature of 10°C. Samples were purified and concentrated prior to submitting for sequencing using a Zymo Research DNA Clean & Concentrator™ kit.

2.8 Bacterial Properties – Materials and Methods

To understand how best to combat the discoverable pathogenic bacteria simple investigations were undertaken to learn a little of the properties of the bacteria.

2.8.1 Gram staining and Microscopy of Bacteria

Preparation of slides to examine bacterial cells under the microscope was done using a heat fixed, Gram-stained smear. 75mm x 27mm x 1mm glass cavity slides were used to mount the bacteria with a 18mm square cover glass approx. 0.15mm thick. The glass was cleaned prior to making the smear with ethanol and then passed through a flame. A single colony was selected from an agar culture and mixed with water on a metal loop and smeared on the slide. This was then air dried then fixed with heat. The slide was then flooded with 2% Crystal violet and left for 1 minute. The

excess liquid was removed and the slide rinsed under water. Gram's iodine was then added for 1 minute and the slide once more rinsed. Drops of ethanol were then added until no colour appeared in the effluent. The slide was then immediately rinsed in water to remove the ethanol. The slide was then counterstained with 2.5% Safranin for 30 seconds, the excess removed and the slide rinsed with water and dried with blotting paper (Jones et al, 2003).

The slides were viewed with an Olympus CX41 microscope. The image was captured with an Olympus SC100 camera and the image manipulated and measured with Cellsens Software.

2.8.2 Growth Curves

Growth curves of the bacteria were plotted using Luria-Bertani broth. A single colony was selected from a streaked plate and shake incubated in 10mL of LB broth for 18hrs at 225rpm. The resultant product was then equalized to reach an Optical density of 1, equivalent to 10^{9} CFU/mL.

1mL of 10^{9} CFU/mL was then added to 100mL of LB broth in a conical flask. The optical density reading was checked at 600NM with an Eppendorf Biophotometer 6131 Spectrophotometer, every 30 minutes for 12 hours and then a final reading after 24 hours. (Hall et al, 2013).

2.8.3 Swimming Motility

Petri dishes were prepared using Luria-Bertani agar at 0.25%. The dishes were air dried in a laminar flow cabinet for 24hrs prior to inoculation. A single colony was selected from a streaked plate and point inoculate onto the centre of the prepared plates. The plates were kept in dark room conditions at 24°C and the resultant halo forming around the inoculation recorded over a 10day period. Motility was then assessed by examining the circular zone created by the bacterial cells migrating away from the inoculation point. (Ha et al, 2014).

2.8.4 Genome Extraction for PacBio Library

A single colony was selected from a streaked plate and shake incubated in 10mL of LB broth for 18hrs at 225rpm. Qiagen Gentra® Puregene® extraction kit was used to extract the DNA from bacteria using the Gentra Puregene Handbook 12/2014. The quantity and quality of the DNA was tested using a Nanodrop Spectrophotometer. The product was required to have an OD260: OD280 ratio of 1.8 to 2.0 and an OD260: OD230 ratio of 2.0-2.2.

Gel electrophoresis was also carried out on the DNA product run on a 1% agarose gel matrix at 20 volts over a 12hour period. This established the purity of the sample as well as allowed the quantity of the sample to be measured by comparison to a Hyper-ladder. (Qiagen Gentra Puregene kit typically produce product of 100-200Kb). The resultant product was then sent for testing to the Centre of Genomic Research at the University of Liverpool.

2.8.5 Antagonistic tests - Reaction zones of bacterial lawns with Tannin solution

An individual colony was selected from a streaked culture and placed in 10ml of LB Broth. The culture was then agitated in a shake incubator for 12 _18 hours at 27°C. 100µl was then added to a plate of LB agar and spread to create an even lawn. A sterile infusion disc (without chemical infusion) was placed in the centre of the plate and 10µl of varying tannin concentrations was added to the infusion disc on each plate. The cultures were then incubated at 27°C for 4 days. Measurements of the distance of the edge of the bacterial growth to the edge of the infusion disc were recorded.

2.8.6 Antagonistic tests - Bacteria versus Bacteria

Antagonistic tests were carried in vitro, under controlled conditions on petri dishes with media deemed most appropriate for testing.

Known, cryogenically frozen colonies of the bacteria commonly associated with the exudate associated with Acute Oak Decline, were re- streaked onto LB agar and grown for 2 days at 27°C. An individual colony was selected from each streaked culture and placed in 10ml of LB Broth. The culture was then agitated in a shake incubator (2,000 rpm) for 12 _18 hours at 27°C.

A petri dish was prepared with 20mL of LB and the area demarcated equally into two with a permanent marker. 10uL of each bacteria was then placed in the middle of the petri dish, close to the demarcation line.

The plates were then incubated at 27°C and monitored over a four day period. The resulting growth of the bacteria and the interaction within the adjacent bacteria was then recorded.

2.8.7 Antagonistic tests - Bacteria versus Antibiotics

As of before an individual colony was selected from a streaked culture and placed in 10ml of LB Broth. The culture was then agitated in a shake incubator for 12 _18 hours at 27°C. 100µl was then added to a plate of LB agar and spread to create an even lawn. Known amounts and concentrations of antibiotics were then placed on the prepared plates. The plates were then incubated for 3 days at 27°C. Measurements were then recorded of the zone of inhibition between the bacterial lawn and the anti-biotic.

2.8.8 Antagonistic tests - Bacteria versus Endophytes

As of before an individual colony was selected from a streaked culture and placed in 10ml of LB Broth. The culture was then agitated in a shake incubator for 12 _18 hours at 27°C. 100µl was then added to a plate of LB agar and spread to create an even lawn.

The fungal endophyte was grown on PDA agar for a period of approximately 21 days in sealed petri dish in darkened conditions at room temperature. A 3mm plug of the endophytic fungi was extracted and placed in the centre of the plate with the prepared lawn of bacteria. The petri dishes

were then sealed with parafilm and incubated at room temperature for 12 days. Measurements were made of the amount of fungal endophyte growth and compared with controls. The amount of fungal endophyte growth was measured and recorded.

2.9 Culture of Fungal Endophytes– Materials and Methods

2.9.1 Collection of Wood Material to sample for Endophytic Fungi

Samples of the inner bark and sap wood material were collected from ten symptomatic and ten non-symptomatic trees from Writtle Forest in May 2018. (Fig 2.4). The samples were taken from trees previously sampled for bacteria, (to reduce damage created by sampling to the Oak tree stock within the woodland). In some instances, where the trees had consequently died, nearby trees of a similar stature were chosen from which to sample. One sample per tree was taken at a height of 1.5m from ground level on the side of the stem exposed to most sunlight. On trees that exhibited bleed symptoms the material was collected away from any area of existent exudate. An area of approximately 40mm x 40mm was cut into using a chisel to a depth of approximately 20mm. The outer bark was discarded, the inner bark and sapwood material were collected and stored at 4°C.

2.9.2 Preparation of Samples for Isolation of Endophytic Fungi

Within the Laboratory the wood material was cut into small pieces of approximately 10mm x 10mm x 5mm. Each piece of wood was then washed under a running tap before being submersed for 60secs in 95% Ethanol, followed by 2 mins in 70% Ethanol and 5 mins in 33% Sodium hypochlorite. Each piece of wood was then subjected to 5 separate washes in sterile water for 1 minute each.

2.9.3 Growth media for Endophytic Fungi

Each piece of wood was then placed on to agar plates. Media used was Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Oak Leaf Agar created by boiling down Oak leaf and mixing the concentrated strained material to Agar. PDA was found to be the most consistent and effective growth media.

The wood sample was placed on the agar, the petri dish was then sealed with parafilm and the dish left in darkened conditions at 20°C. The first growth was generally observed after 10 days. After 20 days each fungal endophyte was re-isolated and grown on for a further 15 days before carrying out the DNA extraction.

2.9.4 DNA extraction

100mg of the sample was crushed with sand using a micro-pestle within a 1.5mL Eppendorf tube. To this was added 1mL of CTAB buffer warmed to 65°C and 10ul Mercaptoethanol. (The CTAB buffer was made up to 10mL as of 500ul Tris HCl pH8 (1M), 400ul EDTA pH8n (0.5M), 1400ul NaCl (5M), 0.1g Cetrimonium bromide (CTAB), 0.1g Polyvinylpyrrolidone (PVP) and 0.2g of Sodium dodecyl sulfate (SDS). The solution is made up to 10mL by adding sterile water).

The solution was vortexed to mix then incubated at 65°C for 15mins with the solution being vortexed to mix every 5mins. The mixture was then transferred to a 2mL tube and 500ul of Chloroform: Isoamyl Alcohol (24:1) added and mixed by inversion for 5 mins. The mixture was then centrifuged at 13,000rpm for 5mins. The top aqueous layer of the mixture (approx. 800ul) was then transferred to 1.5mL tube and centrifuged at 13,000rpm for 3mins. The supernatant is then transferred to a new 1.5mL tube (approx. 700ul) and an equal volume of ice cold isopropanol is added by swirling together. The mixture is then centrifuged at 13,000rpm for 3mins. Discard supernatant. Keep pellet add 500ul of 70% ethanol to wash. Centrifuge for 1min at 13,000rpm, pour off ethanol. Dry remaining pellet in tube in heat block at 37°C for 5 to 10mins. Resuspend in

50ul of TE buffer by vortexing. The product was then tested with a Nanodrop Spectrophotometer. Where the peak reading appears abnormal then 1ul of RNase (1mg/1ul) was added to the product and incubated for 30mins at 37°C.

2.9.5 PCR and Sequencing of Fungal Endophytes

PCR was conducted using Gotaq™ with a Taq DNA polymerase enzyme and Forward primer ITS1_KY02, TAG AGG AAG TAA AAG TCG TAA and Reverse primer 1TS4 TCC TCC GCT TAT TGA TAT GC (Toju et al 2012). The temperature cycle applied was an initial denaturing temperature of 95°C for 5mins. Then 30 cycles of the following: 95°C for 30 seconds, 52°C for 30 seconds (to anneal the primers), and 72°C for 90 seconds. At the end of these 30 cycles the DNA was heated for 1 cycle at 72°C for 5 minutes then left at a holding temperature of 10°C. Samples were purified and concentrated prior to submitting for sequencing using a Zymo Research DNA Clean & Concentrator™ kit.

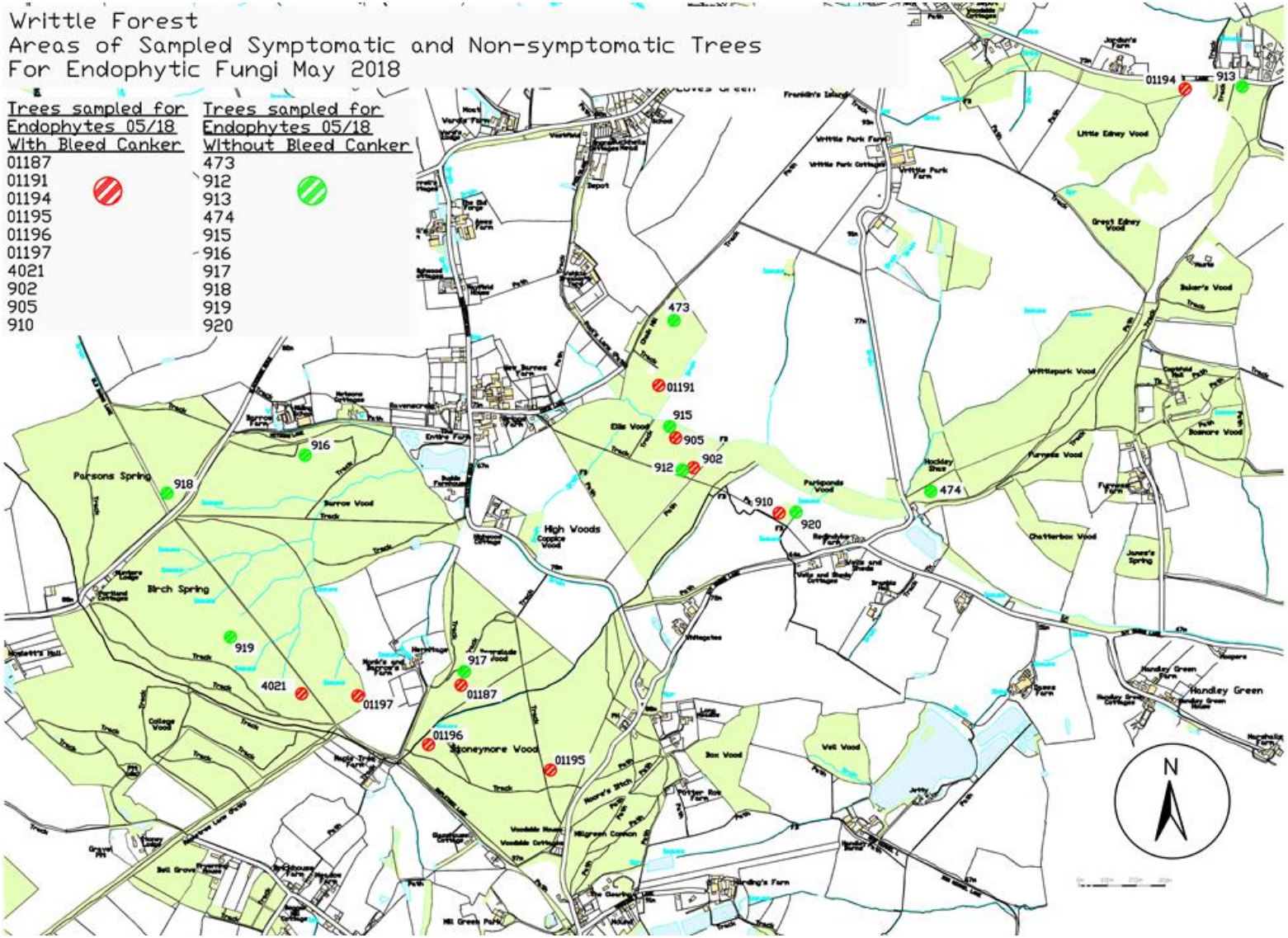

Fig. 2.4: Writtle Forest, Areas of trees sampled for endophytic fungi

Chapter 3: Results – Epidemiological Studies

3.1 Context of research

The emergence of the bleed canker on Oaks within the woodland and the interaction of the pathogen(s) within the host's environment is integral in providing a picture of possible control methods. Management of plant and tree disease will more often involve an integrated approach, considering the management of the host's environment and not solely the host – pathogen relationship. Establishing a picture of the progression of the syndrome/ decline over time will provide insight as to causal relationships (Turchetti & Maresi, 2008). This in turn will allow the development of different strategies to decide when and how it is best to intervene within the growing cycle of the crop or life cycle of the individual tree (Vanderplank, 1963).

3.1.1 Outline of Methodology

The epidemiological studies were undertaken to understand the prevalence and movement of the Oak Bleed Canker within Writtle Forest. Data was gathered to establish the persistence and spread of the signs of the bleed cankers on the Oak trees throughout the woodland.

This approach was based upon adapted, simplified landscape models of scale (Holdenrieder et al, 2004). The Gamma scale extents are considered as of the entire Writtle Forest woodland complex (and dictated by ownership and access). A transect was evolved and reviewed biannually over the 6year period. Information pertaining to the number of Oak trees with bleed canker were recorded, with each tree that had canker being tagged to monitor the progression of the bleed. (Fig 3.1)

The initial transect was evolved from a general sweep of the woodland. This identified 12 symptomatic trees. Ten of these trees were chosen from across the woodland complex that were thought to encompass a broad variability of micro-topography, soil type, management systems and companion tree species. (Fig. 3.2).

The Beta scale consideration focused on a section of 1 hectare woodland predominantly planted with Oak and exhibiting extensive signs of the Bleed Canker. The aim of the beta scale study was to consider the host pathogen interaction, the localized spread of the pathogen as well as the mortality rate of symptomatic trees. (Fig. 3.5).

3.2 Gamma scale Epidemiological Study of Writtle Forest

The woodland of Writtle Forest is a mosaic of ancient semi-natural woodland of approximately 240 Hectares, covering an area of 14 square kilometres. A transect of the woodland was used by which to evaluate the extent and spread of the incidence of exudate on the Oak trees through the area over a period of six years (Peterken & Mountford, 1998). The transect was designed to encapsulate an area of between approximately 5% and 10% within each woodland with established Oak populations. This equates to approximately 6.6% of the total area of the woodland area, this is an area of approximately 15.825 Ha. (Table 3.1). The transect was taken in the same direction each time, toward the end of April beginning of May. All trees with signs of exudate observed on the main stem of the tree within or up to 5m of either side of the transect were included within the count. Trees outside of this zone were not considered due to the unreliability of visibility of the exudate beyond 5m from the transect path.

No other details were recorded from the transect other than the presence of exudate on the tree. Figure 3.1, shows the position of the trees noted with signs of exudate. The circles

represent the area of the trees and are not to scale. Where the circles overlap this represents persistent bleed on the same tree over a six-year period.

3.2.1 Perceivable Infection rates of Bleed Canker over 6years along Transect

It can be seen (Table 3.1) that along the transect the number of symptomatic trees increased from 12 in 2012 to 37 in 2018. An increase of three times as many symptomatic trees.

The transect line accommodates both existent public footpaths and bridleways as well as areas through the woodland with no established path. The concentration of persistent and increasing incident of the bleed cankers is evident in areas of established footpaths.

It was also evident from the transect that the bleed canker persisted over a number of years on the same tree. Where such bleed occurred on trees it was evident that the incidence of bleed canker in that area of woodland was prevalent.

Table 3.1 Size of woodland areas within Writtle Forest and area of transect

Woodland	**Size of woodland (Hectares)**	**Length of transect (Metres)**	**Size of woodland metre squared**	**metres squared transect (5m to either side of the transect)**	**Percentage area of woodland**	**Number of trees with exudate 2012**	**Number of trees with exudate 2015**	**Number of trees with exudate 2018**
Little Edney wood	15.128 Ha	980m	151280m^2	9800m^2	6.5%	0	1	1
Great Edney Wood	6.349 Ha	660m	63490m^2	6600m^2	10.4%	0	1	1
Chelmsford Hill	5.290 Ha	425m	52900m^2	4250m^2	8.0%	2	4	4
Writtle Park Wood	24.177 Ha	2180m	241770m^2	21800m^2	9.0%	1	3	1
Park Ponds Wood	3.009 Ha	300m	30090m^2	3000m^2	10.0%	0	0	0
Ellis Wood	24.387 Ha	1950m	243870m^2	19500m^2	8.0%	2	7	9
Chalk Hill	7.439 Ha	525m	74390m^2	5250m^2	7.1%	2	3	3
Deerslade Wood	15.993 Ha	940m	159930m^2	9400 m^2	5.9%	1	2	3
Stoneymore Wood	33.743 Ha	1950m	337430m^2	19500 m^2	5.8%	0	4	5
Birch Spring	43.215 Ha	2850m	432150m^2	28500 m^2	6.6%	2	5	8
Barrow Wood	32.486 Ha	1475m	324860m^2	14750 m^2	4.5%	1	2	2
Parsons Spring	29.079 Ha	1590m	290790m^2	15900 m^2	5.5%	1	1	0
Totals	**240.295 Ha**	**15825m**	**2402950m^2**	**158250m^2**	**6.6%**	**12**	**33**	**37**

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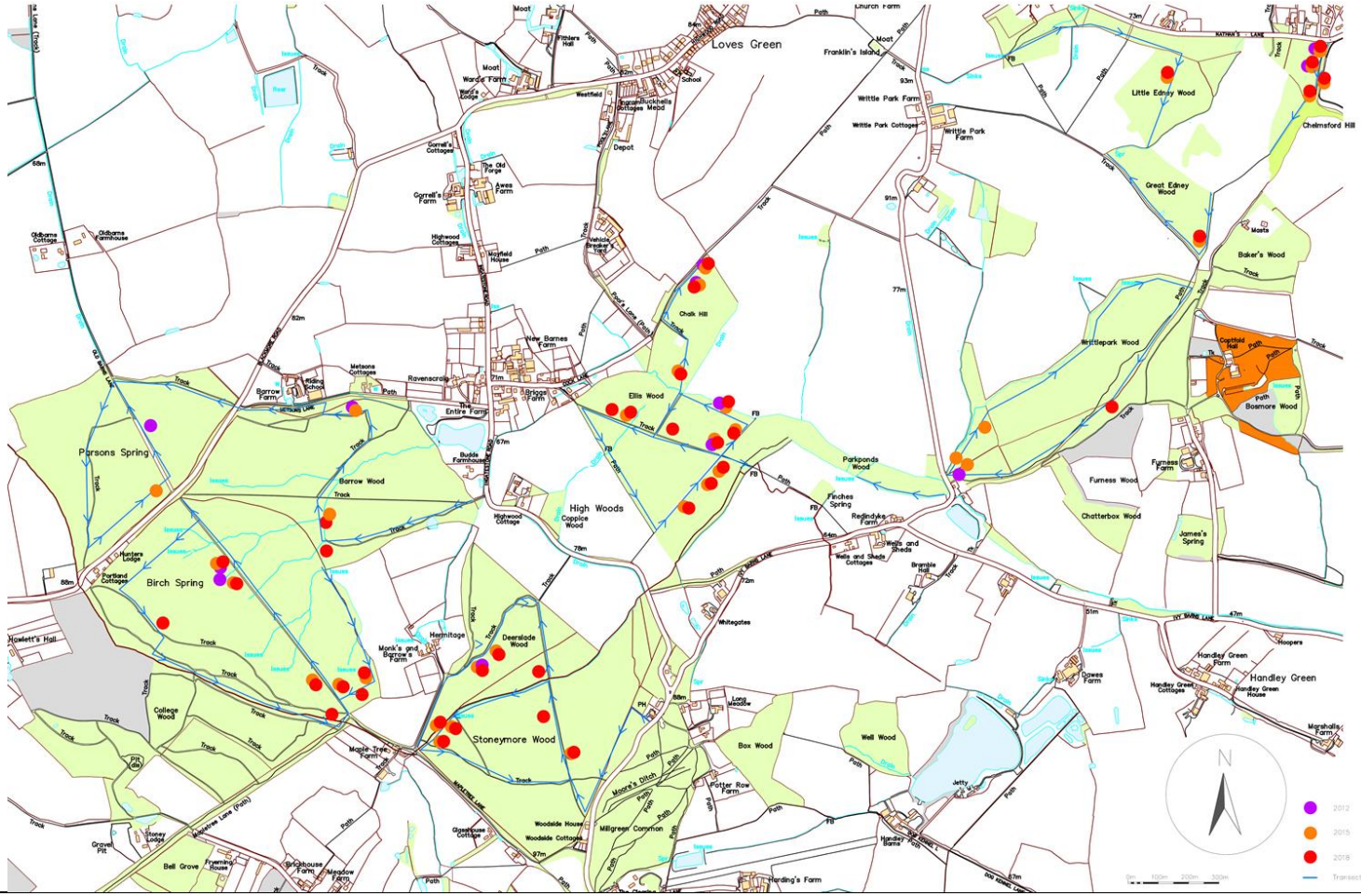


Fig. 3.1: Map showing Transect of Writtle Forest and discoverable Oak trees exhibiting signs of exudate between 2012 and 2018. Blue lines show the transect path. Coloured circles indicate trees displaying exudates in different years: purple (2012), orange (2015), red (2018) – Gamma scale.

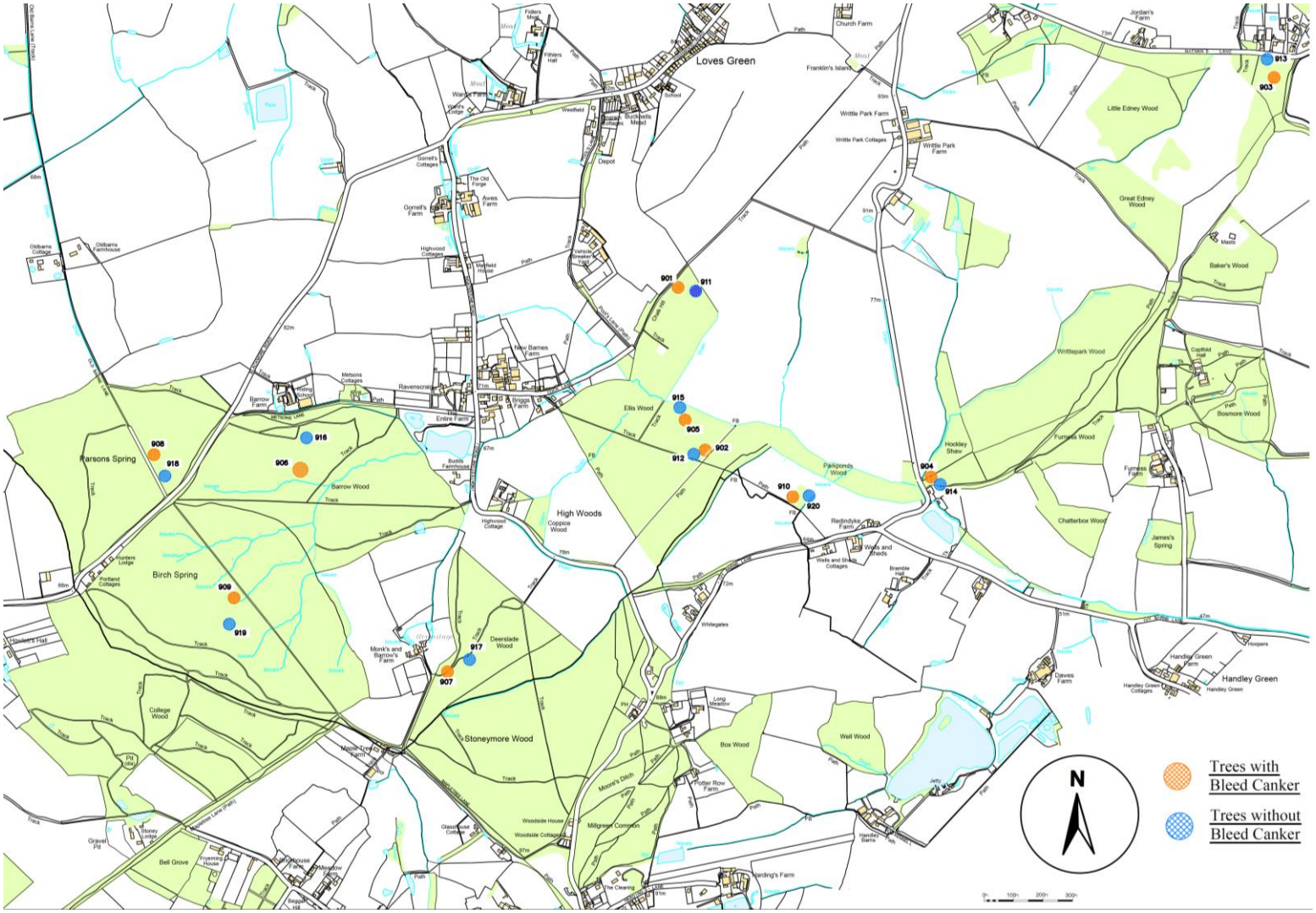


Fig. 3.2 The position of individual symptomatic and non-symptomatic trees throughout Writtle Forest

3.2.2 Ten original symptomatic and non -symptomatic Trees along Transect

Along with gathering samples of wood tissue and soils, data was collected relating to the original ten discoverable symptomatic trees and the related ten non -symptomatic trees. (Fig 3.2). This pertained to the number of bleed cankers, the presence of the Agrilus as well as whether the tree was alive or dead.

The data in Fig 3.3 shows the following information pertaining to the 10 symptomatic trees and 10 Non-symptomatic trees collected over the 6year period. Four of the symptomatic trees died during the period between 2012 and 2018, All four trees exhibited signs of Agrilus and bleed in 2012.

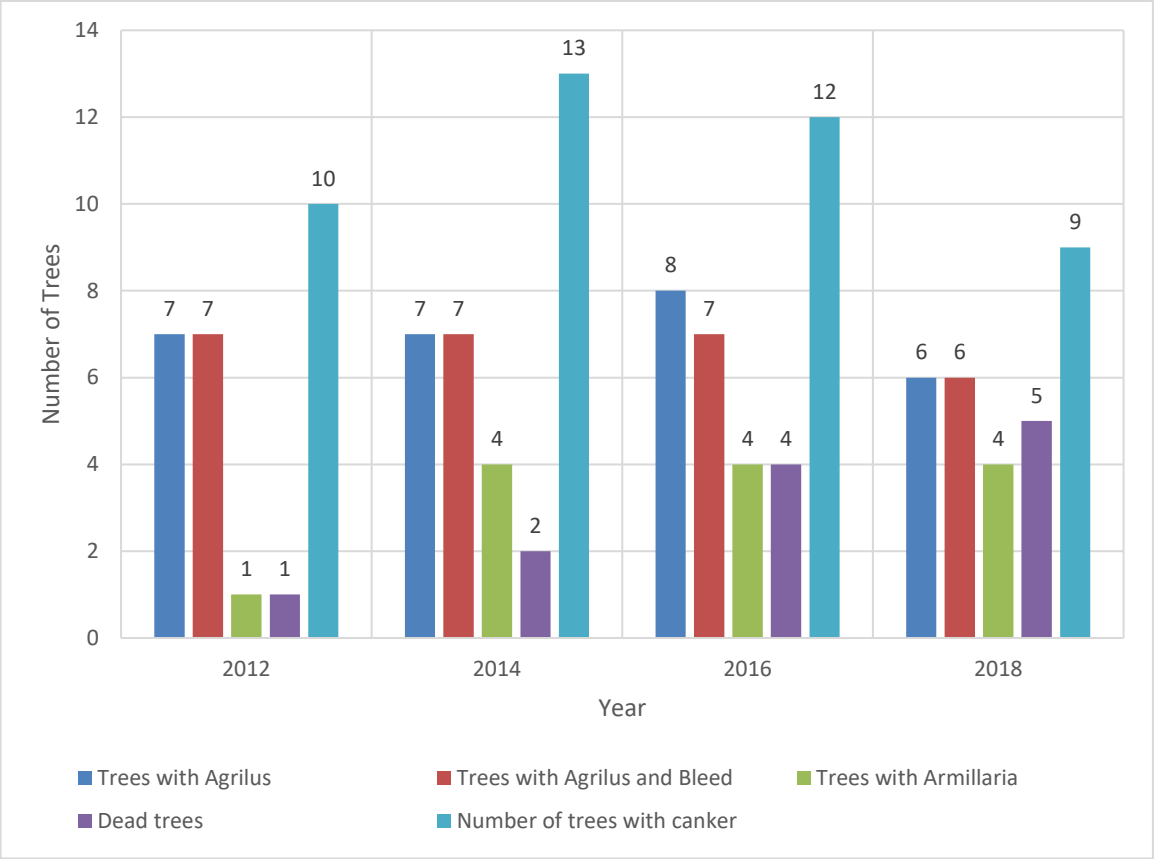


Fig. 3.3: Key observations of 20 trees spread through the Writtle Forest complex.

The progression of the canker on those trees that were alive in 2018, showed a downward trend of cankers with those trees that had previously had the bleed canker. However, three non -symptomatic trees developed bleed symptoms during the 6year period. The incidence of bleed was relatively minor and two trees had recovered within the 6year period. One tree exhibited progressive bleed symptoms that were still current at the end of the study.

One symptomatic tree that died later suffered from windthrow. This meant that data relating to bleed and *Agrilus* were not readily obtainable. It must also be considered that once all vascular conducting tissue has ceased functioning the bleed dries and the exudation ceases. (Fig 3.4)

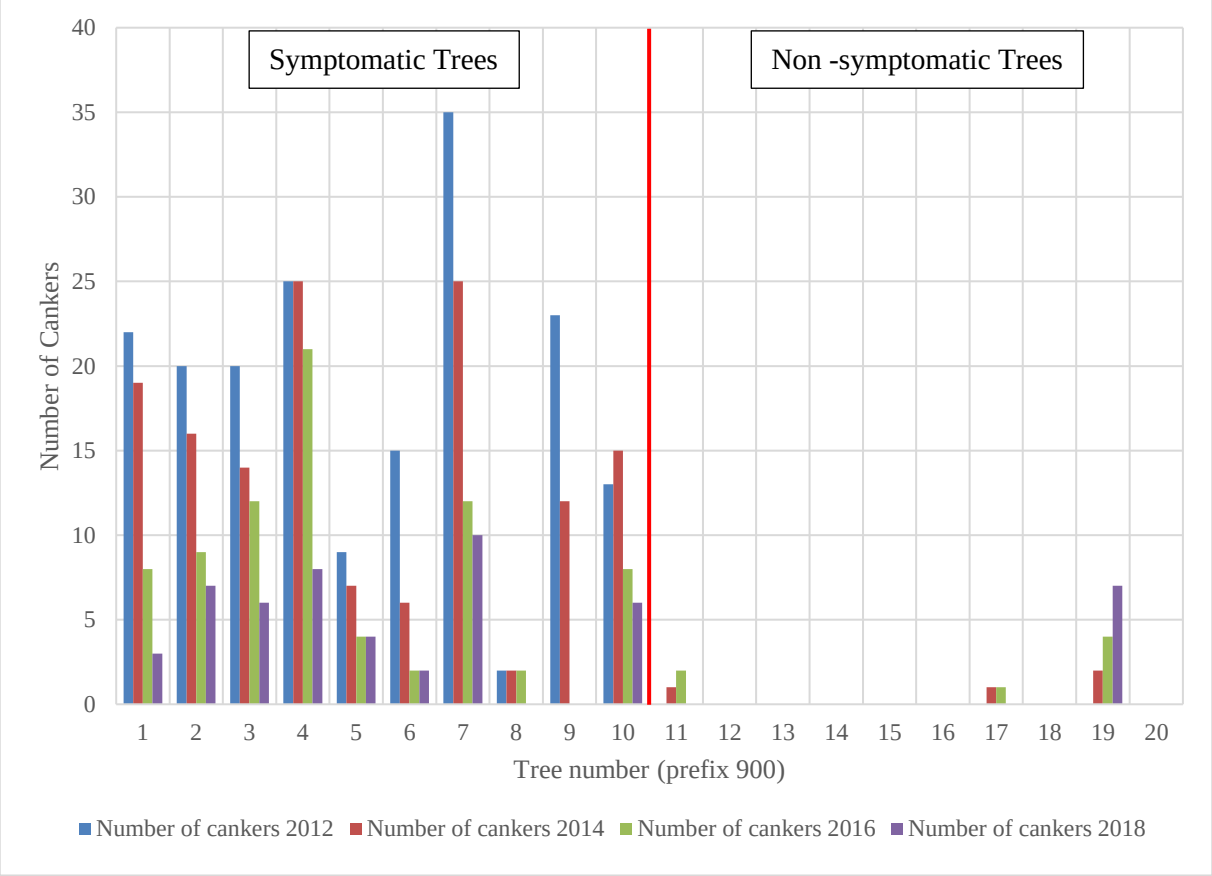


Fig. 3.4: The progression of bleeding cankers on 20 trees spread through Writtle Forest over six years.

3.3 Epidemiological Study of 1 Hectare Oak Woodland – Beta Scale

3.3.1 Area of Study

The Gamma scale considerations are relatively brief and consider 1) the incidence of the symptoms on trees throughout the woodland, 2) provide the basis of general sampling of Oak trees through the woodland and 3) provide facility to consider nutrient deficiencies in soils as a possible pre-disposing factor to the disease event. The beta scale analysis of 1 hectare of woodland is chosen to look at knowledge gaps relating to 1) the relationship of the bleed canker to the Agrilus beetle, 2) the mortality of the trees consequent to bleed symptoms, 3) the spread of the disease within a defined area. These considerations are easier to record over 6years when all the trees within the area can be mapped. Whilst the data set is limited the model over a 6year period provides sufficient evidence of a basic understanding of the disease cycle, such as to conclude basic management guidance. Further, that the trees are within the same area of woodland and subject to similar weather and soil conditions, reduces the variable differences between trees, enabling direct analysis of tree ring growth between trees that have experienced the disease event in different ways (Chapter 5).

For the beta scale a topographical survey of the area was carried out mapping 192 Oak trees. The trees were tagged with numbered aluminum tags. All of the Oak trees were identified as *Quercus robur*, distinguished by the leaf petiole.

3.3.2 Data Collection

Tree data were collected to enable baseline data by which to monitor spread of the canker on the trees over time. Three time points in 2013, 2016 and 2019 were considered to collect data, (a year after each transect date). All data were collected between the last week in April and the first week in May.

The estimated age and age categorisation of the tree is based predominantly on the stem diameter taken at 1.5m from ground level. This applies the consideration that every 12.5mm in circumference equates to approximately 1year of growth for forest grown trees (Mitchell, 1976). This is measured at the stem diameter defined as breast height (DBH), which is 1.5m from ground level.

Trees with a stem diameter of less than 300mm DBH are categorised as Young trees, this is those trees up to an estimated age of 75years old. Trees with a stem diameter of 300mm to 500mm DBH are categorized as Semi-mature trees with an age range of 75years to 125years. Trees with a stem diameter of 500mm to 700mm DBH are categorized as Early mature trees, with an age range of 125years to 175years. Trees with a stem diameter of 700mm to 900mm DBH are categorized as Mature trees, with an age range of 175years to 250years. Trees with a stem diameter of over 900mm DBH are categorized as Over mature trees, with an age exceeding 250years.

The life expectancy of an Oak tree is varied and dependent on a wide range of factors. The categorization of the age of an Oak tree may be based on the safe useful life expectancy of the tree, the ecological benefit the tree provides or the cultural significance within the landscape. The age categorization in this instance, is based on the useful function of the harvested tree for purposes of timber production.

Generally, the ground level was even to all compass points around the trees. Where there are uneven ground levels then the measurement of 1.5m was taken at the highest ground level. The measurement was taken using a general diameter tape.

The height of each tree was estimated, based on measurement of ten of the trees within the plot taken, with a Haglof CI Electronic Clinometer. The crown spread of the trees was taken either from west to east or south to north, whichever gave the better representation of the spread of the crown diameter.

The percentage of deadwood within the crown was based on the amount of deadwood in proportion to the remaining crown. This was categorised as following: between 10 and 15% deadwood/ crown transparency within the crown was categorised as Normal. Between 15 and 45% deadwood/ crown transparency was categorised as Above Normal. Between 45 and 85% deadwood/ crown transparency was categorised as Excessive. Between 85 and 100% deadwood/ crown transparency was categorised as Dead. These categorisations are based upon assessment categories as developed by Innes (Innes, 1993) and Roloff (Roloff, 1989).

The bleed cankers are often hard to see in certain light or weather conditions. A number of different pieces of information were gathered on the cankers. 1) the number of cankers on the main stem up to a height of 3m. Above this height the identification of cankers was less reliable. 2) whether the cankers were fresh or old. This was generally determined by the amount of visible exudate at the time of the survey. 3) the number of cankers that were identified as having developed visible callous tissue that occluded the wound. 4) the main orientation of the cankers on the stem given as compass points. 5) the most reliably visible canker highest on the main stem. The total number of cankers considered to be present at the time of the survey was arrived at by adding the number of recent and old cankers together.

However, those cankers that had begun to form callous tissue, unless exhibiting signs of recent exudation, were not included in the overall number of cankers counted.

Other data as to the health of the trees was taken. This included whether there was any evidence of *Agrilus biguttatus*, which has been postulated as a potential primary vector. This was based purely on whether there were visible 'D' shaped exit holes on the main stem (see Fig.10). Previous or historic wounding was also recorded both to the main stem or the crown of the tree. Evidence of other known pathogens were also recorded, particularly the presence of *Armillaria sp.*

The base line data for years 2013, 2016 and 2019 are detailed within the Appendices. The DBH, height and crown spread readings were only taken as of the first data collection exercise in 2013. During the study 25 trees were used to trial varying soil ameliorants. These trees were generally excluded from results comparing the condition of the trees over the six-year period.

3.3.3 Plotting of Trees and creation of maps

The trees were plotted using Leica TS16 total station with the resultant data processed by applications in CAD n4ce software. All maps were created in Auto CAD 2013 as .dwg files and exported as PDF files. The PDF image was then enlarged to 400% and the snapshot facility in the edit tools selected to copy and paste the map into the body of the text. (Fig 3.5).

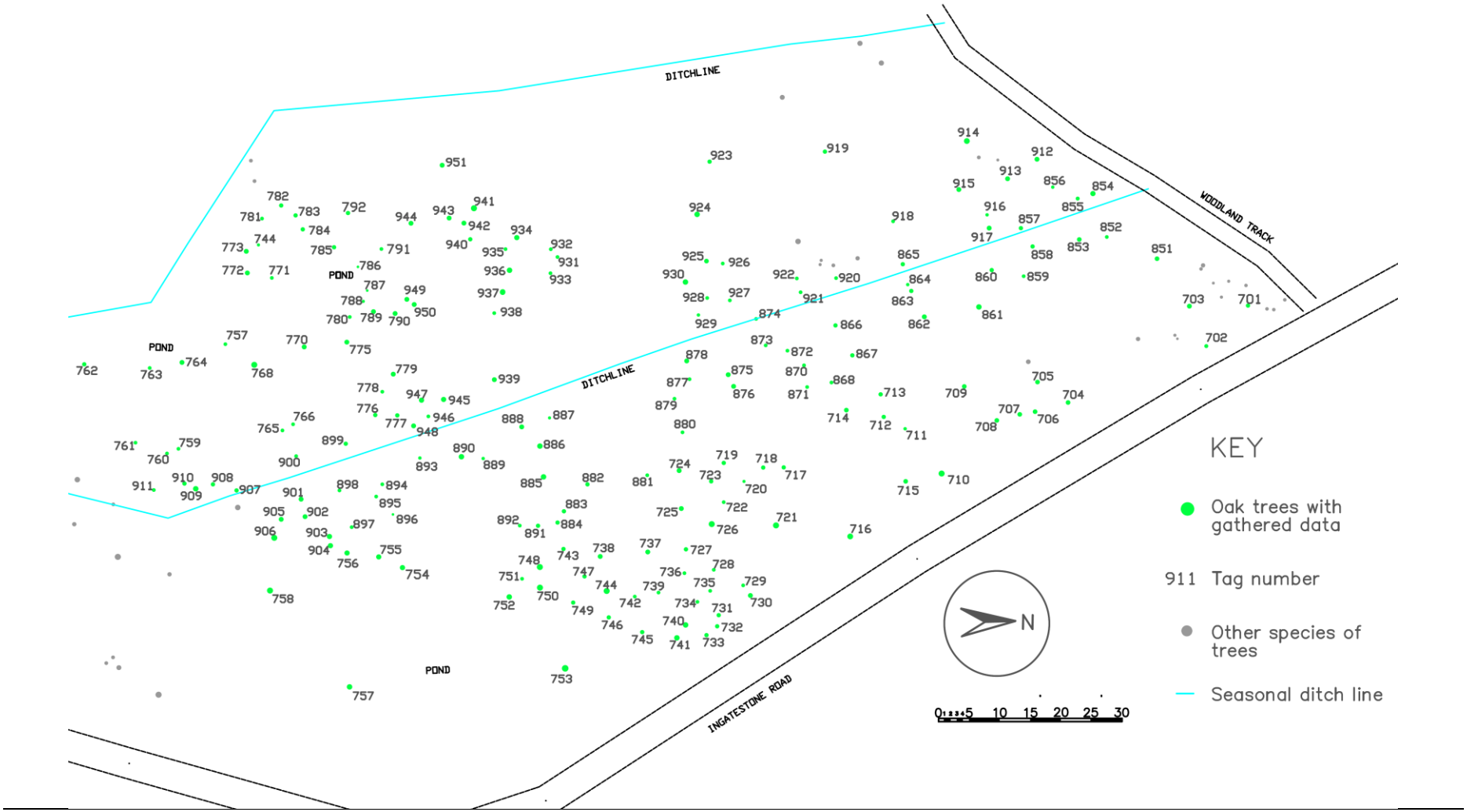


Fig 3.5: Map showing 1 Hectare of Writtle Forest (Compartment 15B Stoneymore Wood) with 192 mapped Oak trees – Beta scale

3.3.4 Data relating to Exudate on Oak Cohort

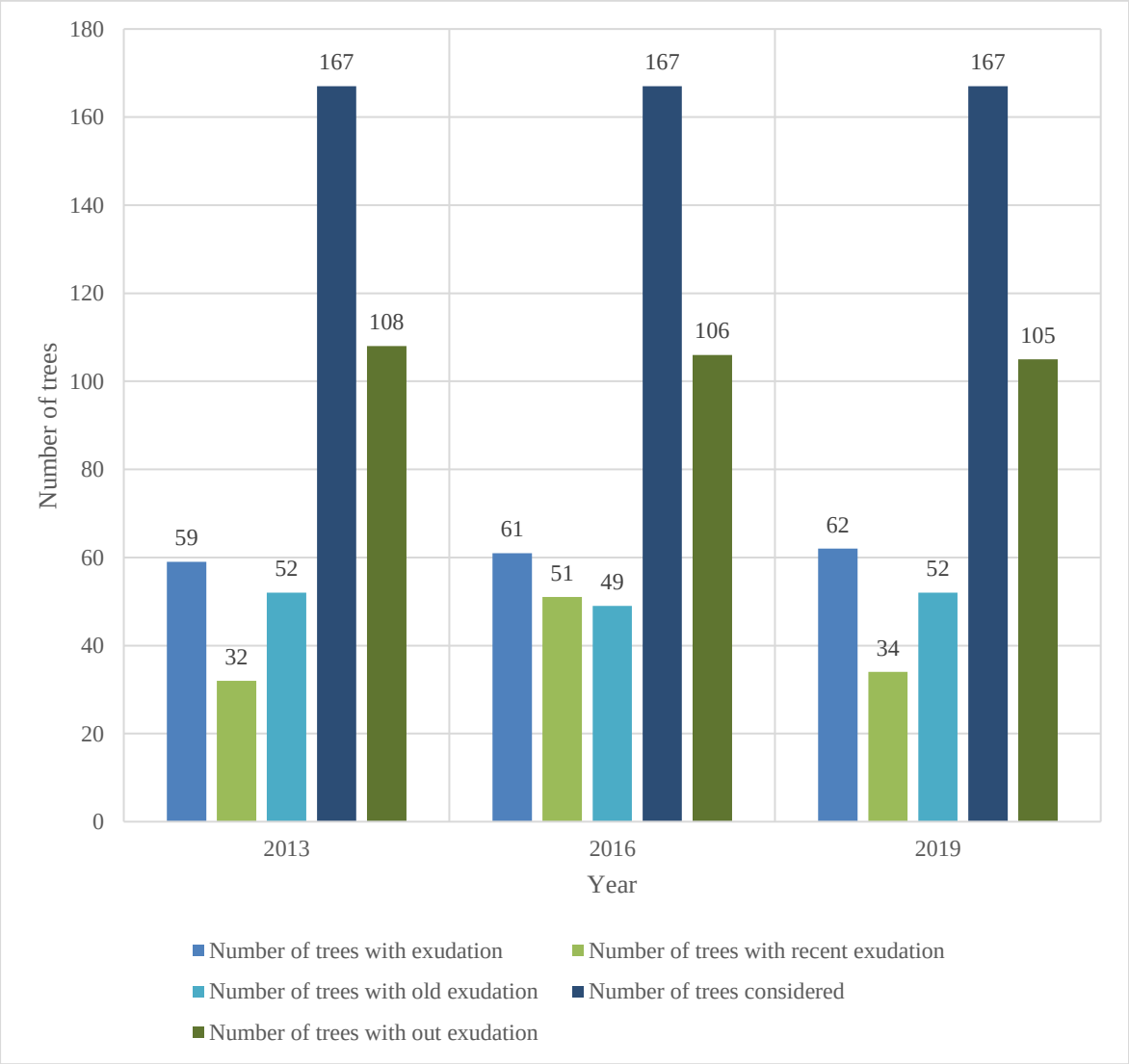


Fig. 3.6: Trees with Bleed Exudate in Stoneymore Woodland 2013 -2019.

The number of trees that exhibited bleed/exudation within the area when the survey was initiated in 2013 amounted to 35.33% of the total number of trees included within the survey data (Fig. 3.6). After a period of six years this percentage shows an increase in the bleed symptoms by 1.79% to 37.12%.

With Chi – square analysis of this data the following null hypotheses were considered;

1) There is no significant increase in trees with exudate over time.

Table 3.2: Chi -squared analysis of the null hypothesis that there is no significant increase in trees with exudate over time.

category	observed	expected		p-value
2013	59	60.67		
2016	61	60.67		
2019	62	60.67		0.96227

The p-value in this instance is significantly large enough that the null hypothesis is accepted and that there is no significant increase in the number of trees with exudate over the period of 2013 to 2019.

2) There is no significant increase in the number of trees with no exudate over time.

Table 3.3: Chi -squared analysis of the null hypothesis that there is no significant increase in trees with no exudate over time.

category	observed	expected		p-value
2013	108	106.33		
2016	106	106.33		
2019	105	106.33		0.978295

Again the p-value in is significantly large enough that the null hypothesis is accepted and that there is no significant increase in the number of trees with no exudate over the period of 2013 to 2019.

3) There is no significant increase in the number of trees with recent exudate.

Table 3.4: Chi -squared analysis of the null hypothesis that there is no significant increase in incidence of recent exudate on trees over time.

category	observed	expected		p-value
2013	32	39		
2016	51	39		
2019	34	39		0.061123

In this instance the p-value is close to 0.05, the value at which the null hypothesis would be rejected. It is believed that there is a significant increase in the incidence of recent exudate over time.

The predominance of trees within the area ranged from 75years to 175years (Fig. 3.7). Semi mature trees aged between 75 to 125 years old constituted 55% of the cohort. Early mature trees, age range between 125 and 175years, constituted 36.5% of the Oak cohort. All trees were considered to be within the same age ranges over the six-year period.

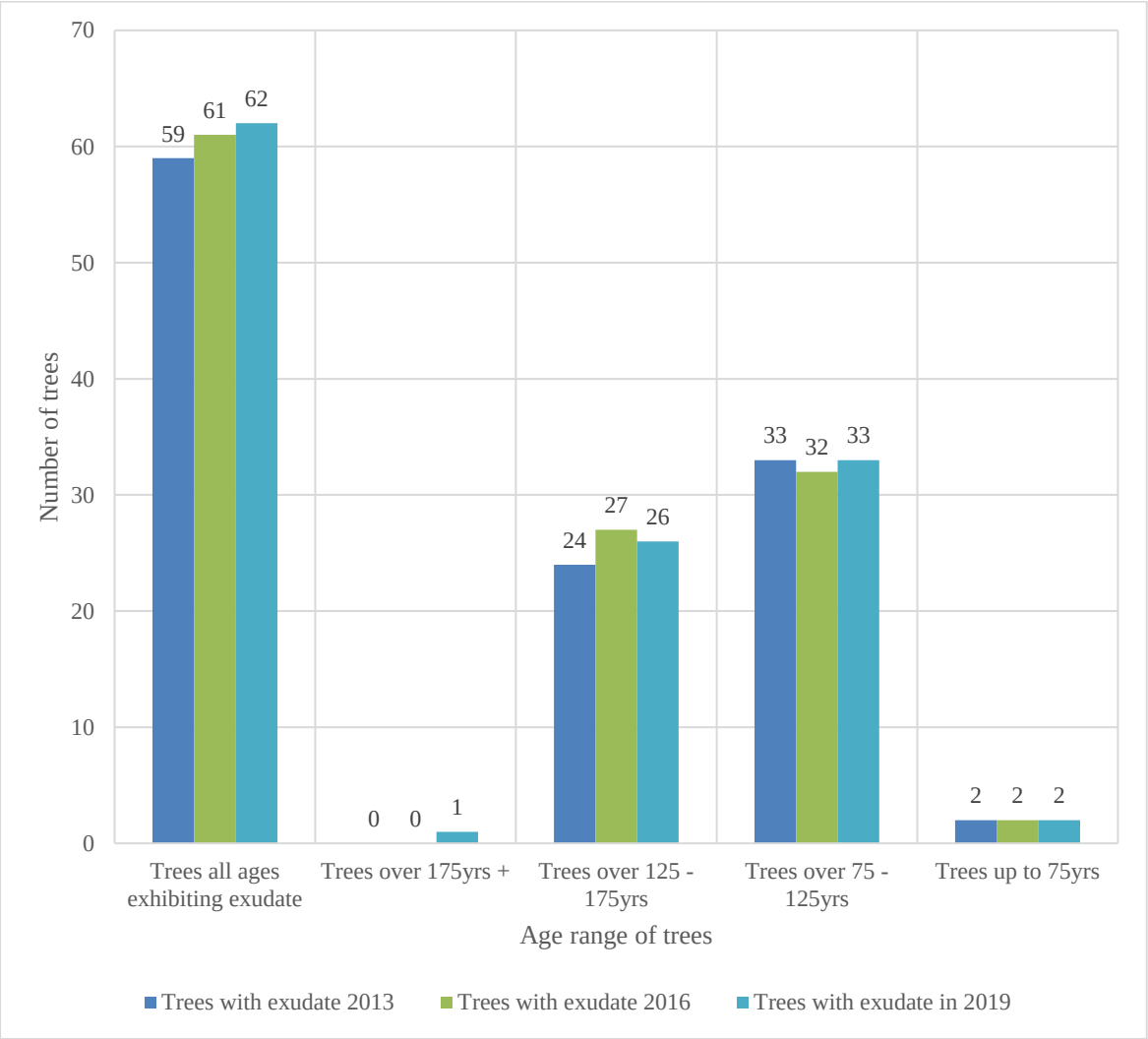


Fig. 3.7 Age range of Trees displaying exudate in Stoneymore Woodland 2013 -2019.

Trees aged between 125 and 175years old constituted 40-44% of the trees that were exhibiting signs of exudate. Trees aged between 75 and 125years old constituted 53-56% of the trees that were exhibiting signs of exudate.

3.3.5 Changes within the Oak Cohort

The trees that were windblown or failed had generally lost their bark. All signs of exudate and staining of the timber consequential to the exudate was not visible. These trees (20 in number) were not considered as part of the overall cohort with respect to bleed symptoms within the consideration of data collection within this section.

The variance in percentage of bleed overall within the area is generally of minor significance over a six-year period (Fig. 3.8). If it were that the bleed symptoms were to persist over a fifty-year period at this percentage rate, this would increase the percentage of trees affected by the condition to 15%.

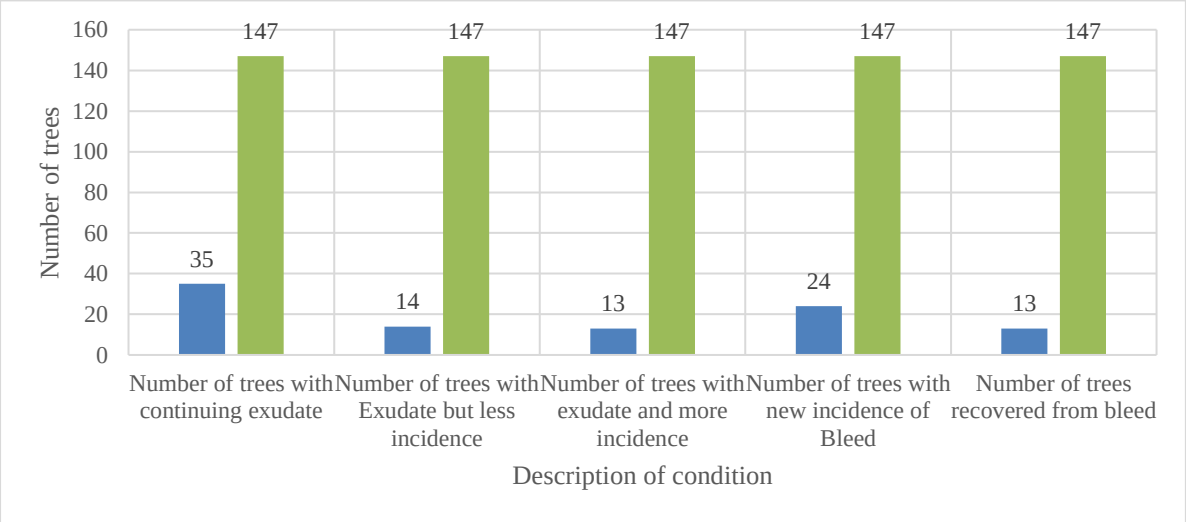


Fig. 3.8 Presence of exudate on Oak trees in Stoneymore Woodland 2013 -2019.

Green columns show the total number of trees considered. However, there is greater variance within the cohort over the 6year period, of the expression of the symptoms (Table 3.2).

Table 3.5: Explanation of percentage variance of exudates on Oak trees

Type of variance over a 6year period	Percentage of variance
Increase in number of trees exhibiting exudation from 2013 to 2019	1.79%. From 59 trees in 2013 to 62 in 2019, out of 147 trees, inclusive of dead trees.
Number of trees with continuing exudate from 2013 to 2019	59%. 35 trees, from the 59 trees exhibiting bleed in 2013
Number of trees with exudate but less incidence from 2013 to 2019	23.7% 14 trees, from 59 trees exhibiting bleed in 2013
Number of trees with exudate and more incidence from 2013 to 2019	22% 13 trees, from 59 trees exhibiting bleed in 2013
Number of trees with new incidence of bleed from 2013 to 2019	27.3% Out of 88 trees not exhibiting bleed in 2013, 24 trees are now exhibiting bleed
Number of trees recovered from bleed from 2013 to 2019	22% 13 trees, from 59 trees exhibiting bleed in 2013

When expressed as percentages of the overall population the variance of movement of the symptoms within the population considered is far greater than the increase in incidence of trees exhibiting bleed symptoms.

The change within a six-year period, between trees exhibiting new signs of bleed/ exudation (27.3%) was far greater than the general overall increase of bleed symptoms within the cohort (1.79%).

3.3.6 Data relating to Tree Mortality

The number of dead trees within the area increased by approximately 4.4% over the six-year period (Fig. 3.9). (This is an increase of 8 tree deaths within a 6year time frame from a cohort of 180 live trees).

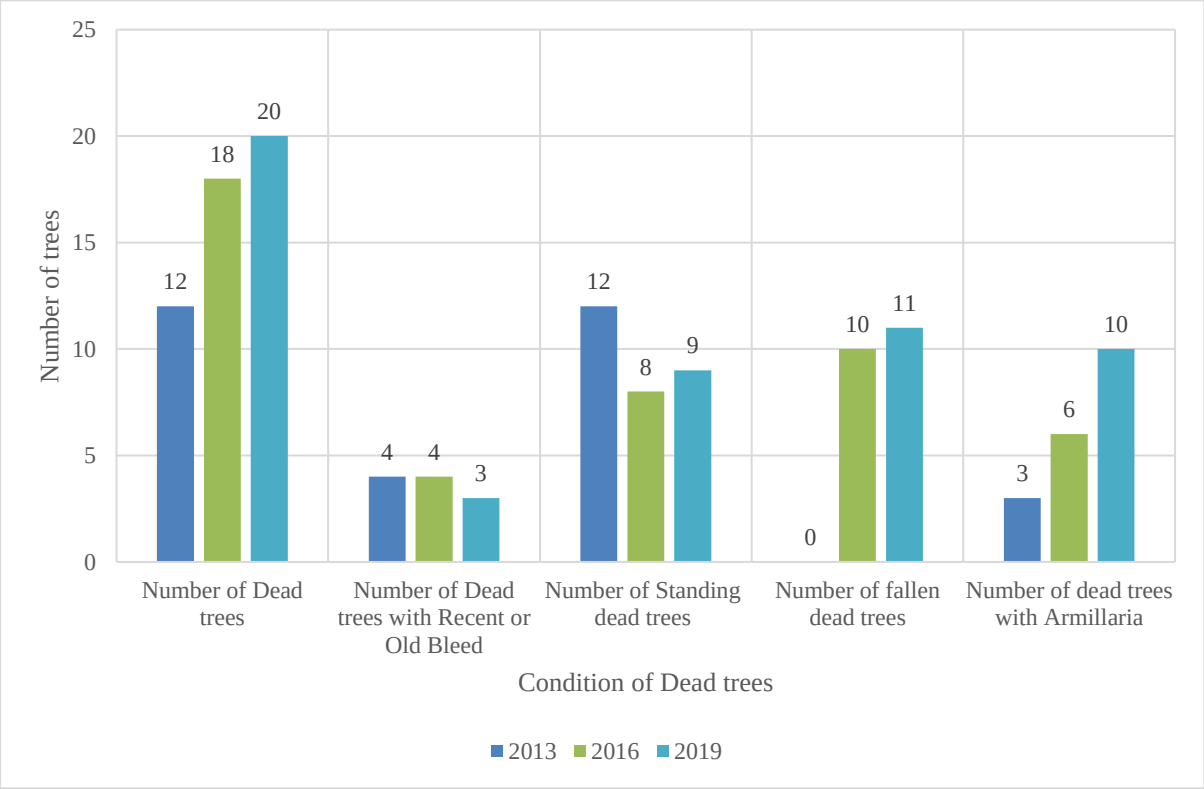


Fig. 3.9 Information pertaining to dead trees in Stoneymore Woodland 2013 -2019.

From the data collected it was not possible to ascertain exactly why individual trees had died. Seven of the eight trees that died between 2013 and 2019 were alive in 2013 with varying crown conditions and exhibiting a varying number of exudates on the main stem. By 2016 five of these trees were dead. *Armillaria mellea* was evident on four of these trees. This was identified through evidence of the mycelium sheets and typical 'bootlace' fungi found beneath the bark of standing trees and to the roots of fallen trees. (The fruiting bodies of this fungus are not evident until the summer months). Thus, these observations indicate some trees exhibited

symptoms of exudates (cankers) and also *Armillaria* infection. From the data in Table 3.3. it can be seen that 3 of the seven trees had 10 cankers or more.

Table 3.6: Observations between dead trees, *Armillaria* and number of bleed cankers.

Tag no.	Age	2013 Deadwood	Number of cankers	2016 Deadwood	Number of cankers	2019 Deadwood	Number of cankers	Comments
709	EM	Excessive	35	Dead	35	Dead	35	Standing dead
738	SM	Above normal	5	Dead	2	Dead	0	Standing Dead, Agrilus and Armillaria
745	SM	Above normal	1	Excessive	0	Dead	0	Fallen - Root decay - Probable Armillaria
749	SM	Excessive	5	Dead	5	Dead	3	Standing Dead, (Agrilus)
788	SM	Normal	2	Dead	0	Dead	0	Standing stem to height of 5m
892	SM	Above normal	10	Dead	0	Dead	0	Wind- blown dead tree – Armillaria
894	SM	Excessive	10	Excessive	4	Dead	0	Standing dead – Armillaria

With Chi – square analysis of the data as shown within Fig 3.9 the following null hypotheses were considered;

1) That there is no significant increase in dead trees over time.

Table 3.7: Chi -squared analysis of the null hypothesis that there is no significant increase in dead trees over time.

category	observed	expected		p-value
2013	12	16.67		
2016	18	16.67		
2019	20	16.67		0.35352784

The p-value in this instance is large enough such that the null hypothesis cannot be rejected and that there is no significant increase in the number of dead trees over the period of 2013 to 2019.

2) There is no significant increase in dead trees with *Armillaria*.

Table 3.8: Chi -squared analysis of the null hypothesis that there is no significant increase in dead trees with *Armillaria*.

category	observed	expected		p-value
2013	3	6.33		
2016	6	6.33		
2019	10	6.33		0.142502388

The p-value in this instance is greater than 0.05. As such the null hypothesis cannot be rejected and there is no significant increase in the number of dead trees with *Armillaria* over the period of 2013 to 2019.

3.3.7 Data relating to *Agrilus* and Exudate

Agrilus has been reported associated with the oak bleed symptoms (Brown et al, 2014). The woodland area considered in this study found that the presence of *Agrilus* was generally low.

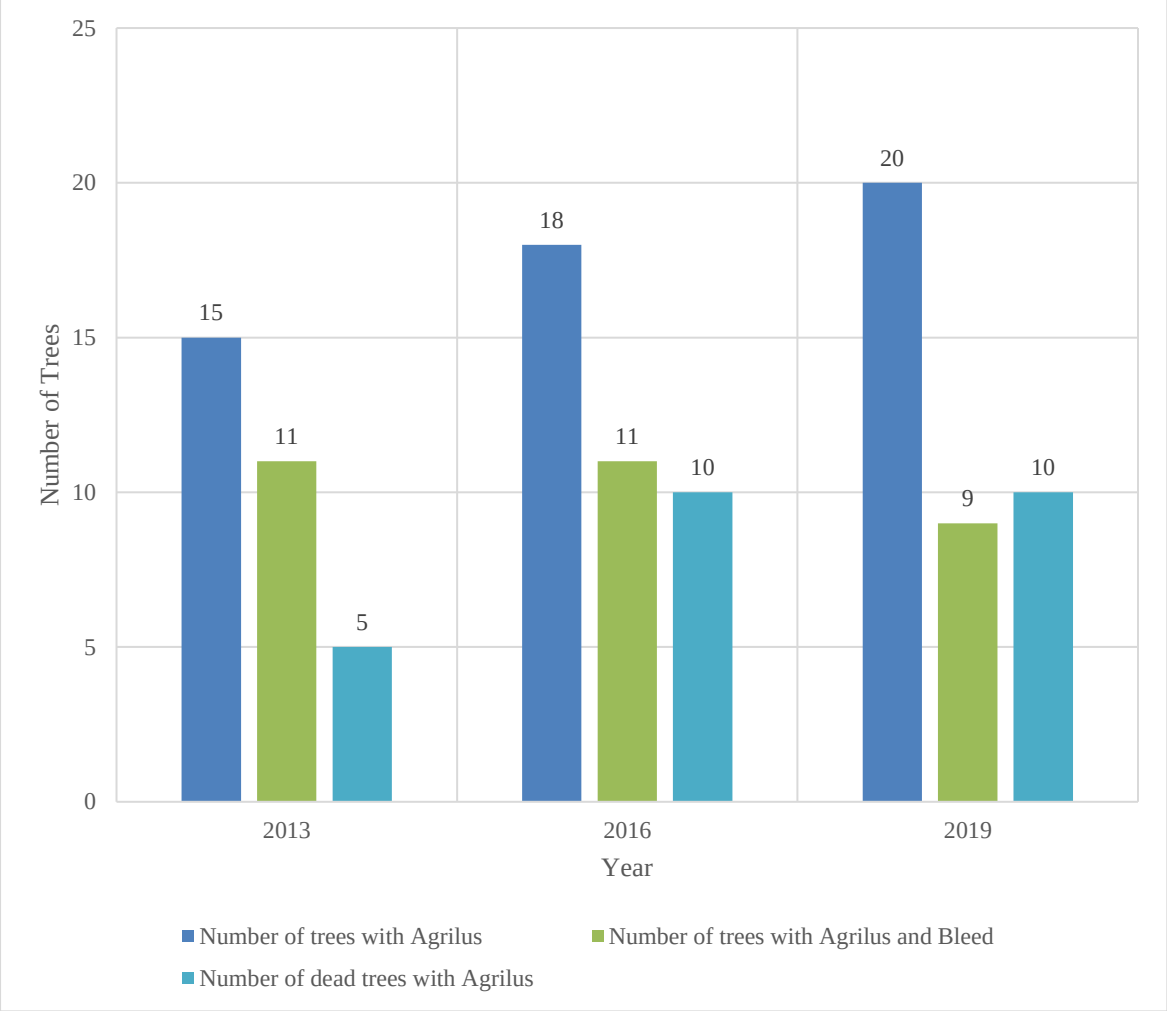


Fig. 3.10 Presence of *Agrilus* in Stoneymore Woodland 2013 -2019.

The percentage of trees with Agrilus in 2013 was 9% and this had increased to 12% by 2019. *Agrilus* can be detected on dead trees once the bark has fallen off, due to the larval galleries. However, on fallen trees these are not always observable as up to 50% of the stem can be hidden once lying on the ground.

3.4 Vectors of Movement for the Bacteria

Most bacterial species that spread through the environment will require a vector for movement. With plant bacteria the vectors are generally insects or water. (Agrios, 2005).

The Bleed canker can occur without the presence of the Agrilus beetle. The spread of the bleed canker between the trees was relatively high with 27% of the trees within the Oak cohort of Stoneymore wood showing new symptoms. The clustering of trees exhibiting signs of new bleed canker in 2019 closely related to those trees with recorded bleed canker in 2013. This would suggest that the mode of transference was related to movement of bacteria through either root connectivity or the soils environment. (Fig 3.11).

3.5 Summary of Findings

The number of trees with Oak bleed canker increased over the study period within the wider area of the Writtle Forest woodland complex increased by three times the amount of trees with visible bleed canker (from 12 trees to 37 trees -Table 3.1). However, the increase in the number of trees exhibiting signs of bleed canker within the densely clustered area of Oak in Stoneymore woodland (the 1 hectare Beta scale study), increased by only 1.79% over the same time period. Notably, the variance between those trees that had bleed canker in 2013 and those that had the bleed canker in 2019 was 27.3%. This was due to remission of the bleed symptoms on trees previously exhibiting signs of the bleed canker and the appearance of the bleed canker on other trees within the area of woodland.

The number of dead trees within the 1 Hectare area increased by 8 over the six-year period. (12 were previously logged as standing dead in 2013). Of a cohort of 180 live trees this represents an increase in dead trees of 4.44% over the six-year period, an annual mortality rate of 0.74%.

The pattern of trees exhibiting bleed symptoms within the 1 hectare of woodland suggests that the transmission is localised and hence transmission is either through the soils or through inter-connectivity of the root systems.

The percentage of trees that exhibited bleed canker within the 1 hectare area in 2013 amounted to 35.33%. Of these trees 9% were infected with *Agrilus*. By 2019 the percentage of trees that exhibited bleed canker increased by 1.79% to 37.12% of the Oak tree population within 1 hectare. Of the Oak trees with bleed symptoms in 2019 12% showed signs of *Agrilus* activity. The increase in *Agrilus* activity was greater than the increase in bleed symptoms. Given the proportionality of *Agrilus* to Bleed symptoms if there were a significant link between the two it would be expected that the increase in bleed symptoms would be far greater.

It appears that the Agrilus may act as a vector for transmission of the bleed canker but that from the study within the 1 hectare area of woodland the primary vector was more localised and most probably related to soils or roots.

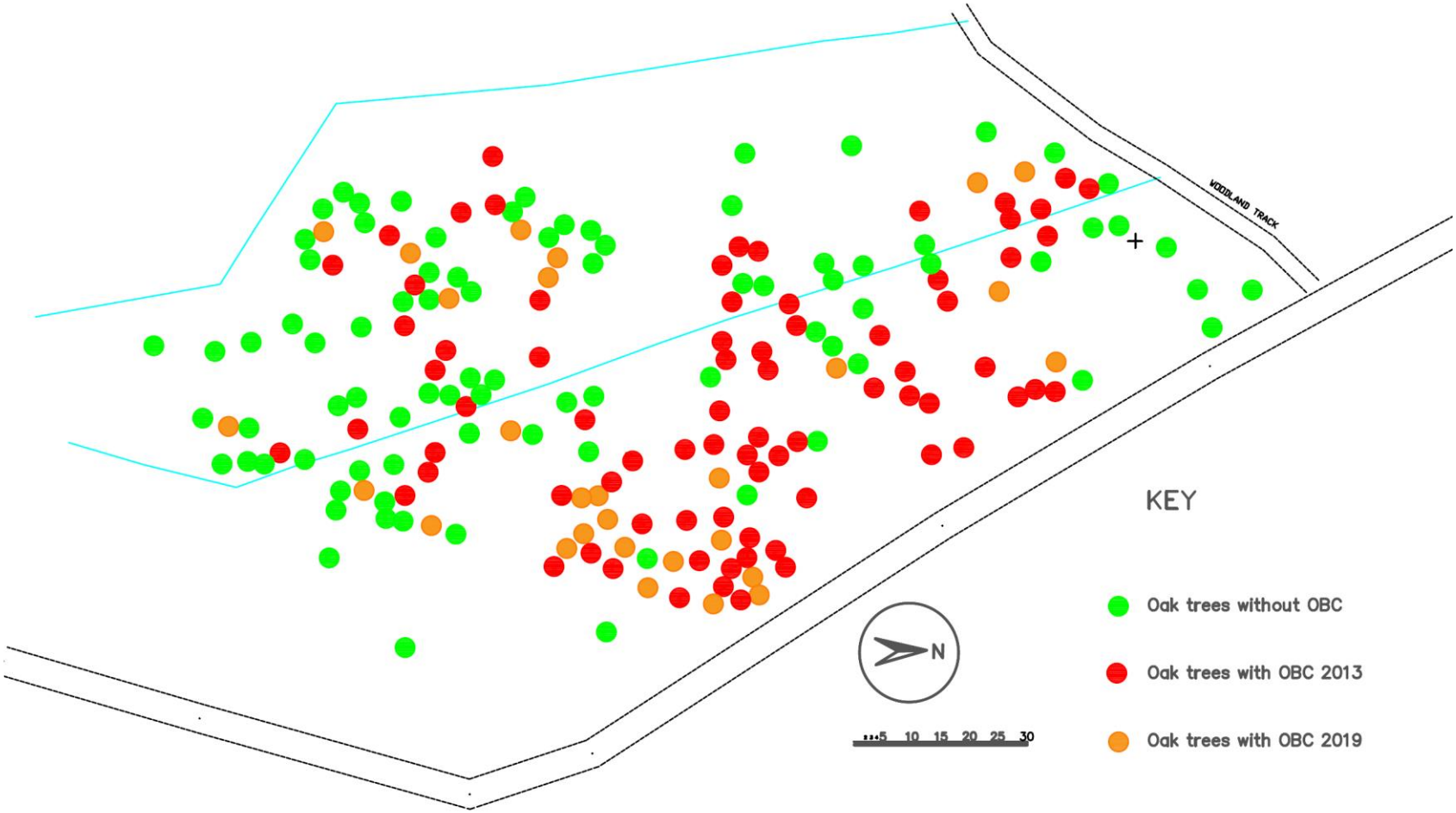


Fig 3.11: Map of Stoneymore wood depicting spread of OBC from 2013 to 2019. (Trees recorded with bleed in 2013 are shown regardless of remission in 2019).

Chapter 4: Results – Relationship of Soils to Oak Bleed Canker

4.1 Context of Research

As part of the study to establish whether there are any environmental factors that pre-dispose the trees to bleed cankers, the nutrient levels in the soils were considered. This was carried out throughout the woodland using the 10 symptomatic and 10 non symptomatic trees as identified, as reference. Consistent reduced levels of Calcium were discoverable and a trial test was carried out to establish the effects of increasing calcium levels within the soils as a means of possible management of the bleed canker.

4.2 Soils data collected from 10 symptomatic and 10 non-symptomatic throughout Writtle Forest woodland complex

From the original 10 symptomatic and corresponding 10 non -symptomatic trees throughout Writtle Forest woodland complex soils were collected from within 2.5m of the base of the tree, at a depth of 300mm below ground level and from the topsoil immediately below the leaf litter. The soils were sent for analysis to A&L Analytical Laboratories.

The results were analysed by Bartlett Tree Research Laboratories to consider deficiencies in comparison to those ranges regarded as best suited to *Quercus robur.* The ideal pH for *Quercus robur* is considered as between 5.0 and 6.5. Where the results are considered deficient the item is highlighted in red within Tables 4.1 -4.4 below.

(As of the tables below The Estimated Nitrogen Release (ENR) is an estimate of the amount of nitrogen that will be released over the season. In addition to organic matter level, ENR may be influenced by seasonal variations in weather conditions as well as physical soil conditions).

Consistent to all soils, regardless of the depth that the sample was taken or the whether the soils were from within the rooting zone of a symptomatic or non-symptomatic tree, was the high acidity levels and the deficiency in Calcium. Other nutrient deficiencies across the woodland were Magnesium, Phosphorus and Manganese.

Forest soils are generally regarded as of poor condition comparative to agronomic soils. This maybe either related to the fact that historically better soils have been adopted for agriculture and that the parameters generally defining soils are related to nutrient content comparable to the acceptable conditions of agronomic soils (Binkley and Fisher, 2013). The primary difference between forest soils and agronomic soils is the root establishment (through a large range of soil horizons) and the consequent development of dynamic mycorrhizal relationships. It is these unique relationships with mycorrhizae that benefits the trees, allowing, in the simplest of expressions, increased surface area for absorbing and utilising water and nutrients. The aim of the soil data was to look at comparisons between the soils within the rooting area of the symptomatic and non -symptomatic trees, to establish whether there were differences that would indicate a pre-disposition relating to the soils and the symptomatic trees.

Table 4.1 Properties of soil samples taken 2.5m from symptomatic trees between 50mm and 200mm below ground level

Tree Number	Soil pH	Nitrogen (ENR)	Phosphorous (P)	Potassium (K)	Magnesium (Mg)	Calcium (Ca)	Sodium (Na)	Iron (Fe)	Manganese (Mn)	Copper (Cu)	Zinc (Zn)	Boron (B)
T00901	4.5	756.0	40.0	224.0	214.0	1390.0	68.0	440.0	126.0	3.0	80.2	0.6
T00902	4.6	234.0	48.0	324.0	168.0	886.0	48.0	882.0	132.0	3.4	3.8	1.0
T00903	4.4	329.0	38.0	306.0	164.0	1032.0	58.0	802.0	128.0	3.8	5.6	1.4
T00904	4.3	140.0	18.0	342.0	164.0	738.0	66.0	606.0	62.0	2.4	29.2	0.6
T00905	4.7	742.0	88.0	334.0	302.0	1602.0	68.0	428.0	504.0	2.4	17.6	1.0
T00906	4.3	67.0	14.0	148.0	104.0	338.0	46.0	1112.0	18	1.8	3.4	0.8
T00907	4.0	681.0	30.0	146.0	108.0	686.0	46.0	368.0	30.0	3.0	6.6	0.6
T00908	4.5	146.0	22.0	460.0	138.0	544.0	50.0	1416.0	34.0	1.8	6.6	0.8
T00909	4.2	83.0	18.0	118.0	78.0	396.0	40.0	746.0	42.0	2.8	4.6	0.6
T00910	4.4	65.0	12.0	114.0	76.0	398.0	42.0	770.0	14.0	2.0	2.6	0.4

Table 4.2: Properties of soil samples taken 2.5m from symptomatic trees between 200mm and 350mm below ground level

Tree Number	Soil pH	Nitrogen (ENR)	Phosphorous (P)	Potassium (K)	Magnesium (Mg)	Calcium (Ca)	Sodium (Na)	Iron (Fe)	Manganese (Mn)	Copper (Cu)	Zinc (Zn)	Boron (B)
T00901	4.2	112.0	136.0	286.0	132.0	742.0	66.0	916.0	76.0	2.8	5.4	1.2
T00902	4.5	89.0	14.0	124.0	80.0	338.0	56.0	804.0	136.0	2.6	3.0	0.8
T00903	4.9	95.0	14.0	268.0	162.0	708.0	62.0	522.0	78.0	3.0	5.0	0.8
T00904	4.6	126.0	16.0	312.0	392.0	888.0	82.0	700.0	14.0	3.4	6.0	1.4
T00905	4.5	72.0	18.0	112.0	132.0	504.0	68.0	320.0	84.0	2.2	2.2	0.6
T00906	4.2	1198.0	42.0	158.0	190.0	1178.0	50.0	262.0	58.0	3.2	10.8	0.4
T00907	5.2	63.0	10.0	100.0	38.0	234.0	38.0	1382.0	6.0	1.4	2.2	1.2
T00908	3.9	110.0	30.0	240.0	128.0	666.0	60.0	860.0	22.0	4.4	3.4	0.8
T00909	4.8	2138.0	72.0	228.0	222.0	1558.0	52.0	264.0	90.0	3.0	13.2	0.6
T00910	4.7	1521.0	28.0	118.0	154.0	1490.0	52.0	468.0	128.0	3.6	16.0	0.6

Table 4.3: Properties of soil samples taken 2.5m from Non-symptomatic trees between 50mm and 200mm below ground level

Tree Number	Soil pH	Nitrogen (ENR)	Phosphorous (P)	Potassium (K)	Magnesium (Mg)	Calcium (Ca)	Sodium (Na)	Iron (Fe)	Manganese (Mn)	Copper (Cu)	Zinc (Zn)	Boron (B)
T00911	3.8	2273.0	34.0	262.0	188.0	1738.0	72.0	300.0	106.0	5.0	17.4	0.8
T00912	4.5	505.0	32.0	126.0	142.0	1160.0	62.0	814.0	70.0	5.8	18.2	0.6
T00913	4.0	627.0	44.0	202.0	198.0	1562.0	52.0	554.0	218.0	4.2	8.6	1.0
T00914	4.6	1521.0	30.0	286.0	252.0	1372.0	62.0	300.0	108.0	3.2	9.0	0.8
T00915	4.4	247.0	36.0	436.0	316.0	1616.0	94.0	804.0	178.0	5.6	32.0	1.2
T00916	3.7	2021.0	28.0	168.0	152.0	1242.0	64.0	410.0	166.0	3.6	11.4	0.6
T00917	3.9	2066.0	20.0	124.0	132.0	614.0	46.0	264.0	42.0	47.6	13.0	0.4
T00918	4.2	1783.0	48.0	116.0	186.0	1168.0	80.0	434.0	70.0	4.6	20.2	0.8
T00919	4.0	1097.0	22.0	98.0	114.0	672.0	64.0	688.0	246.0	4.4	6.0	0.8
T00920	4.6	171.0	34.0	100.0	182.0	608.0	62.0	542.0	158.0	3.0	9.2	0.6

Table 4.4: Properties of soil samples taken 2.5m from Non -symptomatic trees between 200mm and 350mm below ground level

Tree Number	Soil pH	Nitrogen (ENR)	Phosphorous (P)	Potassium (K)	Magnesium (Mg)	Calcium (Ca)	Sodium (Na)	Iron (Fe)	Manganese (Mn)	Copper (Cu)	Zinc (Zn)	Boron (B)
T00911	4.4	72.0	18.0	396.0	94.0	398.0	98.0	732.0	32.0	2.0	4.4	0.8
T00912	4.5	99.0	20.0	110.0	94.0	534.0	54.0	810.0	142.0	2.6	4.4	1.0
T00913	4.0	99.0	20.0	166.0	92.0	592.0	54.0	746.0	22.0	2.8	3.0	1.0
T00914	4.0	144.0	16.0	368.0	274.0	766.0	88.0	638.0	52.0	3.6	7.0	0.8
T00915	4.7	97.0	16.0	208.0	198.0	744.0	94.0	684.0	34.0	4.0	8.6	1.0
T00916	4.1	91.0	12.0	158.0	72.0	286.0	66.0	1366.0	26.0	3.2	3.0	1.0
T00917	3.9	72.0	10.0	166.0	60.0	288.0	60.0	1570.0	16.0	8.0	3.8	1.2
T00918	4.1	76.0	12.0	152.0	70.0	246.0	66.0	1474.0	10.0	2.4	2.0	1.2
T00919	4.5	59.0	12.0	170.0	60.0	268.0	84.0	456.0	134.0	3.2	2.8	0.4
T00920	4.6	72.0	18.0	110.0	184.0	582.0	104.0	506.0	44.0	3.4	5.6	0.6

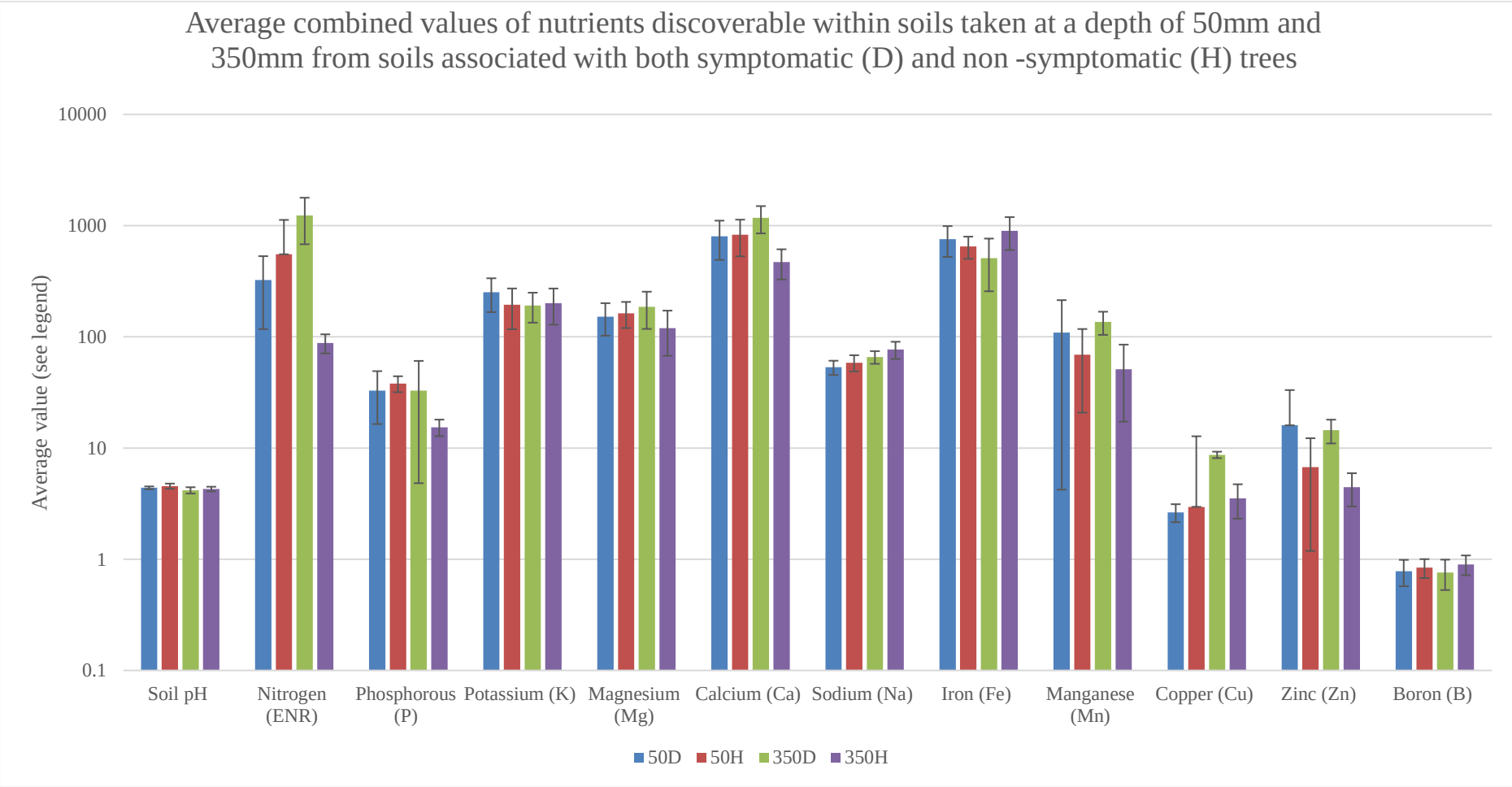


Fig. 4.1: Average combined values of nutrients discoverable within soils taken at a depth of 50mm and 350mm from soils associated with both symptomatic (D) and non -symptomatic (H) trees gathered around Writtle Forest. Error bars = 95% Confidence intervals

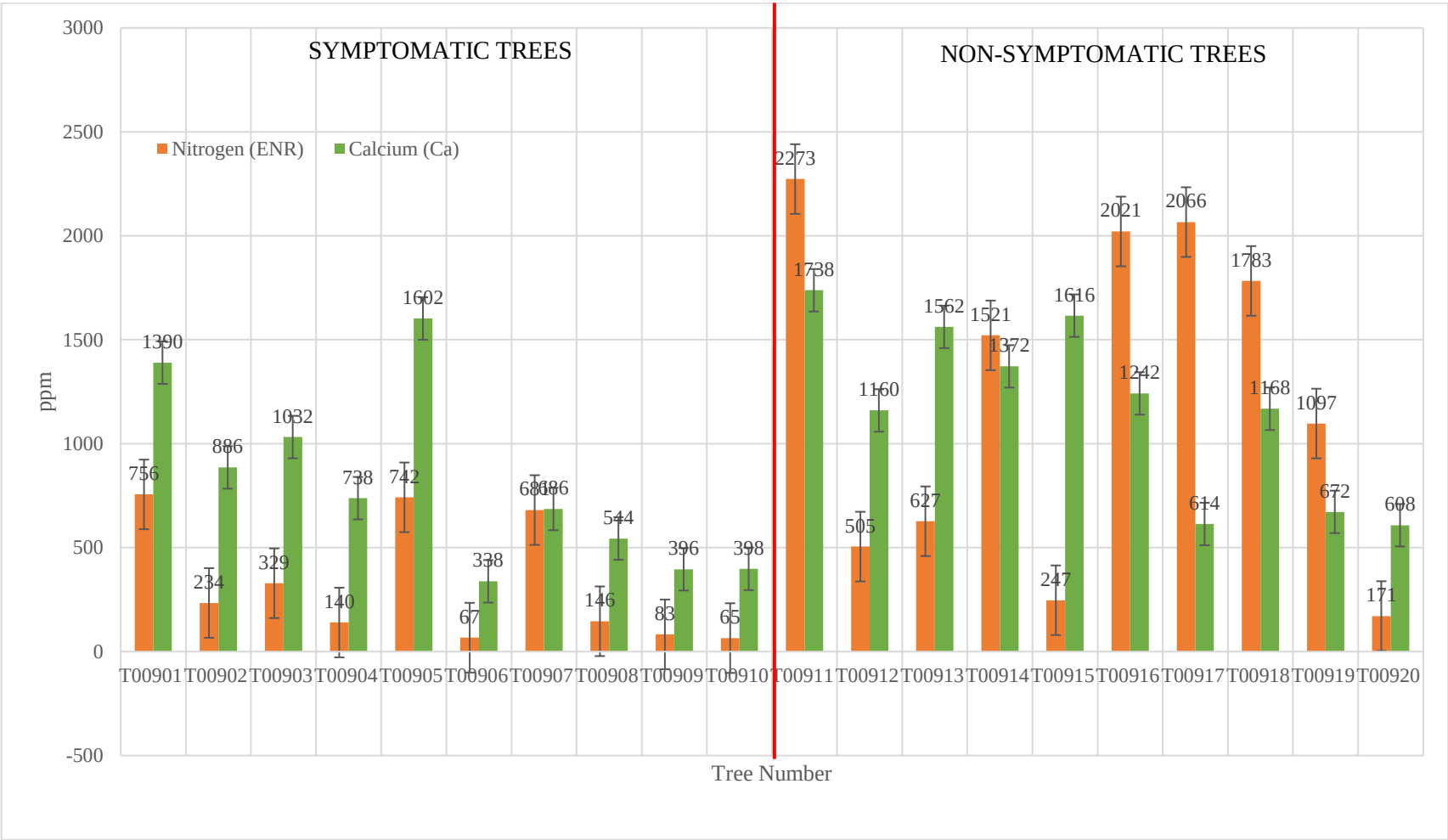


Fig. 4.2: Nitrogen and Calcium concentrations of 50 -200mm soils of symptomatic (T00901-10) and non-symptomatic (T00911-20) trees gathered around Writtle Forest. Error bars = standard error

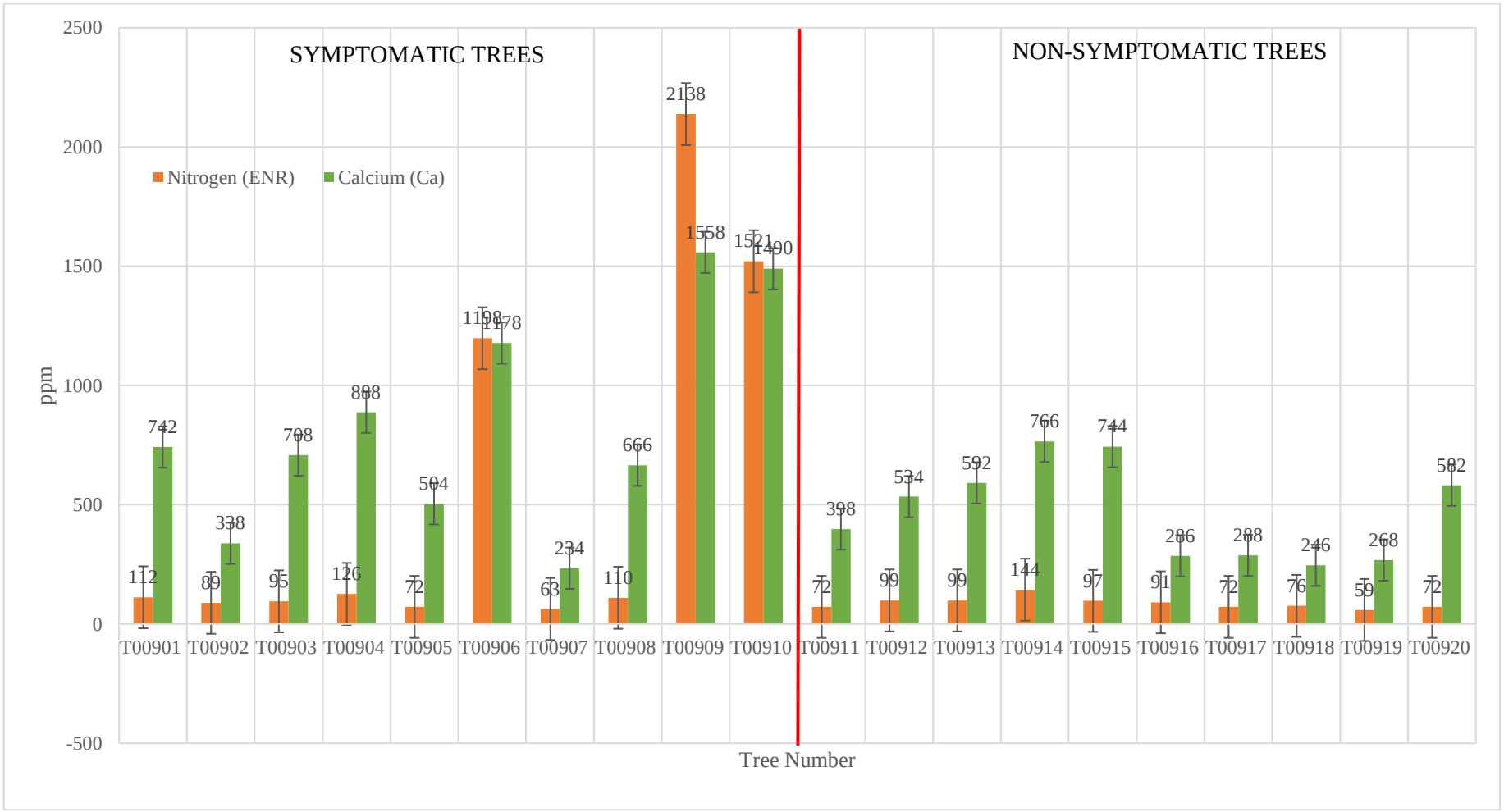


Fig. 4.3: Nitrogen and Calcium concentrations of 200 -350mm soils of symptomatic (T00901-10) and non-symptomatic (T00911-20) trees gathered around Writtle Forest. Error bars = standard error

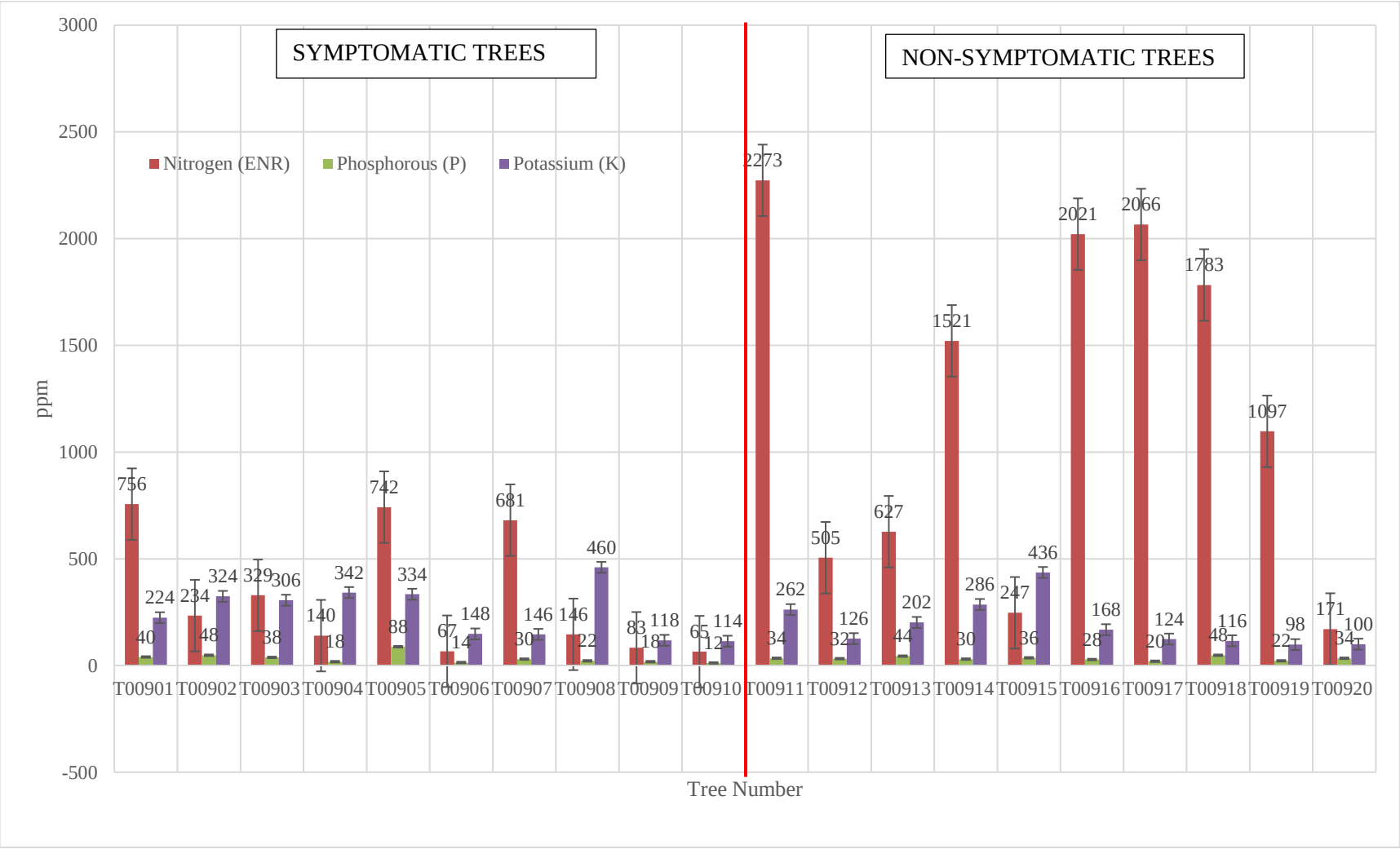


Fig. 4.4: Nitrogen, Phosphorus and Potassium trends within soils at 50 -200mm, gathered around Writtle Forest. Error bars = standard error

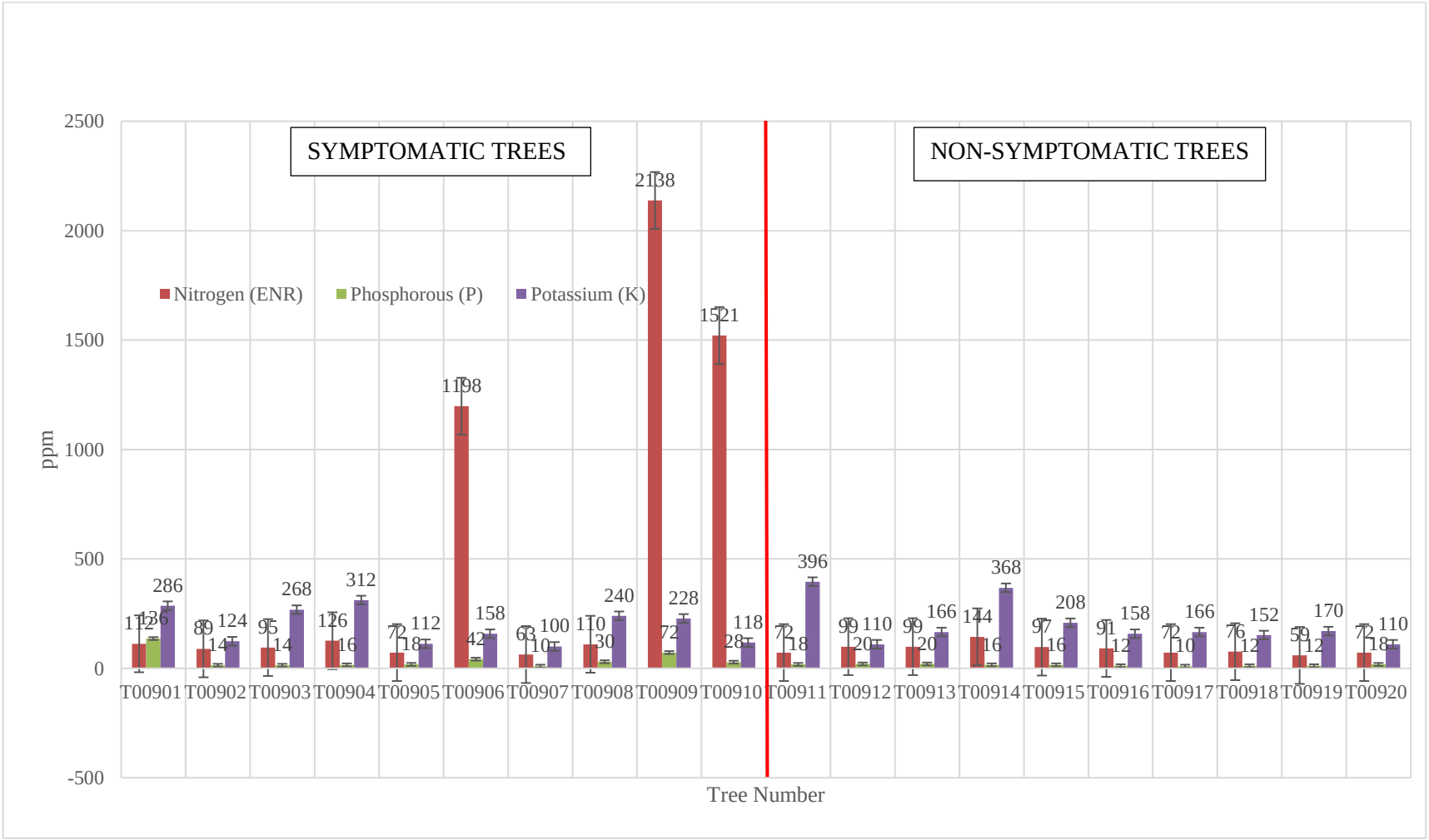


Fig. 4.5: Nitrogen, Phosphorus and Potassium trends within soils at 200-350mm, gathered around Writtle Forest. Error bars = standard error

4.2.1 Differences between nutrient levels in soils from symptomatic and non-symptomatic trees

Differences were explored between soil nutrient levels between non-symptomatic and symptomatic trees, where deficiency or excess of nutrient could be mitigated with accessible, recognised and cost effective soil ameliorant treatments.

An analysis of variance was carried out for all nutrients within soils at depths of 50mm and 350mm associated with symptomatic and non-symptomatic trees. Where the Kurtosis value of the data set falls inside the parameters of 2 to -2 then an analysis of variance, ANOVA (single factor) was undertaken:

Table. 4.5 Analysis of variance of nutrients at soil depths of 50mm and 350mm associated with symptomatic and non-symptomatic trees.

Data sets analysed	**P(T<=t) two-tail**	**Significant difference**
pH values of symptomatic and non-symptomatic trees at a depth of 50mm	0.093619456	No
pH values of symptomatic and non-symptomatic trees at a depth of 350mm	0.091256064	No
N values of symptomatic and non-symptomatic trees at a depth of 50mm	0.003479155	Yes
N values of symptomatic and non-symptomatic trees at a depth of 350mm	n/a	Kurtosis value exceeds 2 for non-symptomatic trees in 350mm data set
P values of symptomatic and non-symptomatic trees at a depth of 50mm	n/a	Kurtosis value exceeds 2 for non-symptomatic trees in 50mm data set
P values of symptomatic and non-symptomatic trees at a depth of 350mm	0.085458434	No
K values of symptomatic and non-symptomatic trees at a depth of 50mm	0.254232906	No
K values of symptomatic and non-symptomatic trees at a depth of 350mm	0.88817486	No
Mg values of symptomatic and non-symptomatic trees at a depth of 50mm	0.246797765	No

Data sets analysed	P(T<=t) two-tail	Significant difference
Mg values of symptomatic and non-symptomatic trees at a depth of 350mm	0.271348009	No
Ca values of symptomatic and non-symptomatic trees at a depth of 50mm	0.065165699	No
Ca values of symptomatic and non-symptomatic trees at a depth of 350mm	0.033384491	Yes
Na values of symptomatic and non-symptomatic trees at a depth of 50mm	0.034564228	Yes
Na values of symptomatic and non-symptomatic trees at a depth of 350mm	0.019280784	Yes
Fe values of symptomatic and non-symptomatic trees at a depth of 50mm	0.05888985	No (marginal)
Fe values of symptomatic and non-symptomatic trees at a depth of 350mm	0.165528854	No
Mn values of symptomatic and non-symptomatic trees at a depth of 50mm	n/a	Kurtosis value exceeds 2 for symptomatic trees in 50mm data set
Mn values of symptomatic and non-symptomatic trees at a depth of 350mm	0.394583517	No
Cu values of symptomatic and non-symptomatic trees at a depth of 50mm	n/a	Kurtosis value exceeds 2 for non-symptomatic trees in 50mm data set
Cu values of symptomatic and non-symptomatic trees at a depth of 350mm	n/a	Kurtosis value exceeds 2 for non-symptomatic trees in 350mm data set
Zn values of symptomatic and non-symptomatic trees at a depth of 50mm	n/a	Kurtosis value exceeds 2 for symptomatic trees in 50mm data set
Zn values of symptomatic and non-symptomatic trees at a depth of 350mm	0.195322946	No
B values of symptomatic and non-symptomatic trees at a depth of 50mm	0.865527381	No
B values of symptomatic and non-symptomatic trees at a depth of 350mm	0.65026097	No

Whilst both Na data sets show significant difference between symptomatic and non-symptomatic trees, at both soil depths, none of the readings are not deemed of concern to the functional growth of trees within the parameters set by Bartlett Tree Expert Laboratories.

The lack of significance of difference in Fe levels were only marginal between symptomatic and non- symptomatic trees at a depth of 50mm in the soils, where the p-value parameter was 0.05.

Calcium levels in symptomatic soils were generally consistent within the topsoil and sub soil levels, with an average range between 801 to 830 ppm across both horizons. Calcium levels in non -symptomatic trees was higher in the leaf litter, averaging 1175 ppm but lower in sub soils averaging 470 ppm. (Figs 4.2 and 4.3 and Tables 4.2 to 4.5). As of Table 4.5, there is a significant difference between Ca levels in soils at 350mm depth between the symptomatic and non -symptomatic trees. There is not a significant difference in Ca levels at 50mm, although this is marginal when the p-value is set at 0.05. The Ca levels in all soil samples are generally considered deficient.

Other divergence relates to the measurable, available nitrogen in the soils. Nitrogen levels within the top -soils of symptomatic trees were generally of acceptable levels and averaged 324 ppm. Similarly, within the sub soils Nitrogen levels were generally acceptable and averaged 552ppm. Nitrogen levels within the top -soils of non -symptomatic trees were high and averaged 1231 ppm. Yet in sub soils the nitrogen levels were low with an average of 88ppm. There was significant difference in the levels of available Nitrogen within soils at 50mm between symptomatic and non-symptomatic trees.

The pattern is of wide divergence in availability of Calcium and Nitrogen between the top -soil/ leaf litter and the sub soils in those trees that are non -symptomatic. However, soils relating to symptomatic trees did not generally display this wide divergence in availability of the nutrients between the leaf litter and the sub soils.

There was no consistent ratio between the Nitrogen and the Phosphorus and Potassium in either the upper soils or sub soils relating to either of the symptomatic or non - symptomatic trees. Generally, higher ratios of Nitrogen to Potassium and Phosphorus are favourable to tree growth (Herbert, 1983).

4.3 Soil Properties within Stoneymore Wood

As well as information pertaining to the health of the trees in the Beta study of Stoneymore, soils were also sampled and analysed relating to those trees exhibiting Bleed canker. Soils were collected as of the previous trees throughout the Writtle Forest woodland complex, at approximately 5m from the stem to the north of each tree. Soils were collected below the leaf litter, between a depth of 150mm and 300mm below ground level. The soils were sent for analysis to A&L Analytical Laboratories, Inc. 2790 Whitten Rd Memphis, USA to consider soil acidity, Nitrogen, Phosphorous, Potassium, Magnesium and Calcium. The results were analysed by Bartlett Tree Research Laboratories to consider deficiencies in accord to those ranges best suited to *Quercus robur.*

Table. 4.6 Analysis of selected areas of soils in Stoneymore Woodland 2013 -2019.

Tree Number	**pH**	**Nitrogen (Estimated Nitrogen Release)**	**Phosphorous**	**Potassium**	**Magnesium**	**Calcium**
708	4.1	114	56	438	202	756
Analysis	Soil pH too acidic, Phosphorous, Magnesium – deficient. Calcium very low					

Tree Number	pH	Nitrogen (Estimated Nitrogen Release)	Phosphorous	Potassium	Magnesium	Calcium
710	4	99	30	576	240	1072
Analysis	Soil pH too acidic, Nitrogen, Phosphorous, Magnesium – deficient. Calcium very low					
711	4.2	106	18	376	466	1252
Analysis	Soil pH too acidic, Phosphorous– deficient. Calcium very low					
715	4	99	30	576	240	1072
Analysis	Soil pH too acidic, Nitrogen, Phosphorous, Magnesium – deficient. Calcium very low					
718	4.1	106	20	378	222	682
Analysis	Soil pH too acidic, Phosphorous, Magnesium – deficient. Calcium very low					
722	4.1	97	14	470	414	1016
Analysis	Soil pH too acidic, Nitrogen, Phosphorous– deficient. Calcium very low					
727	4.3	120	20	498	304	978
Analysis	Soil pH too acidic, Phosphorous, Magnesium – deficient. Calcium very low					
729	3.9	99	14	542	284	932
Analysis	Soil pH too acidic, Nitrogen, Phosphorous, Magnesium – deficient. Calcium very low					
733	4.1	243	56	480	296	1438
Analysis	Soil pH too acidic, Phosphorous, Magnesium – deficient. Calcium very low					

Tree Number	pH	Nitrogen (Estimated Nitrogen Release)	Phosphorous	Potassium	Magnesium	Calcium
855	4.3	124	32	356	456	1354
Analysis	Soil pH too acidic, Phosphorous – deficient. Calcium very low					

4.3.1 Overview of soil deficiency within Stoneymore Wood

Common to all soils sampled near or adjacent to symptomatic trees revealed from laboratory analysis was that the soils have a very low pH value ranging from pH 3.9 to 4.3 as well as low amounts of Calcium (Table 4.6).

Rackham (2003) notes that Essex Oak woodlands generally have acidic soils with pH values of 4.8 to 5.5, these data date back to the 1950s. pH is known to be an important driver of microbial communities, which can influence plant health. Also, calcium is known to have a number of important functions in tree metabolism, particularly signaling functions in plant defence and repair of damage from biotic and abiotic stress. Calcium is also important to the structural chemistry and function of woody tissues. (Lautner & Fromm, 2010).

Given the importance of calcium to plant health, experiments were conducted on symptomatic trees within the epidemiological study area, Stoneymore woods. To see if different calcium amendments to roots could change the number of bleeds observed on trees over a six -year period, two sets of trees were tested with 2 different calcium amendments. Five trees were treated with the product ‘Root – Gyp’ and 5 trees with the product ‘Calcifert’.

4.3.2 Root-Gyp' and 'Calcifert' Soil Amendments

'Root-Gyp' was applied at 400g/ m^2, within a radius of 2m of the main stem. This meant that 4.8 kg per 12m^2, was applied to soils. This was incorporated into the first 300mm of soils using an Air-spade. This tool is a lance using compressed air to tilth the soil to incorporate the product whilst ensuring that roots are not damaged. Root-Gyp is a product naturally mined within England and is verified for use as a fertiliser within organic farming systems. Root-Gyp is produced from the processing of the Gypsum mineral, Calcium Sulphate Dihydrate ($CaSO_4.2H_2O$). It provides a source of Calcium and Sulphate which is highly soluble and pH neutral. Typical chemical analysis is Ca: 22%, SO_3: 44%. (www.rootwise.co.uk/agriculture/gypsum/root-gyp/)

'Calcifert' was applied at 165g/ m^2, within a radius of 2m of the main stem. This meant that 1.98 kg per 12m^2 was applied to soils. This was again incorporated into the first 300mm of soils using an Air-spade. 'Calcifert' granulated lime is made from fine limestone flour combined with a water-soluble organic binder. It is produced in the UK from limestone mined in the Peak District and is suitable for use on organic systems and forestry (www.calcifert.co.uk).

Table 4.7 'Root-Gyp' and 'Calcifert' Soil Amendments and Consequent Bleed Canker Activity

Tree	**Product Applied**	**Application rate m^2**	**Area applied m^2**	**Number of Cankers April 2013**	**Number of Cankers April 2016**	**Number of Cankers April 2019**
708	Root-Gyp	400g/ m^2	12 m^2	2	4	5
710	Root-Gyp	400g/ m^2	12 m^2	9	4	5
711	Root-Gyp	400g/ m^2	12 m^2	9	5	2
715	Root-Gyp	400g/m^2	12 m^2	3	1	0
855	Root-Gyp	400g/m^2	12 m^2	8	8	7
718	Calcifert	165g/ m^2	12 m^2	7	6	6
722	Calcifert	165g/ m^2	12 m^2	5	4	1
727	Calcifert	165g/ m^2	12 m^2	5	3	0
729	Calcifert	165g/ m^2	12 m^2	11	11	6
733	Calcifert	165g/ m^2	12 m^2	5	2	0

The results show that generally the progression of the bleed cankers is reduced by the introduction of the soil ameliorants.

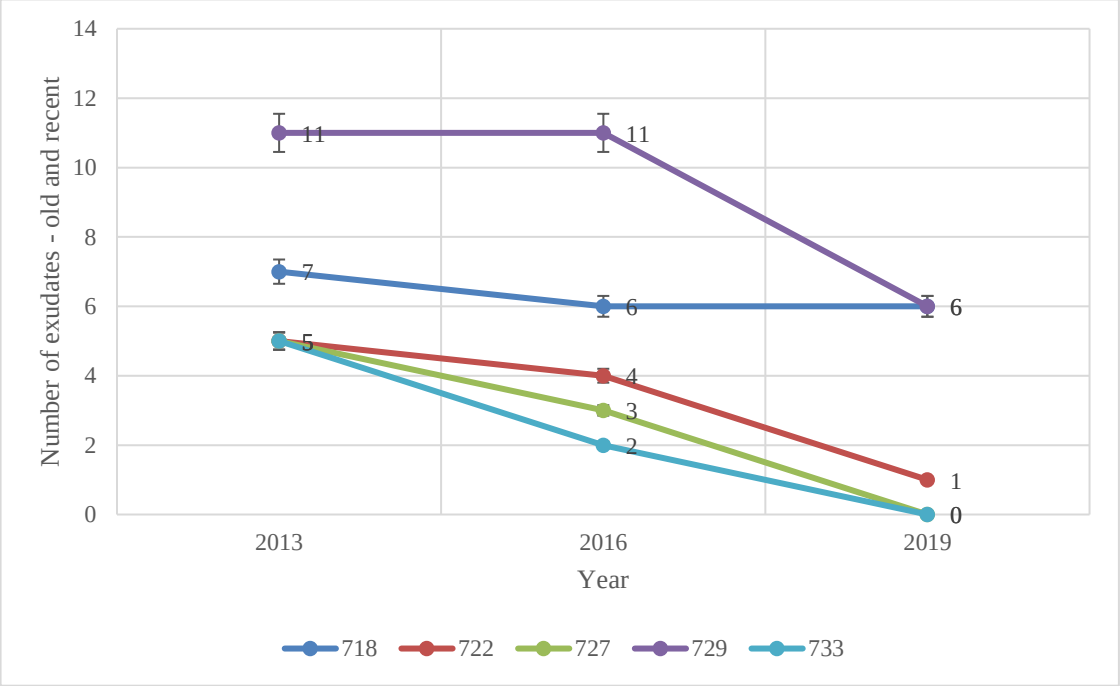


Fig. 4.5: Bleed Canker Activity observed on five trees over six years after 'Calcifert' Soil Amendments. Error bars = Percentage error of 5%

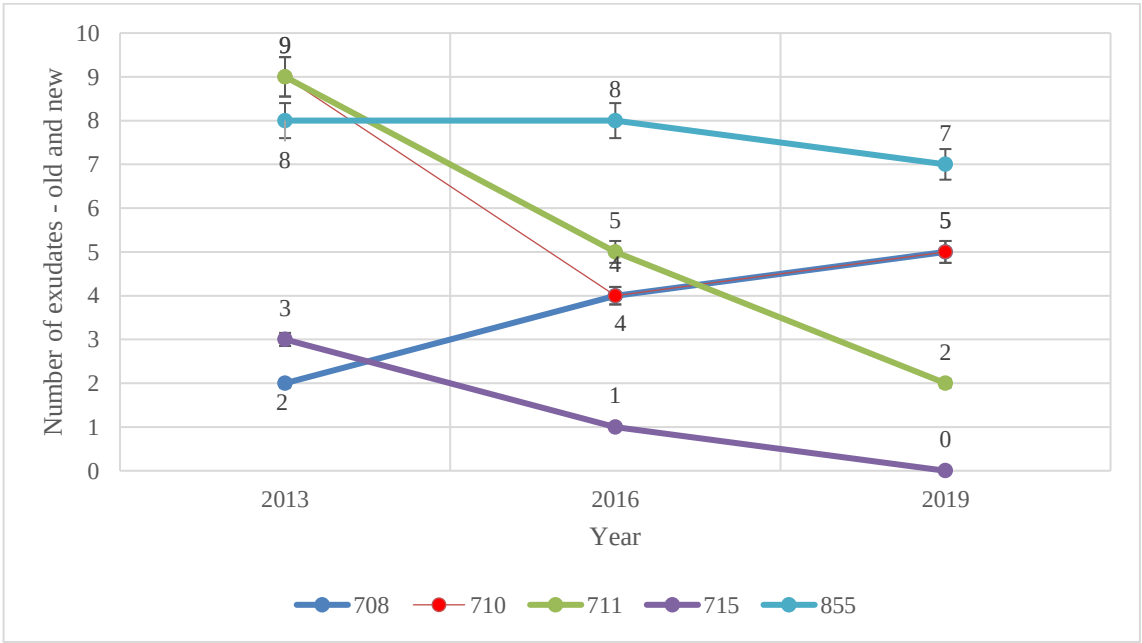


Fig. 4.6 Bleed Canker observations on five trees over six years after 'Root-Gyp' Soil Amendments**.** Error bars = Percentage error of 5%

These results can be seen in relation to 5 control trees within the same area of woodland (i.e. similar variables). The 'Calcifert' has had the greater impact on the bleed canker. This experiment was carried out as a test model to understand whether it could be used to test the efficacy more efficiently on a larger number of trees, field grown with reduced environmental variables. (Ultimately this was not practicable due to the varying success of inoculating the trees to emulate the bleed canker).

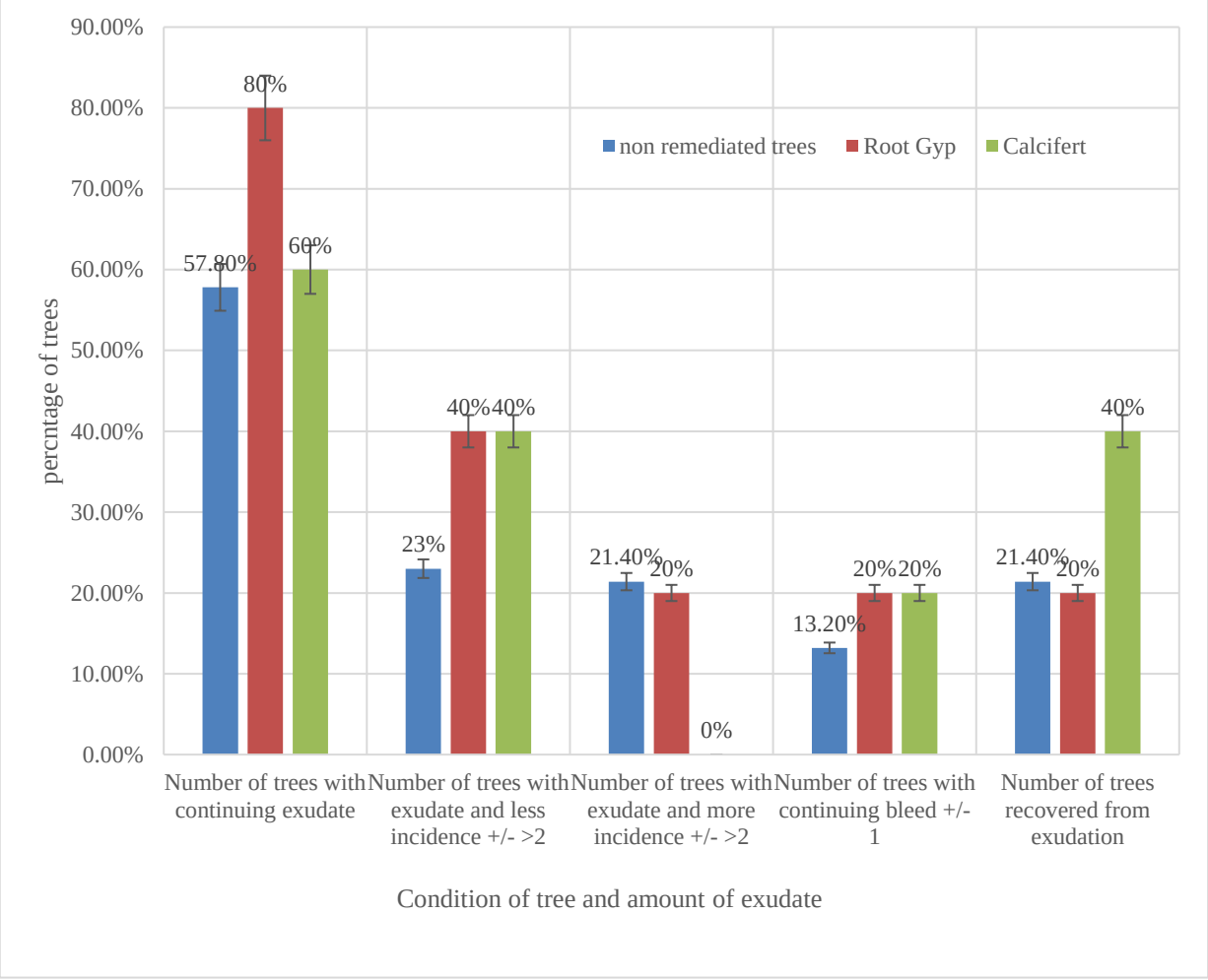


Fig. 4.7: Percentage Bleed Canker observations on five trees over six years after 'Root-Gyp' & 'Calcifert' compared with five non-remediated trees in Stoneymore Wood. Error bars = Percentage error of 5%

4.4 Summary of Findings

From the soil analysis, all soils across the woodland were of greater acidity than expected, minimally ranging between pH 3.9 to 5.2. Calcium was also very low within all the soils analysed. These considerations may contribute to an environment that is conducive to disease. Soils were ameliorated within the rooting environment of selected Oaks to increase the calcium content. It was seen that this had a significant effect on the Oak Bleed Canker, reducing the bleed and encouraging callousing at the bleed area.

Discrepancy was found relating to soils, between those trees that were currently suffering from the Oak Bleed Canker and those that were not. The main difference that could be detected was in relation to the amount of available Nitrogen within the top 200mm of soils. This was generally higher for those trees that were not suffering from the bleed symptoms. However, at a subsoil depth of 200mm to 350mm the difference in available Nitrogen was not found between symptomatic and non-symptomatic trees. Experiments considering the remediation of soils with Nitrogen were not undertaken as part of this study.

Chapter 5: Results – Tree ring growth of Symptomatic and Non- symptomatic trees in Stoneymore Wood

5.1 Context of Research

To establish whether there are any pre-disposing conditions relating to the condition of the trees prior to the Bleed cankers, an analysis of the tree rings were undertaken.

5.1.1 Principles and Application of testing

Tree growth involves a number of different processes. These include bud development, foliage growth, root growth, seed development, production of secondary metabolites as well as growth of storage tissue and stem growth. Measurement of these areas of development provide an indication of the vitality of the tree (Dobbertin, 2005; Tulik, 2014).

When a tree is under stress functional aspects such as photosynthesis are reduced and allocation of carbon within the tree is altered. The incremental stem growth of the tree will reflect such changes. (Dobbertin, 2005). Generally, if a tree is under stress, the production of growth tissue increasing the stem diameter is sacrificed for allocation of resources to more important growth requirements such as root and foliage development. This is reflected within the pattern of annual growth that occurs which can be seen within the growth ring development of the tree.

In broadleaf ring porous trees such as *Quercus sp*., the differences within the annual growth are visibly distinguishable due to the differences in the development of cell tissue laid down by the tree. Earlywood cells are generally (though not exclusively) formed at the beginning of the growth season. They are characterized by the thickness of the cell wall. The thinner cell wall is associated with less dense wood growth of earlywood. As the cell wall increases in size, the wood becomes denser and is generally referred to as latewood (Pallardy, 2008). The transition between these tissues is markedly distinct within many broadleaf species lending to the categorization of what are commonly described as ring porous trees.

The link between tree ring growth and the health and condition of the tree has been used to study decline and disease within trees (Romagnolia et al., 2018; Calleret et al., 2017). Comparative analysis of the tree ring growth of trees with symptomatic and non-symptomatic bleed were considered to assess whether there is a link between decline in growth and the bleed canker symptoms observed.

5.1.2 Measuring Equipment and software analysis

Twenty trees were selected from the 1 Hectare Oak cohort within Stoneymore Wood. Economically, it was not a viable option to take core samples of the trees. A non-destructive wood density assessment of trees was made using a Resistograph R650. This is an electronic, high-resolution, needle drill-resistance measurement device. The thrust and mechanical drive of the drilling device is measured. This provides high correlation between the measured values and the density of the penetrated wood (Fundova et al., 2018).

Drill readings were taken at a height of approximately 1.5m on the main stem, at a 90degree angle to the growth rings, at positions of drilling from north to south and south to north on

each tree. The consequent measurement readings were analysed using TSAP software (Cufar et al, 2008; Koprowski et al., 2010). This enables identification of the tree rings and cross referencing of data readings from the drill readings from one tree. It further allows for the incremental area of the tree rings to be estimated.

5.1.3 Reference markers provided by climatic conditions

The link between tree ring growth and climatic conditions is well established. Climatic events can be used as points of reference when interrogating tree ring data; (Fritts, 1973, Nabeshima, 2010). To this end the environmental conditions for Writtle Forest were considered for the last 32 years.

5.2 Environmental conditions relating to Writtle Forest

Rainfall data was provided on request from the National Meteorological Library & Archive. The information provided was for each day since 1959 from the area of Writtle, Essex.
NGR = 5678E 2066N. Altitude = 32 metres. Latitude = 51:73N Longitude = 00:43E.
Information related to mean temperature for day and night as well as rainfall per day was provided, but information relating to sunlight was incomplete.
Data for rainfall was processed by considering the total rainfall for each year. Data was considered since 1986, a date of twenty years prior to the first noted outbreak of Acute Oak Decline.

5.2.1 Consideration of Rainfall in Writtle Forest

Figure 5.1 shows the polynomial trend line (order 2) for rainfall over a thirty two-year period. The average rainfall for this period is 582mm. The trend line shows reduced rainfall of

approximately 40mm over the period. This represents an average 7% reduction of rainfall within the last 32 years for the trees within Writtle Forest.

5.2.2 Consideration of Temperature in Writtle Forest

Figure 4.2 shows the average temperature each day for temperatures recorded between March and October inclusively over a 32year period. The average temperature over this time was 13^{o}C. The polynomial trend line (order 2) shows an increase of 1 ^{o}C over the last 32 years for the trees within Writtle Forest.

5.2.3 Climatic signals

From the details derived from the data over a 32year period, the year which has the highest temperature and highest rainfall is 2014. Such conditions would be conducive to tree growth. The year with the coldest temperature and lowest rainfall is 1996. Such conditions would not be conducive to tree growth. Thus, there would be an expectation of associated physiological changes being detected with the drill method for these years. These peaks were clearly identifiable within the drill readings.

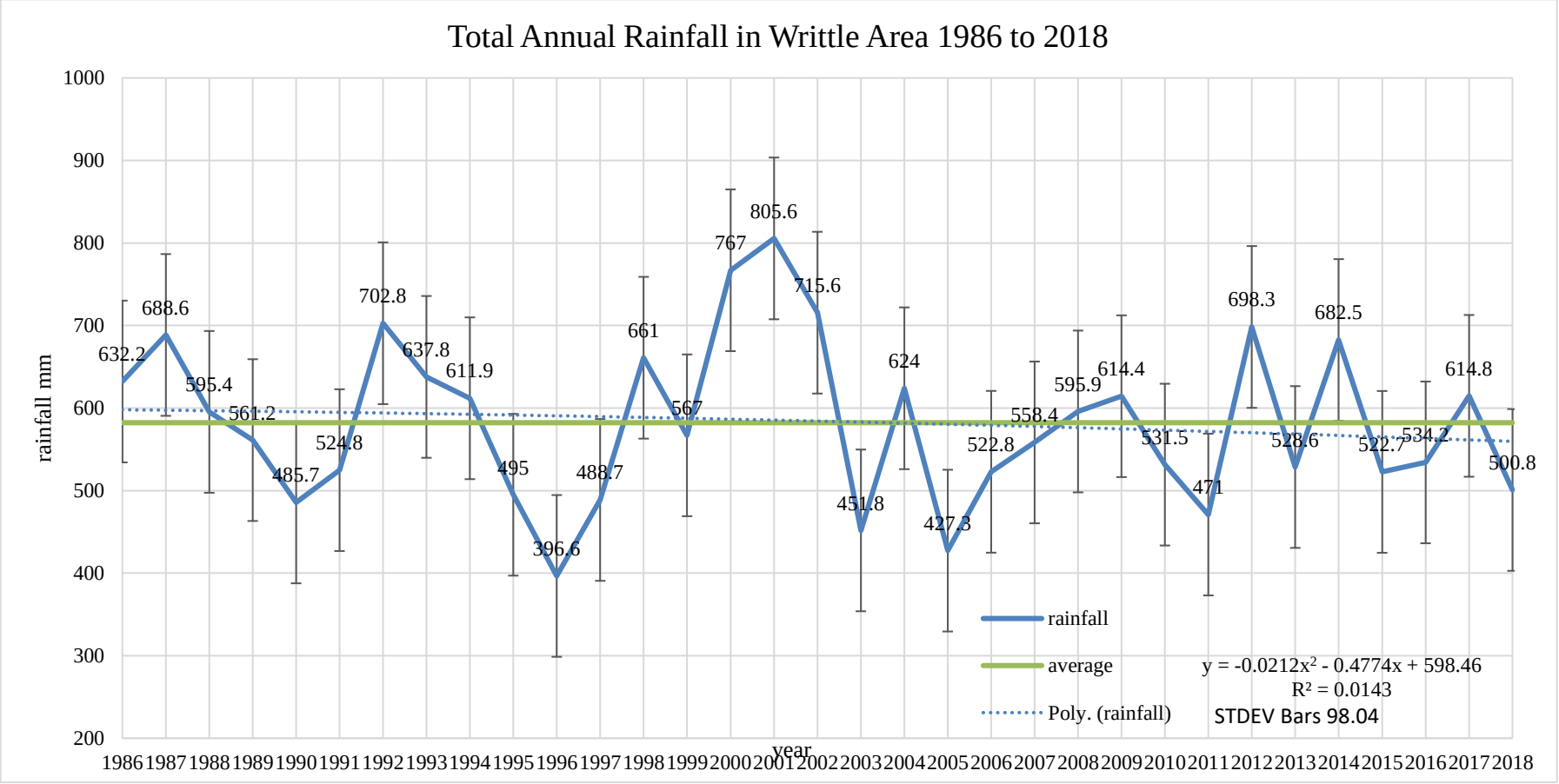


Figure 5.1. Rainfall for Writtle Forest 1986-2018. Error bars = standard deviation

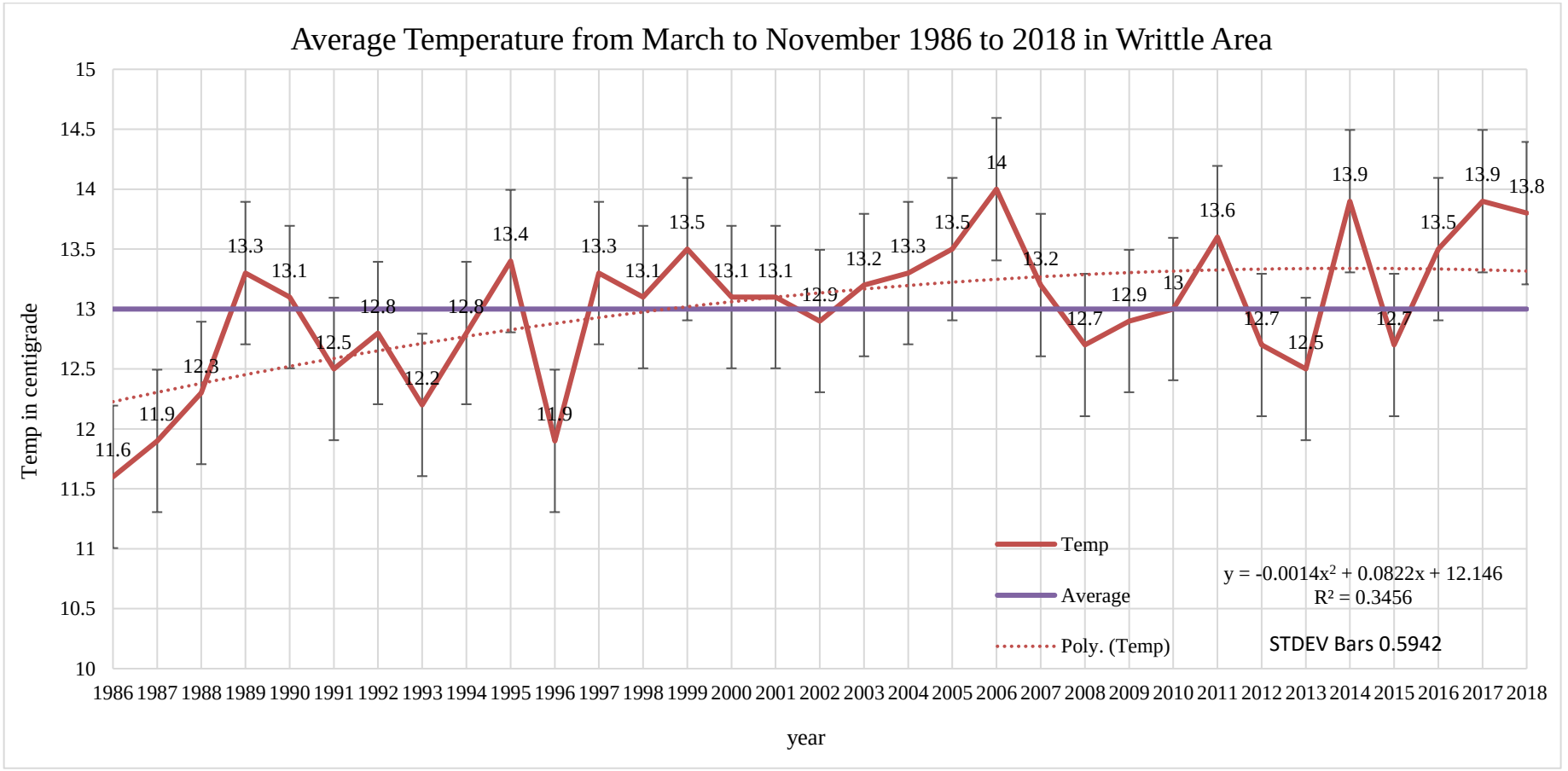


Figure 5.2. Average temperatures recorded for Writtle Forest 1986-2018. Error bars = standard deviation

5.3 Tree Growth ring analysis

5.3.1 Trees categorisation

From the 1 Hectare of Oak trees within Stoneymore Wood four categories of tree condition were considered:

1. Trees that did not exhibit any bleed symptoms over the six-year period, described as Zero Oak Bleed Canker (OBC).
2. Trees that exhibited a consistent pattern of bleed and were still alive, described as Consistent OBC.
3. Trees that showed new incidence or a significant increase in bleed symptoms, described as New OBC.
4. Trees that showed a significant decrease or remission of bleed symptoms, described as Remissory OBC.

From each of these categories, five trees were chosen that were most aligned to the categories and were similar in stem girth. (The tree ring graphs for all 20 trees is provided within the Appendices).

Trees with Zero OBC between 2013 and 2019

Age: Over Mature - OM, Mature -M, Early Mature – EM, Semi Mature - SM, Young – Y

DBH: Diameter at Breast Height measured at 1.5m

Tag no.	Age: OM, -M, EM, SM, Y)	DBH 1.5m (mm)	2013 % Deadwood	2013 Evidence of *Agrilus*	2013 Number of Cankers	2013 Recent Cankers	2013 Old Cankers	2019 % Deadwood	2019 Evidence of Agrilus	2019 Number of Cankers	2019 Recent Cankers	2019 Old Cankers
701	SM	460	Normal	N	0	0	0	Above normal	N	0	0	0
702	EM	540	Above normal	N	0	0	0	Normal	N	0	0	0
703	EM	640	Above normal	N	0	0	0	Above normal	Y	0	0	0
898	EM	605	Normal	N	0	0	0	Normal	N	0	0	0
899	SM	465	Above normal	N	0	0	0	Above normal	N	0	0	0

Trees with Consistent OBC between 2013 to 2019

Age: Over Mature - OM, Mature -M, Early Mature – EM, Semi Mature - SM, Young – Y

DBH: Diameter at Breast Height measured at 1.5m

Tag no.	Age: OM, -M, EM, SM, Y)	DBH 1.5m (mm)	2013 % Deadwood	2013 Evidence of *Agrilus*	2013 Number of Cankers	2013 Recent Cankers	2013 Old Cankers	2019 % Deadwood	2019 Evidence of Agrilus	2019 Number of Cankers	2019 Recent Cankers	2019 Old Cankers
771	SM	400	Normal	N	6	3	3	Normal	N	6	0	6
779	SM	450	Normal	N	2	0	2	Normal	N	1	0	1
785	SM	460	Above normal	N	2	0	2	Excessive	N	2	1	1
925	EM	540	Excessive	Y	25	15	10	Excessive	N	25	7	18
939	EM	600	Above Normal	N	4	2	2	Normal	N	4	1	3

Trees with New OBC between 2013 to 2019

Age: Over Mature - OM, Mature -M, Early Mature – EM, Semi Mature - SM, Young – Y

DBH: Diameter at Breast Height measured at 1.5m

Tag no.	Age: OM, -M, EM, SM, Y)	DBH 1.5m (mm)	2013 % Deadwood	2013 Evidence of *Agrilus*	2013 Number of Cankers	2013 Recent Cankers	2013 Old Cankers	2019 % Deadwood	2019 Evidence of Agrilus	2019 Number of Cankers	2019 Recent Cankers	2019 Old Cankers
746	SM	495	Normal	N	0	0	0	Above normal	N	6	2	4
751	SM	325	Normal	N	0	0	0	Excessive	N	22	18	4
861	EM	680	Normal	N	0	0	0	Normal	N	4	2	2
915	EM	610	Above normal	Y	0	0	0	Above normal	Y	30	5	25
944	EM	515	Above normal	N	1	1	0	Above normal	N	11	0	11

Trees with Remissory OBC between 2013 and 2019

Age: Over Mature - OM, Mature -M, Early Mature – EM, Semi Mature - SM, Young – Y

DBH: Diameter at Breast Height measured at 1.5m

Tag no.	Age: OM, -M, EM, SM, Y)	DBH 1.5m (mm)	2013 % Deadwood	2013 Evidence of *Agrilus*	2013 Number of Cankers	2013 Recent Cankers	2013 Old Cankers	2019 % Deadwood	2019 Evidence of Agrilus	2019 Number of Cankers	2019 Recent Cankers	2019 Old Cankers
724	SM	495	Above normal	N	10	3	7	Normal	N	1	1	0
733	SM	430	Normal	N	5	3	2	Normal	N	0	0	0
875	EM	520	Above normal	N	17	7	10	Above normal	N	0	0	0
880	SM	405	Normal	N	5	0	5	Normal	N	0	0	0
882	EM	540	Above normal	N	11	1	10	Above normal	N	0	0	0

Table 5.1. Data pertaining to trees drilled with the Resistograph.

5.3.2 Area of tree rings considered

Given that the outbreak of Acute Oak Decline was first noted in 2006 it was considered that an overview of between 37 and 40 years would be sufficient to review the recent growth patterns of the trees. This would account for time lag of tree responses to environmental conditions, as well as providing information on circa. the last third of the trees' lifespan. (Wilson et al, 2008).

Generally, the first 35mm to 55mm of reading from the Resistograph drill was information pertaining to bark and phloem tissue. This information was disregarded.

The drill readings were carried out in April 2019. For reliability of read the first definitive annual ring was used and considered to be as of the growth season from 2018.

It was estimated that the incremental growth of stem girth for forest grown trees would be approximately 12.5mm (Mitchell, 1976). This would attribute to a growth ring width of approximately 4mm. To this end an area of approximately 180mm was considered extending from the bark toward the centre of the tree. After cross referencing the data from two drill readings from each tree through the TSAP-win software, it was generally found that an area of 110mm to 115mm covered a period of 36years, equating to an annual growth ring width of approximately 3mm.

5.3.3 Average Annual Ring width growth of all 5 trees within each of the 4 groups considered

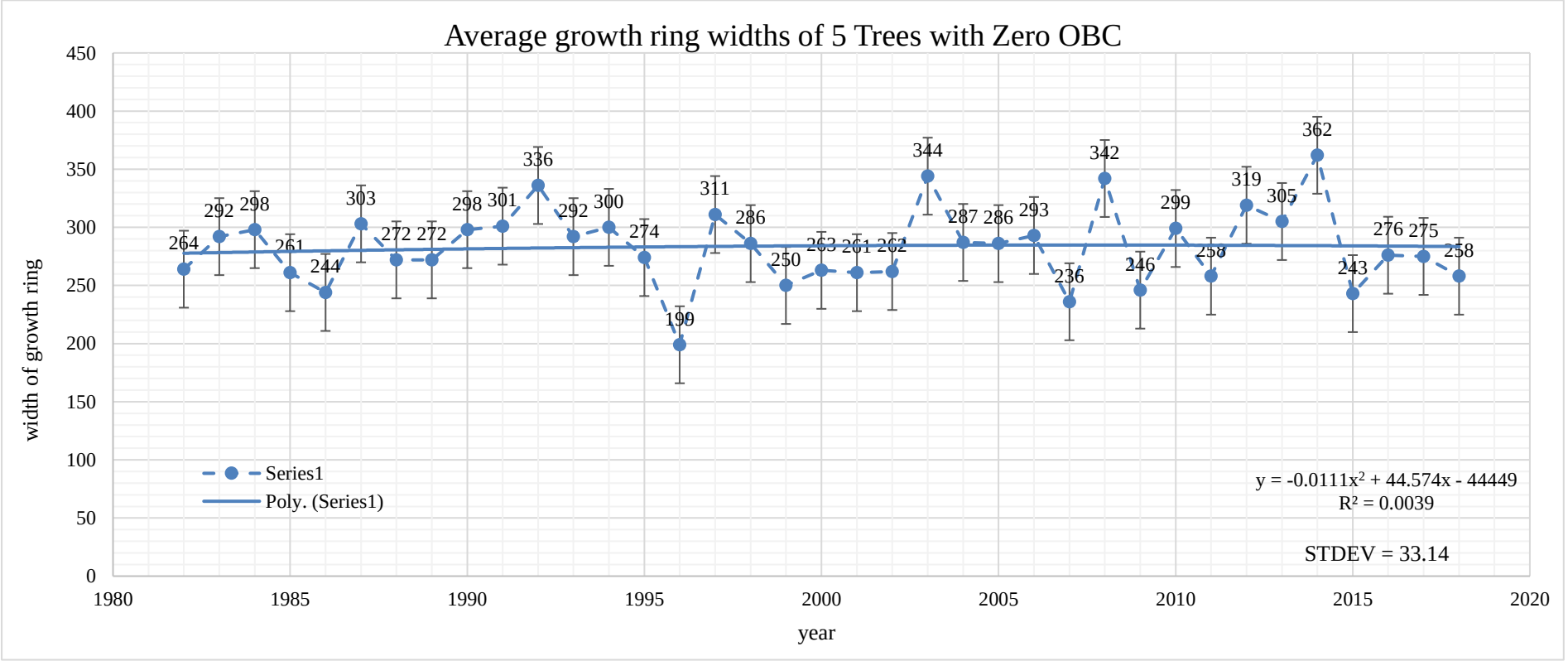


Figure 5.3. Average growth ring widths of 5 trees with Zero OBC combined. Error bars = standard deviation

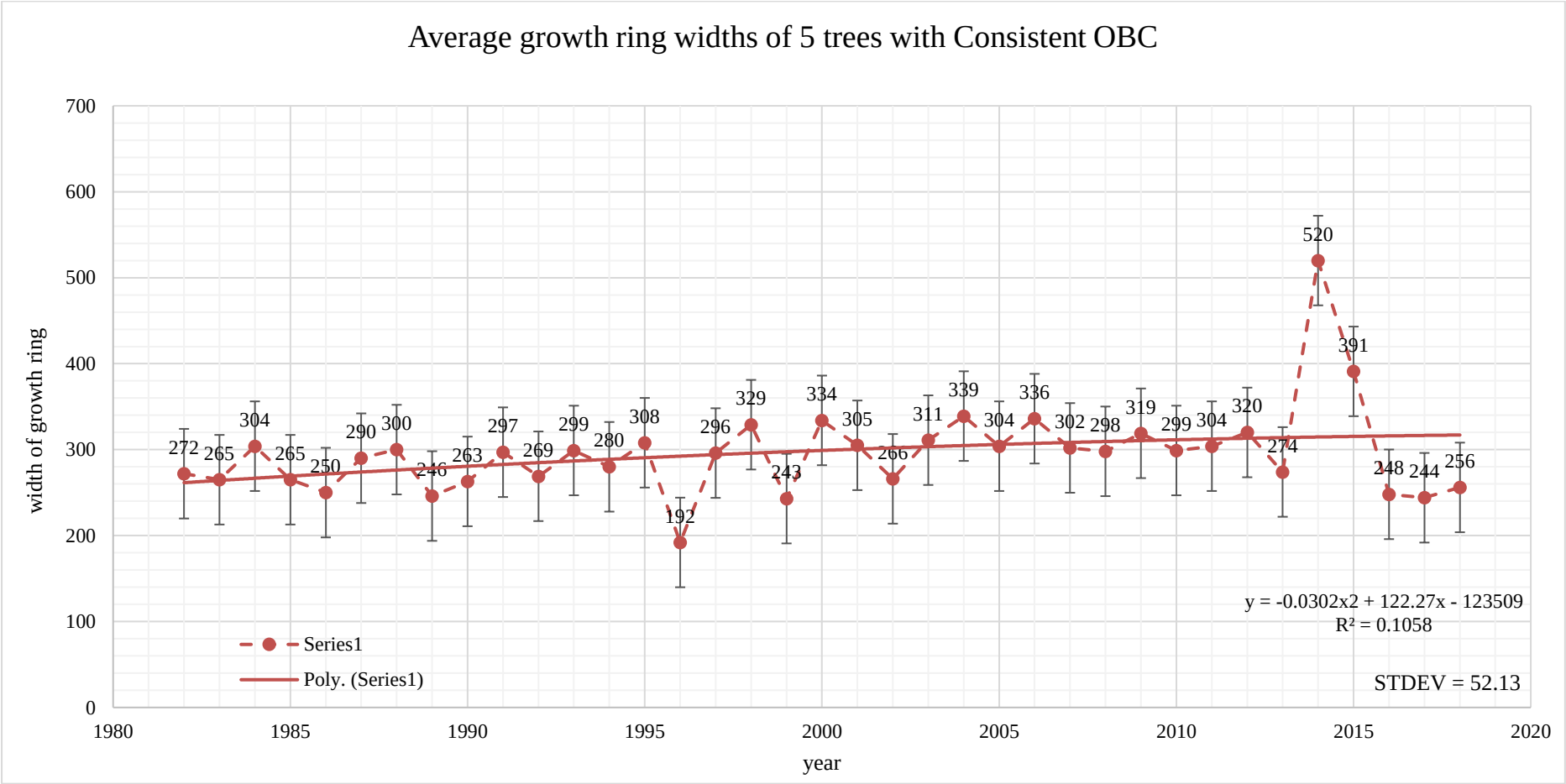


Figure 5.4. Average growth ring widths of 5 trees with Consistent OBC combined. Error bars = standard deviation

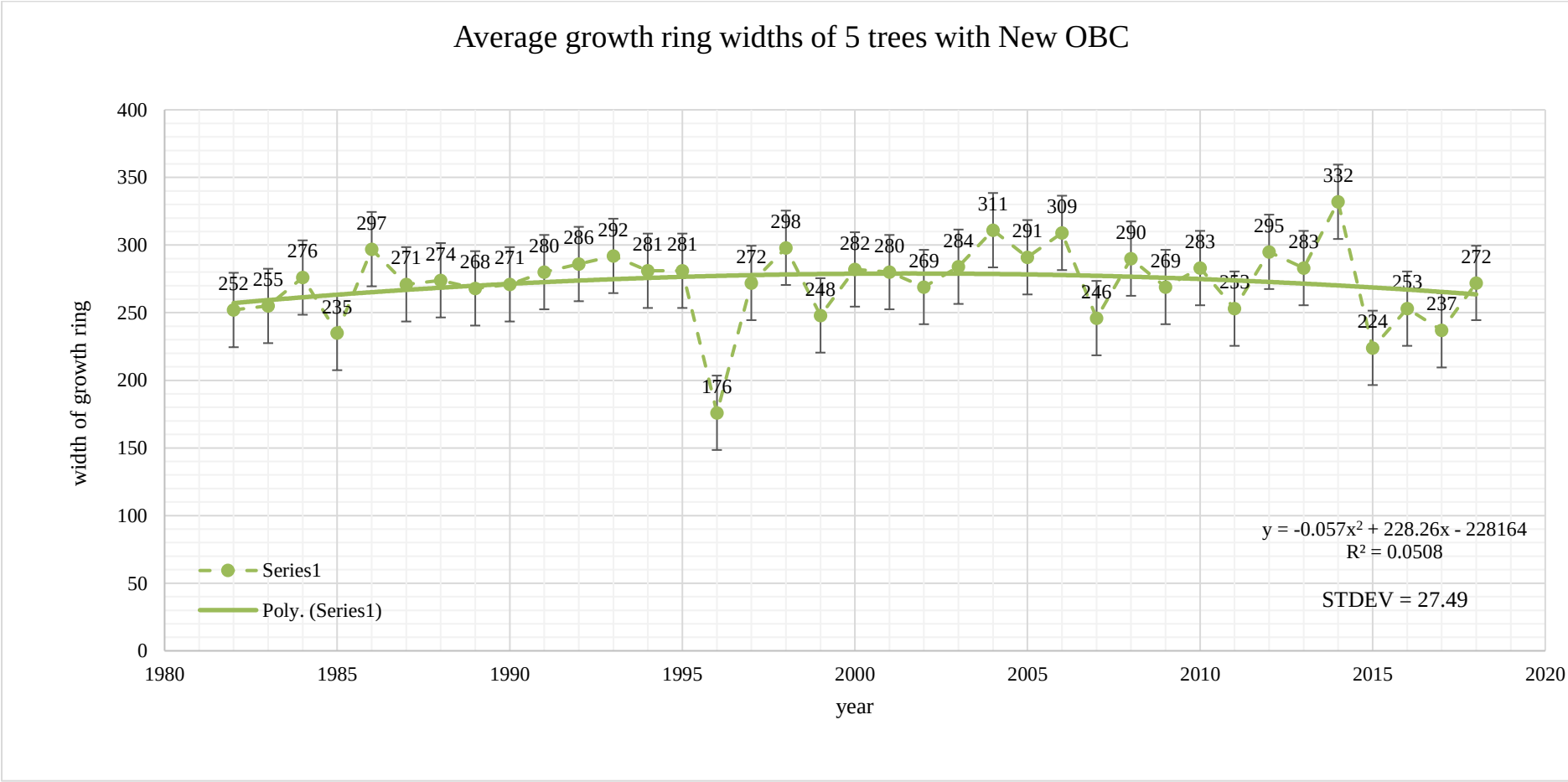


Figure 5.5. Average growth ring widths of 5 trees with New OBC combined. Error bars = standard deviation

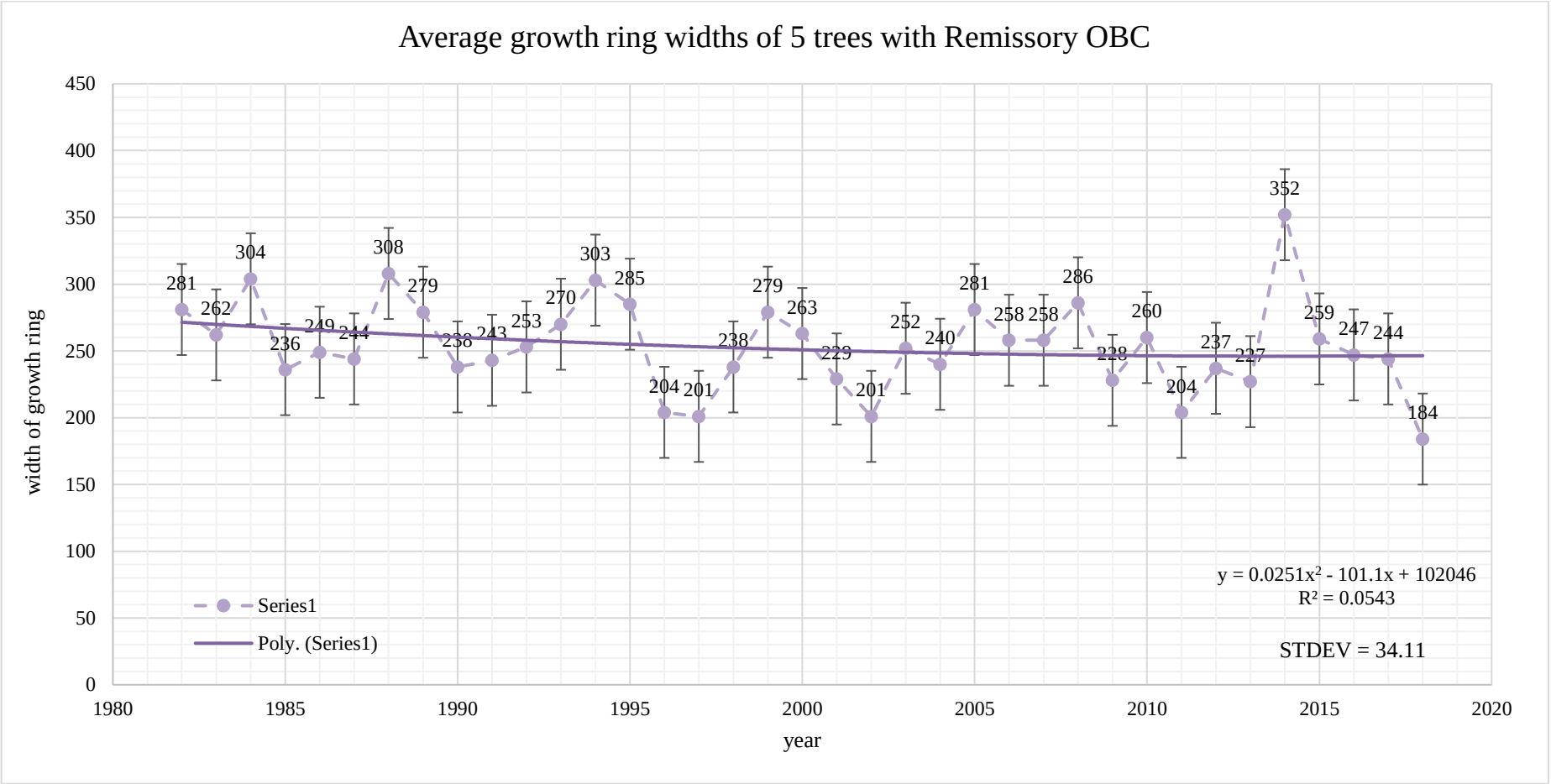


Figure 5.6. Average growth ring widths of 5 trees with Remissory OBC combined. Error bars = standard deviation

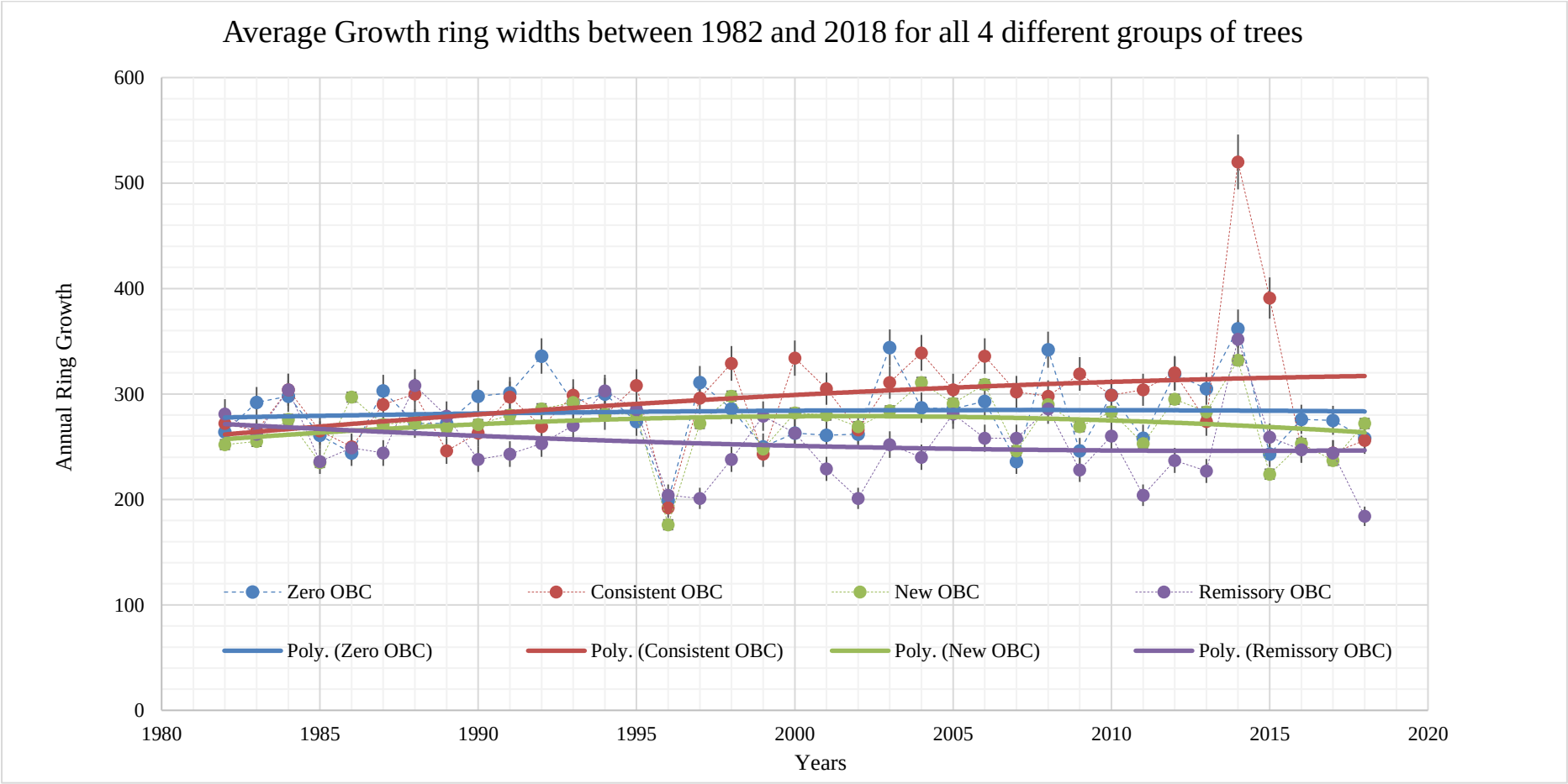


Figure 5.7. Comparison of the average annual growth ring widths of all 4 groups considered. Error bars = Percentage error of 5%

5.3.4 Patterns observed within Annual Ring width growth

For each of the trees within the group an average of ring width growth was calculated within the TSAP -win software.

The average size of growth ring widths rank as follows:

1. Highest for those trees that exhibited Consistent OBC from 2013 to 2019.
2. Those trees that exhibited Zero OBC from 2013 until 2019.
3. Those trees that exhibited New OBC between 2013 and 2019.
4. Those trees that exhibited Remissory OBC between 2013 and 2019.

The climatic time points relating to the 1996 poor growth season and the 2014 good growth season were evident within all categories of tree considered.

All categories of trees had small growth ring widths during the drought with reduced temperature in 1996. The category of trees whose ring width growth in 1996 diverged least from the average ring width growth over a 37yr period (14 years prior to 1996 and 22 years preceding), were those trees that exhibited signs of Remissory OBC between 2013 and 2019.

Group	Average ring growth over 37yrs	Ring growth in 1996	Percentage reduction in average ring growth
Zero	282.92	199	-29.6%
Consistent	295.62	192	-35%
New	272.32	176	-35.4%
Remissory	253.7	204	-19.6%

Table 5.2. Data pertaining to comparative decrease in Tree Ring widths between all 4 groups in 1996. Percentage =s (Ring growth - Average ring growth) / Average ring growth x 100

All trees showed increased growth in 2014 consequent to improved warmth and rainfall. The most prominent increase in width of growth ring was within those trees that exhibited Consistent OBC through the 6year period (from 2013 to 2019). It was least prominent in those trees that exhibited New OBC.

Group	Average ring growth over 37yrs	Ring growth in 2014	Percentage increase in average ring growth
Zero	282.92	362	27.95%
Consistent	295.62	520	75.9%
New	272.32	332	17.98%
Remissory	253.7	352	38.75%

Table 5.3. Data pertaining to comparative increase in Tree Ring widths between all 4 groups in 2014. Percentage =s (Ring growth - Average ring growth) / Average ring growth x 100

5.3.5 Statistical Analysis of Ring width growth of all Four Groups

The ‘null hypothesis’, in this consideration, is that all growth ring widths between the groups selected, do not exhibit a statistical difference in size. The ‘alternative hypothesis’ is that the ring width growth between the trees as grouped have statistically significant difference.

From an analysis of variance, ANOVA (single factor) it was established:

1) That the F-value is greater than the F-critical value for the alpha level selected (0.05). Therefore, there is evidence to reject the null hypothesis and conclude that at least one of the four groups have significantly different means and so a significance of difference between the growth of ring widths.

2) That the p-value is less than the alpha level selected (0.05) also provides evidence of statistically significant difference between the groups. A small p-value (typically ≤ 0.05) indicates strong evidence against the null hypothesis.

Further statistical testing using the Bonferroni approach was used to establish which groups exhibited significant differences between each other. T-Test: Two-Sample Assuming Equal Variances was carried out between each group in turn to ascertain difference between all groups. (Please see Appendices: Chapter 5, for all statistical data analysis).

5.3.6 Summary of differences between groups in relation to tree ring growth

Comparative Groups	**P(T<=t) two-tail**	**Comparative to alpha level p-value 0.05**	**Description**
Remissory and Consistent	0.00011	< 0.05	The greatest difference shown between the 4 groups lies between those trees that exhibit signs of consistent bleed and those that exhibit signs of remission. The former having wider growth rings, the latter narrower.
Remissory and Zero	0.00037	< 0.05	There was a similarly significant difference between those trees that exhibited no signs of OBC and those in remission within the 6year monitoring period
Remissory and New	0.01174	< 0.05	The growth rings of the group of trees in remission of OBC were significantly distinct from the group of trees identified

Comparative Groups	P(T<=t) two-tail	Comparative to alpha level p-value 0.05	Description
			with new symptoms of OBC, during the 6year period.
New and Consistent	0.01877	< 0.05	New and consistent groups also showed significant difference. This may be explained by the consideration that those trees chosen that exhibited signs of new OBC may be a mixture of trees that will exhibit signs of consistent OBC or trees that will exhibit signs of recovery/ remission in the future.
Zero and New	0.13883	> 0.05	Those trees with zero OBC and new OBC did not show a significant difference in ring width growth between each other.
Zero and Consistent	0.21504	> 0.05	The difference in ring width growth of trees with zero bleed and consistent bleed (over the 6year monitoring period), was not significant.

Table 5.4 Summary of differences in ring width between all 4 groups of trees.

It had been expected to see that the trees that exhibited signs of remission and had zero bleed would have more vitality (and hence wider growth rings), as the vitality would confer some

resistance to the OBC. Yet the group of trees that show remission from OBC have a ring width that is significantly different from all the other tree groups that exhibit either new incidence, consistent symptoms or no symptoms of OBC. The ring width growth of those trees which exhibited remission from OBC were significantly smaller.

5.4 Annual incremental growth area

5.4.1 Consideration of Incremental growth of trees

Whilst tree ring growth provides a picture of the trees' growth on an annual basis, it does not necessarily reflect the overall growth trend of the trees. Whilst individual tree growth ring widths may diminish over consecutive years, the area of incremental growth should need to take account of the ever-increasing girth of the main stem of the tree. The TSAP-win software allows for this calculation to be considered.

5.4.2 Average Incremental growth of each of the 4 groups considered

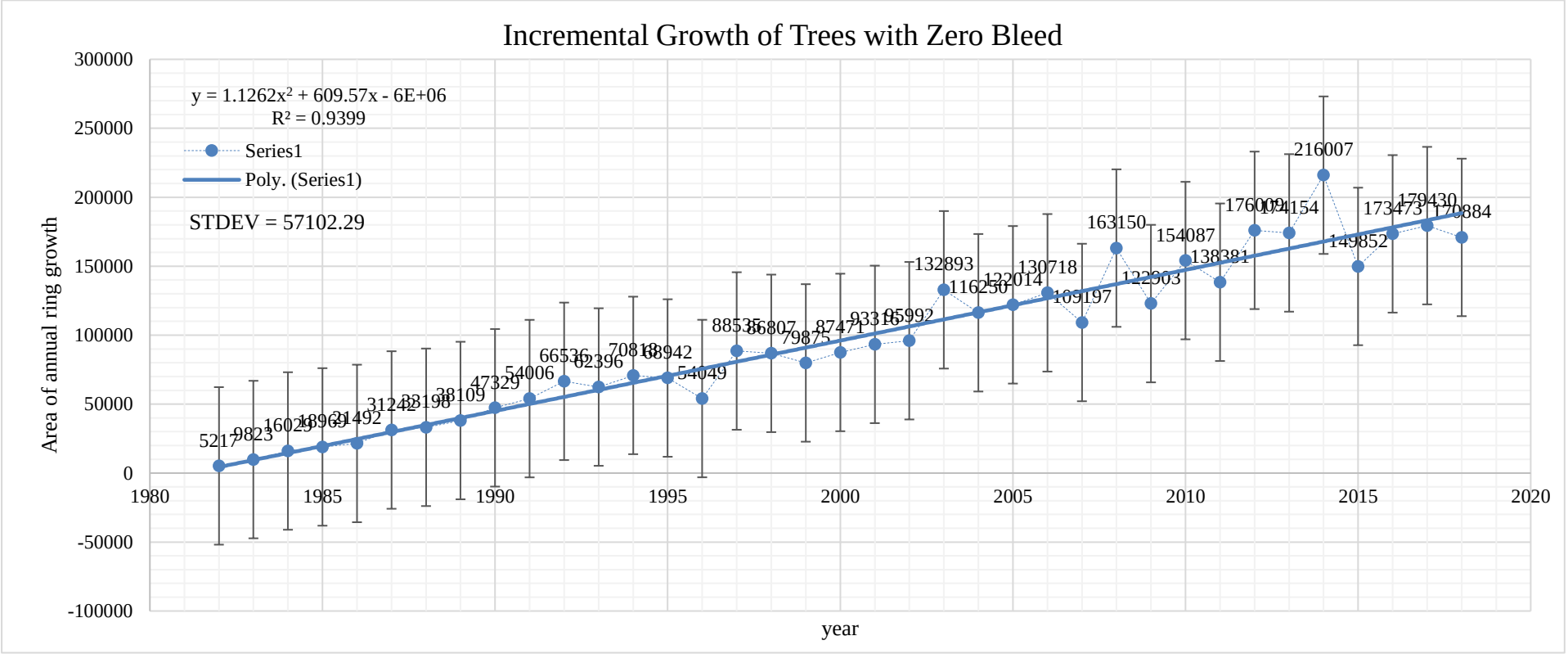


Figure 5.8. Incremental growth of trees with Zero OBC. Error bars = standard deviation

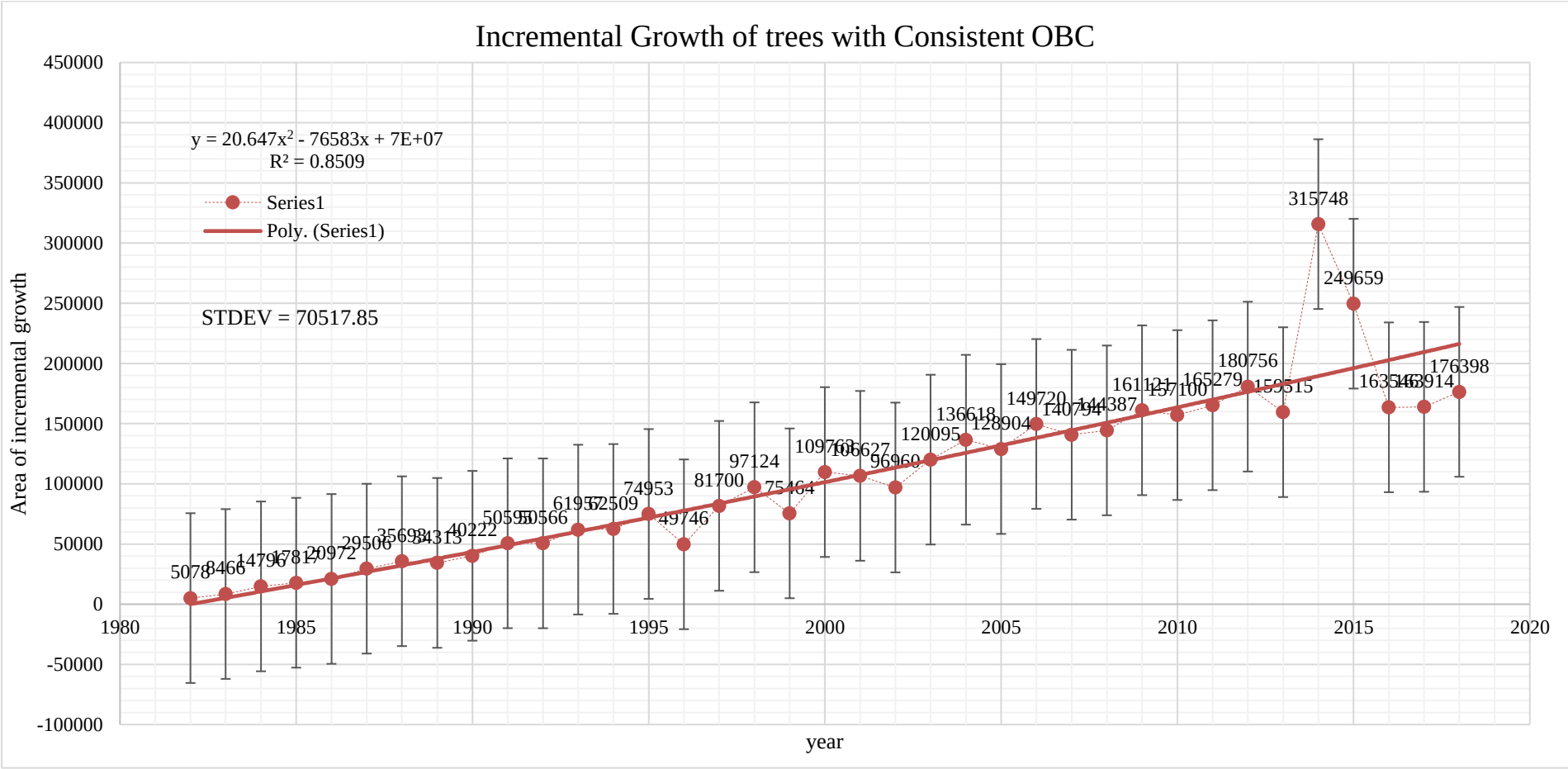


Figure 5.9. Incremental growth of trees with Consistent OBC. Error bars = standard deviation

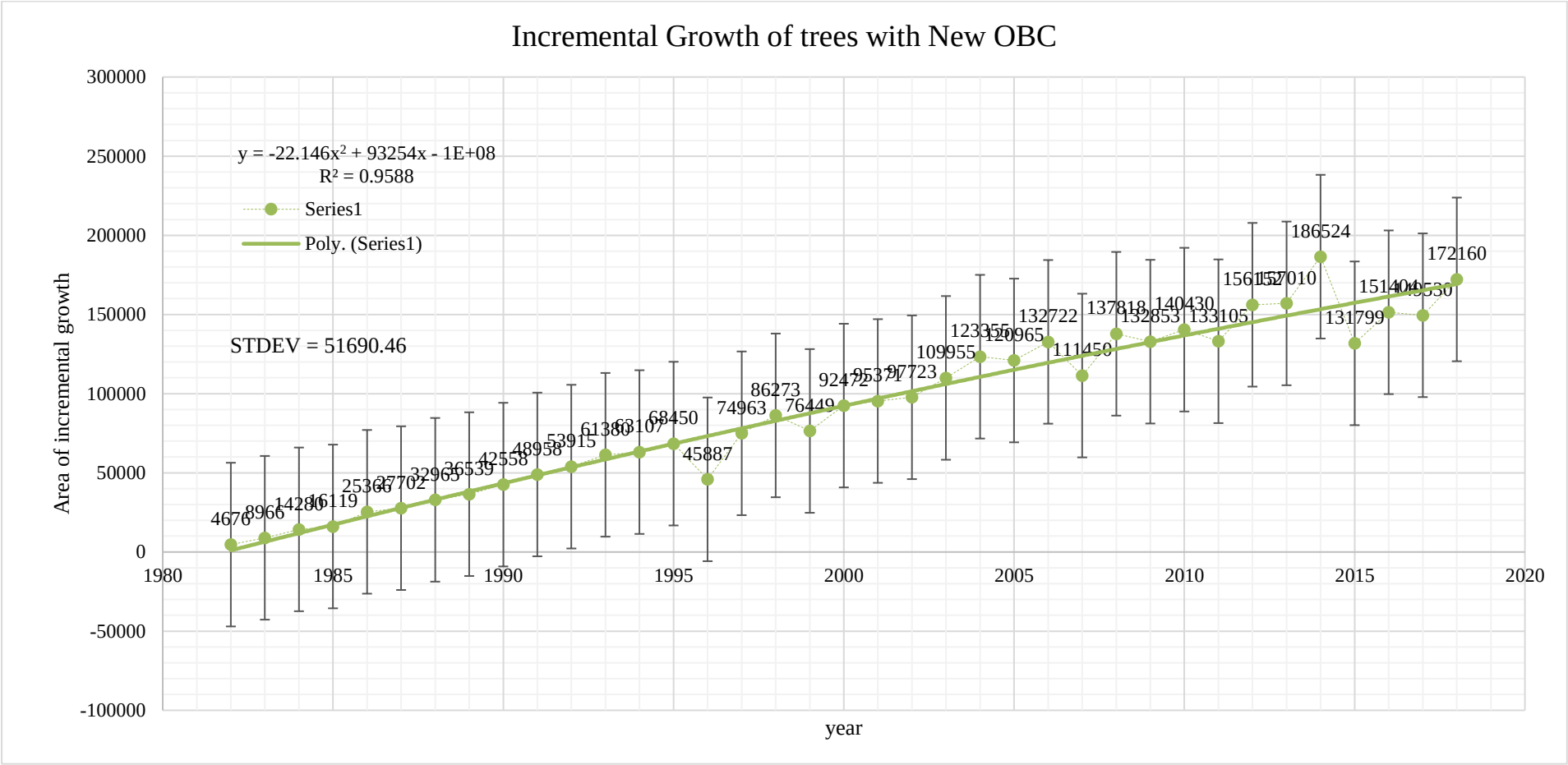


Figure 5.10. Incremental growth of trees with New OBC. Error bars = standard deviation

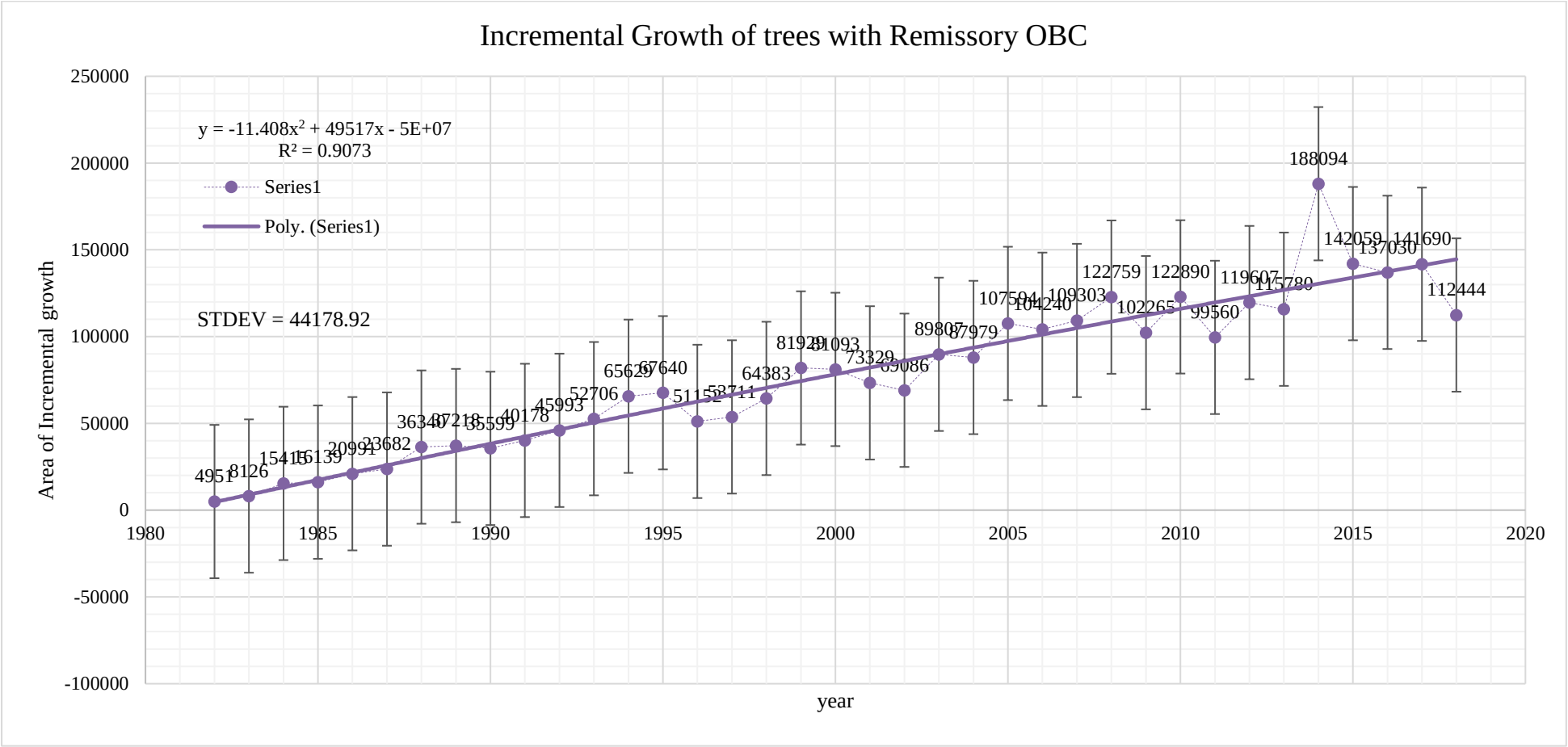


Figure 5.11. Incremental growth of trees with Remissory OBC. Error bars = standard deviation

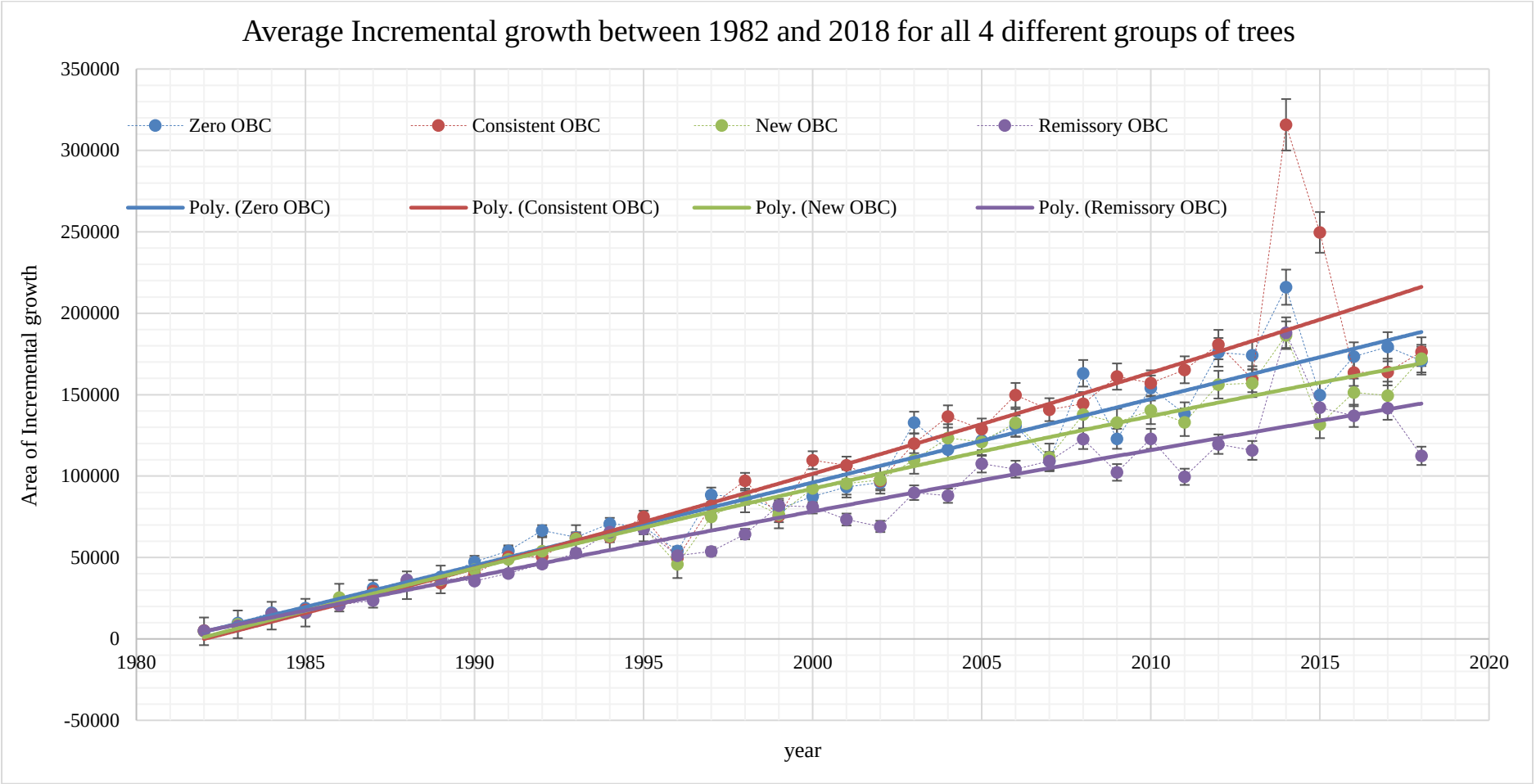


Figure 5.12. The comparative average incremental growth for all 4 tree groups. Error bars = Percentage error of 5%

5.4.3 Statistical Analysis of Incremental growth of all Four Groups 1982 - 2018

The ‘null hypothesis’, in this consideration, is that all incremental growth between the groups selected, does not exhibit a statistical difference. The ‘alternative hypothesis’ is that the incremental growth between the trees as grouped has statistically significant difference.

From an analysis of variance, ANOVA (single factor) it is established:

1. That the F-value is less than the F-critical value for the alpha level selected (0.05). Therefore, there is no evidence to reject the null hypothesis. The four groups do not have significantly different means and so do not have a significant difference between incremental growth over the time period since 1982.
2. The p-value is more than the alpha level selected (0.05). This provides no evidence of statistically significant difference between the groups. A small p-value (typically ≥ 0.05) indicates evidence for the null hypothesis.

5.4.4 Statistical Analysis of Incremental growth of all Four Groups modelled Post 1996

It can be seen from Figure 5.12 that the slower rate of incremental growth of the group of trees with Remissory OBC can be seen to develop around 1990, a year of low rainfall. The different rates of incremental growth between Remissory OBC and the other three groups appears most pronounced as of 1996. There is significantly reduced size in growth rings across all groups. However, the recovery of incremental growth of the group of trees with Remissory OBC is slower than the other three groups after 1996. This difference was considered statistically. The null and alternate hypotheses are as of before but in this instance the data is considered as of post 1996.

With the set of data post 1996, from the analysis of variance ANOVA (single factor), it is established that F-value is more than the F-critical value for the alpha level selected (0.05). The p-value is less than the alpha level selected (0.05). This provides evidence that there is statistically significant difference between the incremental growth of the groups post-1996. Further statistical testing using the Bonferroni approach was used to establish which groups exhibited significant differences between each other.

5.4.5 Differences between Incremental growth of all Four Groups post 1996

Comparative Groups	**P(T<=t) two-tail**	**Comparative to alpha level p-value 0.05**	**Description**
Remissory and Consistent	0.00381	< 0.05	As of the differences in annual ring growth the differences between incremental growth of the Remissory group and the Consistent OBC group were greatest
Remissory and Zero	0.01392	< 0.05	As above the groups Remissory and Zero reflect similar differences as were found in the annual tree ring growth. Between the Remissory group of trees and those trees showing Consistent OBC and Zero OBC the difference in incremental ring growth appears in 1996 with the first significant decrease in precipitation.

Comparative Groups	P(T<=t) two-tail	Comparative to alpha level p-value 0.05	Description
Remissory and New	0.05732	> 0.05	This comparative group failed to show significant difference in incremental growth. The reduced rainfall in 1996 has not shown a significant divergence between Remissory trees and all groups considered at this point.
New and Consistent	0.11117	> 0.05	As above, whilst significant differences were detected between these groups in relation to the overall tree ring growth this is not seen post 1996 in relation to the incremental growth
Zero and New	0.35030	> 0.05	The differences between Zero OBC and New OBC remain statistically non-significant.
Zero and Consistent	0.43852	> 0.05	The differences between Zero OBC and Consistent OBC remain statistically non-significant.

Table 5.5 Summary of differences in incremental growth between all 4 groups of trees from 1996 – 2018.

5.4.6 Statistical Analysis of Incremental growth of all Four Groups modelled Post 2002

1996 showed a significant reduced rainfall over the time period considered. Subsequent to this the period 2002-2003 shows a steep decline in precipitation. This is reflected in the incremental growth of the trees in 2002, (Figure 4.12). This appears to show the cumulative effect of reduced rainfall over a relatively short succession of time (6years), on the incremental growth of the four tree groups.

Statistical analysis considered whether there was an increase in divergence of differences between the 4 groups. As previously, the hypothesis considered the differences of incremental growth between the group of 4 trees.

With the set of data post 2002, from the analysis of variance ANOVA (single factor), it is established that F-value is more than the F-critical value for the alpha level selected (0.05). The p-value is less than the alpha level selected (0.05). This provides evidence that there is statistically significant difference between the incremental growth of the groups post-2002. Further statistical testing using the Bonferroni approach was used to establish which groups exhibited significant differences between each other.

5.4.7 Differences between Incremental growth of all Four Groups post 2002

Comparative Groups	**P(T<=t) two-tail**	**Comparative to alpha level p-value 0.05**	**Description**
Remissory and Consistent	0.001148	< 0.05	As of comparative analysis post 1996 ring growth is significantly different between these 2 groups.

Comparative Groups	**P(T<=t) two-tail**	**Comparative to alpha level p-value 0.05**	**Description**
Remissory and Zero	0.002662	< 0.05	As above, given the consistent significant difference between these 2 groups this is very much as expected.
Remissory and New	0.014909	< 0.05	The differences between the incremental ring growth of the groups became more prominent after 2002. The reduced precipitation post 1996 did not exhibit a significant difference in incremental ring growth between these two groups. However, there is significant difference post 2002.
New and Consistent	0.048857	< 0.05	As above there is significant difference between these two groups.
Zero and New	0.266188	> 0.05	As before there is no significant difference between these 2 groups.
Zero and Consistent	0.251252	> 0.05	As before there is no significant difference between these 2 groups.

Table 5.6 Summary of differences in incremental growth between all 4 groups of trees from 2002 – 2018.

5.5 Summary of Findings

5.5.1 Conclusions drawn from analysis of Tree ring and Incremental growth of trees

From the preceding statistical analysis and comparative graphs, the following inferences are made from the tree ring analysis:

1) The consideration of the annual tree ring growth reveals significant time points in relation to the growth of the trees and the climatic conditions.
2) The variance in annual tree ring growth reveals significant differences between the groups considered over the time scale of 1982 – 2018. However, such variance reduces the significance of analysis of variance, at specific time points, to allow a qualified understanding of the relation between the growth of the trees and the climatic conditions.
3) A consideration of the incremental growth of all the trees shows progressive incremental growth within all groups considered. Between 1982 and 2018 there was no statistically significant differences between the groups.
4) Statistically significant difference was found when considering the incremental growth in relation to environmental triggers, specifically reduced precipitation.
5) The group with greatest divergence in growth was the group with Remissory OBC.
6) The 2 groups that did not reveal any statistically significant differences between them were the group of trees that suffered from Consistent OBC and those trees that had Zero OBC.
7) From the above it can be concluded that the presence of the OBC does not affect the incremental growth of the trees.

Incremental growth is generally taken as a sign of tree vitality, it would appear therefore that the vitality of trees that exhibit consistent symptoms of the OBC are not affected by the pathogen.

5.5.2 Conclusions drawn in relation to the group of trees with Remissory OBC

Further inference can be drawn in relation to the group of trees that showed Remission of the OBC:

1) The group of trees with Remissory OBC showed significant difference in incremental growth from either the trees with Consistent or Zero OBC after 1996.
2) This reduction in incremental growth appears to have been triggered by reduced precipitation as well as successive accumulative effect of reduced precipitation.
3) If it is assumed that the group of trees with Remissory OBC show resilience to OBC then this is related to the incremental growth of the tree. It is theorized that this may be related to:
    i) a reduced conducive environment for the pathogen due to the reduced width of annual growth ring.
    ii) that the tree is expending more resource on other biological activity other than incremental growth (e.g. secondary metabolites, combative of disease)

If the growth ring width of the four groups of trees is considered between 2012 and 2018 no statistically significant difference is to be found between these groups. Thus, the hypothesis that the reduced width of the annual growth ring affects the environment of the OBC is not substantiated. It is probable that the Remissory group of trees are expending more resource on other biological activity triggered by historic drought stress.

Chapter 6: Results – Bacterial sampling & Profiling

6.1 Context of Research

The primary aim of this study was to identify the causal agents relating to the bleed canker. The morphological characteristics of the bleed canker closely resembled the characteristics of other bleed cankers on trees all of which were caused by pathogenetic bacteria. i.e. Horse Chestnut Bleed Canker caused by bacterial pathogen *Pseudomonas syringae pv. aesculi* (Webber et al, 2008), Bleeding canker of pears caused by bacterial pathogen *Dickeya fangzhongdai* (Chen et al,2020), Walnut Bleed Canker caused by bacterial pathogen *Brenneria rubrifaciens* (McClean et al, 2008), Bleeding Canker of Cherry trees caused by bacterial pathogen *Pseudomonas syringae* (Weaver, 1978).

Bleed cankers may also be caused by Oomycetes and fungus. Phytophthora bleed canker on numerous tree species (Oomycete – *Phytophthora ramorum),* Honey fungus (*Armillaria mellea)* – the bleed canker in this instance is a secondary effect of a root decay. However, in these instances the bleed canker symptoms do not extend over a height of 2m on the main stem.

6.1.1 Initial Isolation of Bacteria from Writtle Forest April 2012

Samples were collected from symptomatic and non- symptomatic trees in April 2012. (Chap. 2, Fig. 2.2). The wood samples were taken to isolate microbial populations that could then be tested as to their pathogenicity and virulence. (As of Chapter 2 section 2.5.1).

Samples were taken in the first instance directly from the bleed canker. Individual colonies were obtained at the 10^3 dilution onward but were more readily isolated at 10^4 and 10^5 dilutions. Plates of pure culture to 10^2 were generally too heavily populated to be able to obtain pure colonies. Plated dilutions were incubated for 3 days at 20°C and were assessed for total number of colonies and different morphotypes (Table 5.1) Colonies were considered only from dilutions of between 10^3 and 10^6. Total bacterial colonies were most commonly isolated at 10^5. Colonies at this dilution ranged from 4 to 12 single colonies. This would give a range of between 4 and 12,000,000 cfu/ml.

Representative samples of the commonest culturable bacterial colony were selected to relate colony morphology to identification through the 16S gene. The colony morphology of 1-2mm in size with a circular form, raised elevation, entire margin, smooth surface and cream coloured was identified as *Brenneria goodwinii*. Sequence analysis was carried out on-line through the National Center for Biotechnology Information utilizing BLAST (Basic Local Alignment Search Tool), to match sequences within the Standard Nucleotide database.

Table 6.1; Samples taken directly from canker on Symptomatic trees

Tree Number	**Condition of Tree**	**No. of individual isolates cultured**	**No. of different colony Morphologies identified**	**No. of different colony morphologies ≥ 10% of isolates taken**	**% of commonest colony morphology observed from isolates cultured**	**Identification of commonest morphology observed**
T00901	Symptomatic	80	16	3	40%	*Brenneria sp.*
T00902	Symptomatic	12	5	2	50%	*Brenneria sp.*
T00903	Symptomatic	15	5	3	46.7%	*Brenneria sp.*
T00904	Symptomatic	16	8	3	31.25%	*Brenneria sp.*

Tree Number	Condition of Tree	No. of individual isolates cultured	No. of different colony Morphologies identified	No. of different colony morphologies ≥ 10% of isolates taken	% of commonest colony morphology observed from isolates cultured	Identification of commonest morphology observed
T00905	Symptomatic	37	10	3	35.7%	*Brenneria sp.*
T00906	Symptomatic	39	13	2	43.6%	*Brenneria sp.*
T00907	Symptomatic	32	16	2	25%	*Brenneria sp.*
T00908	Symptomatic	40	13	3	42.5%	*Brenneria sp.*
T00909	Symptomatic	51	17	1	33.3%	*Brenneria sp.*
T00910	Symptomatic	55	17	1	41.8%	*Brenneria sp.*

Sampling was also undertaken on non -symptomatic Oak trees within proximity to the sampled symptomatic tree. The area sampled was approximately correlated to the most common area sampled from symptomatic trees (generally 1.5m from ground level on the south side of the tree). These data were gathered to distinguish differences in bacterial populations. The main difference noted between symptomatic and non -symptomatic trees was the consistent isolation from non -symptomatic trees of fungi. As before, representative samples of the commonest culturable bacterial colony were selected to relate colony morphology to identification through the 16S gene. However, in this instance the commonest representative sample of the fungal isolates cultured were not sequenced.

Table 6.2; Samples taken from Non- Symptomatic trees

Tree Number	Condition of Tree	No. of individual isolates cultured	No. of different colony Morphologies identified	No. of different colony morphologies ≥ 10% of isolates taken	% of commonest colony morphology observed from isolates cultured	Identification of commonest morphology observed
T00911	Non-Symptomatic	30	9	3	46.7%	*Brenneria sp.*
T00912	Non-Symptomatic	7	5	1	43.5%	fungal
T00913	Non-Symptomatic	13	4	2	61.5%	fungal
T00914	Non-Symptomatic	27	14	1	40.8%	fungal
T00915	Non-Symptomatic	37	19	1	24.3%	*Brenneria sp.*
T00916	Non-Symptomatic	23	11	2	26.1%	fungal
T00917	Non-Symptomatic	21	14	2	23.8%	fungal
T00918	Non-Symptomatic	20	14	2	25%	fungal
T00919	Non-Symptomatic	25	10	4	28%	fungal
T00920	Non-Symptomatic	34	13	4	23.5%	*Brenneria sp.*

6.1.2 Isolation of culturable Bacteria from the Margin of the Canker

The information gained from isolating bacteria directly from the canker revealed a consistent bacterial isolate, however, there were a large number of other bacteria identified by differing colony morphologies. The consistent morphology was no assurance of the pathogenicity of the bacteria. Furthermore, when later excising larger areas below the bark, beyond the area of the

visible exudate it became apparent that the cankers were far more extensive than the visible exudate outside of the bark (See Chapter 1 Fig. 1.8).

Lelliot and Stead (1987), recommend that specimens of diseased material should include the edge of the lesion and several centimetres of healthy material. Samples were then gathered from the margin of the necrotic tissue of three trees. (As of Chapter 2 section 2.6.1).

Table 6.3 Samples taken from margin of canker on Symptomatic trees in Writtle Forest

Tree Number	No. of isolates taken	No. of different morphologies recorded	No. of morphologies >l 10% of population	% of isolates with dominant morphology	Ident. of dominant morphology	Ident. of secondary morphology
T00902	14	4	3	50%	*Brenneria sp*	*Pseudomonas sp*
T00903	12	3	2	66.7%	*Brenneria sp*	*Pseudomonas sp*
T00910	19	4	3	52.6%	*Brenneria sp*	*Pseudomonas sp*

As before representative isolates were selected to relate colony morphology to identification through the 16S gene. The colony morphology of 1-2mm in size with a circular form, raised elevation, entire margin, smooth surface and cream coloured was identified as *Brenneria goodwinii*. The colony morphology of 1-2mm in size with an irregular form, umbonate elevation, undulate margin, smooth surface and cream coloured was identified as *Pseudomonas flavescens.* The colony morphology of 4-5mm in size with an irregular form, flat, undulate margin, smooth surface and cream coloured was identified as *Pseudomonas xinjiangensis.*

Having found fewer colony morphologies at the margin of the cankers 16S rRNA sequencing was carried out on a greater number of isolates from the margin canker of one tree to establish a better understanding of the bacteria isolated (Table 6.4).

Table 6.4 16S rDNA sequencing of isolates recovered from T00903 at margin of canker.

Tree Number	Isolate number	Query length	Query coverage	% Maximum identity	Primary identification	Related identifications within 1% of max identity
T00903	18	1386	82%	99%	*Pseudomonas flavescens*	*Pseudomonas pseudoalcaligenes*
T00903	21	1361	87%	98%	*Pseudomonas sp.*	*P. xinjiangensis & P. sabulinigri*
T00903	22	1456	82%	98%	*Brenneria goodwinii*	*None*
T00903	23	1414	78%	99%	*Brenneria goodwinii*	*None*
T00903	24	1383	86%	97%	*Brenneria goodwinii*	*Enterobacter sp. & Brenneria rubrifaciens*
T00903	25	1428	80%	98%	*Brenneria goodwinii*	*None*
T00903	26	1401	87%	97%	*Pseudomonas xinjiangensis*	*Pseudomonas sabulinigri*
T00903	27	1332	89%	95%	*Brenneria goodwinii*	*Enterobacter sp.*

6.1.3 Sampling from the margin of the canker from Trees in Epping Forest and Writtle Forest in early Summer 2013

To establish that the isolates found at the margin of the canker were not unique to the bleed cankers found on the Oak trees at Writtle Forest further samples were taken from symptomatic trees in Epping Forest in June 2013. Four trees were sampled in Epping Forest. Sampling from

the canker margin from one tree revealed a bacterial isolate previously not identified, namely *Gibsiella quercinecans* (Table 6.5).

A further sample was then taken from a bacterial bleed canker in Writtle Forest also in early Summer (previous isolations were done in Spring) and this also revealed the presence of *Gibsiella quercinecans.*

Table 6.5 Sampling from the margin of the canker in early summer from Epping Forest & Writtle Forest.

Tree Number	**No. of isolates taken**	**No. of different morphologies recorded**	**Ident. Of dominant morphology**	**% of isolates with dominant morphology**	**Ident. Of secondary morphology**
T00931 Writtle Forest	29	4	*B.goodwinii*	42%	*G.quercinecans*
T0Q1 Epping Forest	11	4	*B.goodwinii*	60%	*Pseudomonas sp*
T0Q2 Epping Forest	9	4	*B.goodwinii*	55%	*Pseudomonas sp*
T0C1 Epping Forest	10	3	*B.goodwinii*	70%	*Pseudomonas sp*
T0C2 Epping Forest	9	3	*B.goodwinii*	66%	*G.quercinecans*

As before sequencing was carried out on a greater number of isolates from the margin canker of one tree to establish a better understanding of the bacteria isolated (Table 6.6). To this end tree T00931 as of the summer sampling of 2013 was chosen.

Table 6.6; 16S rDNA sequencing of isolates recovered from T00931 (Writtle Forest) at Margin of Canker

Tree Number	Isolate number	Query length (letters)	% of Query coverage	% of Maximum identity	Primary identification	Related identifications within 1% of max identity
T00931	1	1282	86%	98%	*Brenneria goodwinii*	none
T00931	7	1274	85%	97%	*Brenneria goodwinii*	none
T00931	13	1287	84%	98%	*Brenneria goodwinii*	none
T00931	22	1284	81%	98%	*Gibbsiella quercinecans*	*Brenneria quercina*
T00931	23	1272	82%	98%	*Brenneria goodwinii*	none
T00931	28	1188	84%	96%	*Brenneria goodwinii*	none

As before the dominant, culturable bacterium isolated from the canker was *Brenneria goodwinii*. However, the *Pseudomonas* strains were not consistently isolated from these collected samples. Why the *Gibbsiella quercinecans* bacterial isolate was not previously identified may be explained by the following reasons:

1) The descriptions of the colony morphology of *Brenneria goodwinii* and *Gibbsiella quercinecans* are almost identical other than in some instances the size of the colonies formed by *Gibbsiella quercinecans* are larger, 4-5mm in diameter as opposed to 2mm in diameter. Thus, it may have been that previously the bacteria had been isolated, but not distinguished from the *Brenneria* colonies.
2) The type of bleed cankers from which the *Gibbsiella quercinecans* bacteria was isolated were very similar in nature. When excavating a bleed canker there is often a release of pressurized liquid, but not always and generally the amount of liquid released is limited.

The bleed cankers from where *Gibbsiella quercinecans* was more readily isolated, released a continuous flow of pressurized liquid. The amount of liquid was not quantified but the pressurized flow of liquid lasted for approximately 3 to 4 minutes.

3) The timing of sampling was in early summer instead of spring. This might have influenced the effect of liquid pressure build up beneath the canker but may have also affected the population balance of bacteria present at the margin of the canker (Shakya et al, 2013, Shen and Fulthorpe, 2015).

6.2 Bacterial samples from Writtle Forest July 2016

6.2.1 Collection and Preparation of Samples

Samples were gathered again during July 2016 from ten symptomatic trees. Samples were taken from different trees throughout the woodland. (Chapter 2 section 2.5.2). Firstly, a swab was taken of the exudate using sterilized cotton wool. This was placed in Phosphate Buffer Saline (PBS) kept at between 2 -5°C. Then an area of approximately 40mm x 30mm was cut into using a sterilized chisel. Bark and wood tissue sample were collected separately, stored in PBS and kept at between 2 -5°C prior to preparation. (As of Chapter 2, section 2.5.2).

Wood samples were selected and processed under sterile conditions within the laboratory. Wood was selected that exhibited dark staining and was considered to be diseased. Wood tissue sampled that was not stained was considered to be healthy wood tissue. All samples were placed in 2 ml bead-beating tubes with 1mL of PBS. The tissue was homogenized at high speed (4m/s) for a total of 40 seconds. The resultant liquid was streaked on MacConkey Agar (with Crystal violet, Sodium chloride and 0.15% Bile salts). The plates were incubated for 24 hours at 27°C. This medium allows for preferential growth of Gram-negative bacteria associated with

the bleed symptoms. The varying ability of the bacterial species to ferment lactose meant that distinct morphological differences were discernable.

6.2.2 Selection and identification of culturable isolates

From each plate a representative bacterial colony was selected based on variance in morphology. (As of Chapter 2, section 2.6.3). Individual colonies were selected and re-plated using the three-point streak method onto MacConkeys agar. The cultures were incubated for 2 days at 27°C from which DNA was extracted to be used for PCR. The 16S gene was targeted for amplification and consequentially sequenced. The sequences identified for the bacteria are shown in Table 6.7. This showed clearly that Brenneria and Gibbsiella could be found in the healthy wood tissue of the symptomatic tree, as well as diseased wood and exudate. Other bacteria, also enterics, were also isolated from diseased wood, including *Rahnella*, *Raoultella*, *Pantoea* and *Serratia*, When the total numbers of bacteria recovered from the plate were counted, it was observed a predominance of *Rahnella* and *Raoultella* (Table 6.8).

Table 6.7 16S rDNA sequencing of isolates recovered from exudate, diseased and healthy wood of ten number trees in July 2016

Tree	**Number of Isolates identified for sequencing**	**Bacteria in diseased wood**	**Bacteria in healthy wood**	**Bacteria from swab**
01191	2 + 2 + 2	*Brenneria* (x2)	*Brenneria* *Pantoea*	*Pseudomonas* *Erwinia sp.*
01192	2 + 2 + 2	*Pantoea* *Erwinia*	*Pantoea* (x2)	*Pseudomonas* *Erwinia*
01193	3 + 2 + 3	*Rahnella* (x3)	*Rahnella* (x2)	*Kluyvera* *Gibbsiella* *Rahnella*

Tree	**Number of Isolates identified for sequencing**	**Bacteria in diseased wood**	**Bacteria in healthy wood**	**Bacteria from swab**
01194	4 + 1 + 1	*Raoultella* *Pantoea* *Serattia* *Gibbsiella*	*Gibbsiella*	*Brenneria*
01195	4 + 2 + 2	*Raoultella* *Rahnella* *Brenneria* *Gibbsiella*	*Rahnella* (x2)	*Raoultella* *Yersinia* sp.
01196	3 + 1 + 2	*Enterobacter* *Rahnella* *Brenneria*	*Gibbsiella*	*Brenneria* *Rahnella*
01197	3 + 2 + 2	*Rahnella* (x2) *Gibbsiella*	*Rahnella*	*Serratia* *Ewingella*
01198	3+ 2 + 2	*Rahnella* (x2) *Gibbsiella*	*Gibbsiella* (x2)	*Enterobacter* *Pseudomonas*
01199	3 + 2 + 2	*Raoultella* (x2) *Gibbsiella*	*Rahnella* *Gibbsiella*	*Raoultella* (x2)
01187	3 + 1 + 2	*Raoultella* *Rahnella* *Brenneria*	*Brenneria*	*Raoultella* *Brenneria*

6.2.3 Range of Bacterial species identified

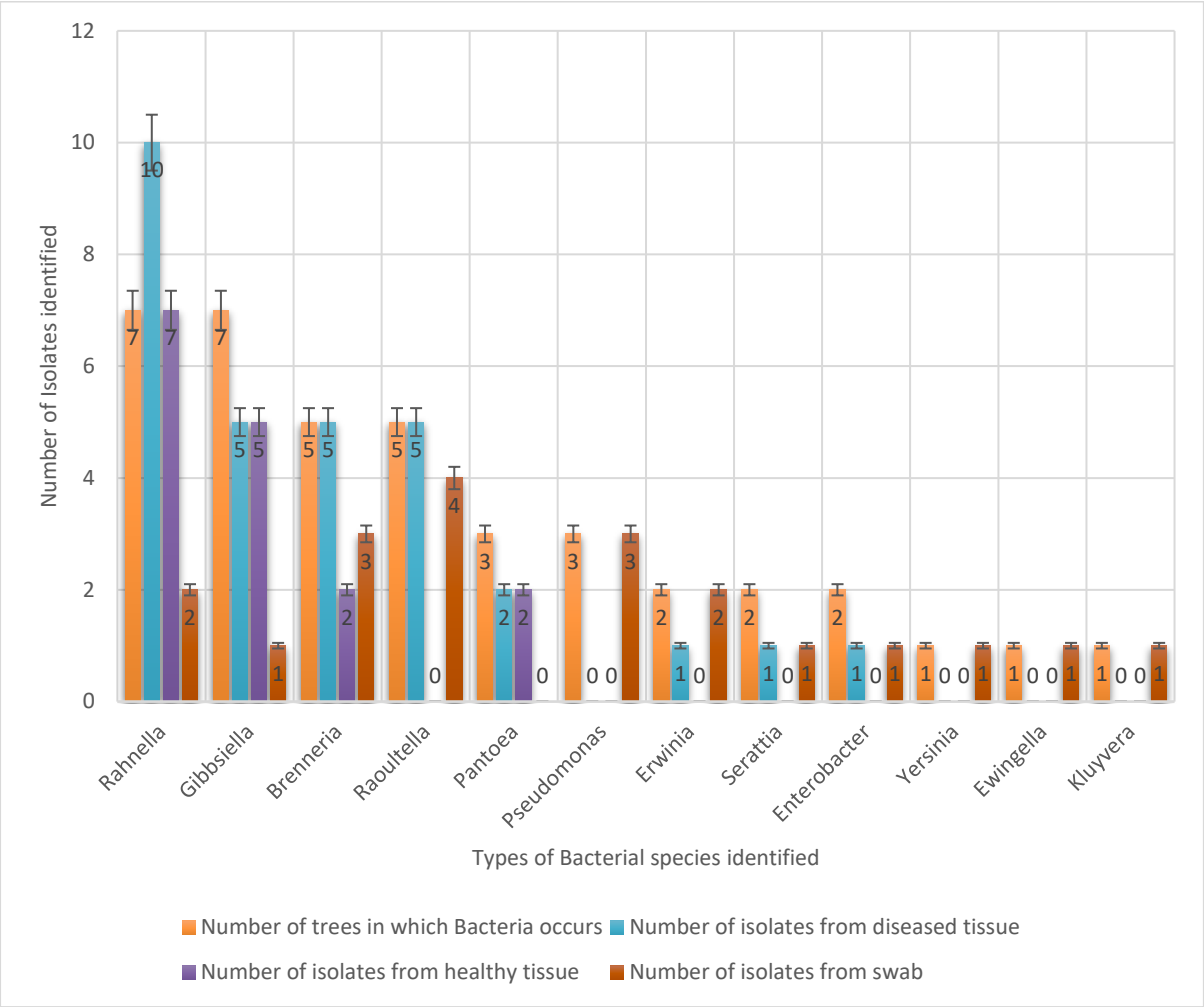


Fig. 6.1: The range of bacterial species cultured from exudate, diseased wood and healthy wood of ten trees in July 2016. Error bars = Percentage error of 5%

Table 6.9 The range of bacterial species cultured from exudate, diseased wood and healthy wood of ten trees in July 2016.

Bacteria	Number of trees in which Bacteria occurs	Number of isolates from diseased tissue	Number of isolates from healthy tissue	Number of isolates from swab	Total number of isolates	% retrieval from 10 trees	% of total 67 isolates recovered
Rahnella	7	10	7	2	19	70%	28.3%
Gibbsiella	7	5	5	1	11	70%	16.4%
Brenneria	5	5	2	3	10	50%	14.9%
Raoultella	5	5	0	4	9	50%	13.4%
Pantoea	3	2	2	0	5	30%	7.5%
Pseudomonas	3	0	0	3	3	30%	4.5%
Erwinia	2	1	0	2	3	20%	4.5%
Serattia	2	1	0	1	2	20%	3%
Enterobacter	2	1	0	1	2	20%	3%
Yersinia	1	0	0	1	1	10%	1.5%
Ewingella	1	0	0	1	1	10%	1.5%
Kluyvera	1	0	0	1	1	10%	1.5%

Of the 68 isolates sequenced, 1 returned a non- conclusive read.

6.3 Summary of Findings

A wide range of bacterial species were discoverable from the sampling. However, the sampling revealed the change in cultivatable bacteria due to seasonality, possibly related to temperature rises in the soils and woody material. These bacteria (in this instance *Rahnella victoriana*), maybe dormant within the woody material as viable but non-culturable (VBNC) bacteria.

The biggest challenge when investigating for suspected bacterial pathogens through cultivation is whether the causal organism can be cultivated. Within soils for example, only 1% of bacterial species can be readily cultivated by traditional methods. (Pham & Kim, 2012). Laboratory methods such as Simulation and Coculturing (Stewart, 2012) can be used to improve the recovery VBNC bacteria. To this end initial trials, not discussed, experimented by addition of infected material to wounding on trees (both within the field grown younger trees and on mature trees exhibiting signs of the bleed canker). None of these tests revealed replication of the bleed symptoms.

The sampling did not reveal a single bacterial species that was consistently isolated from all of the trees sampled with bleed cankers. Neither did it isolate a consistent group of bacteria.

Chapter 7: Results – Pathogenicity testing

7.1 Context of Research

The identification of the causal agents relating to the bleed canker is essential to the understanding and management of the syndrome. The aim of this study was to establish the pathogenic bacteria associated with the Oak Bleed Canker by satisfying Koch's postulates.

7.1 Pathogenicity Testing – Field and Glass House Tests

7.1.1 Initial inoculations to ascertain potential for testing

The first inoculations were carried out in January 2013 to establish whether the bleed symptoms could be replicated on the younger trees from the isolated bacteria from the symptomatic trees. (As of Chapter 2, section 2.7.1). Three 5-8year old *Quercus robur* trees with a stem diameter of between 40mm and 60mm were lifted in dormancy from University of Reading experimental grounds in November 2012. The soils around the roots were removed and the trees placed in 20 litre pots with John Innes Potting Compost number 3. The trees were placed in green house conditions at a temperature of between 18-22°C with approximately 16 hours of light. Under the imposed growing conditions, the trees came out of dormancy and were inoculated when the leaf had fully emerged in January 2013.

The initial inoculations were made with *Pseudomonas flavescens* strain 903_18, *Pseudomonas xinjiangensis* strain 903_21 and *Brenneria goodwinii* strain 903_22. The inoculations were carried out cutting with a scalpel into the main stem of the tree at a 45degree angle to an approximate depth of 5mm. Two incisions were made approx. 50mm apart, at a height of approx. 400mm from soil level. All cuts were made on the same side of the tree stem. 30uL of

inoculum was added directly to the exposed tissue of the bottom cut and 30uL of PBS added to the second cut. The resultant wounds were then sealed with Parafilm wrap.

After 60 days those trees infected with P. flavescens strain 903_18, P. xinjiangensis strain 903_21 showed no signs of exudate or bleeding at the area of the wound suggesting these strains were not pathogenic. After paring away the outer bark, cambium and sapwood the only evidence of stained wood tissue was directly within the area of the incision (Figs 7.1 & 7.2). B. goodwinii strain 903_22 showed signs of exudate after 30 days, leaving a watery black residue on the stem which later dried. Beads of black exudate approx. 20mm above the area of incision were also visible (Fig. 7.3). On paring away the stem tissue adjacent to the wound area, necrotic tissue was revealed extending beyond the wound site. (Fig's.7.3 and 7.4). This suggested this strain might be pathogenic on the trees.

Fig 7.1: Pseudomonas flavescens strain 903_18 after 60 days

Fig 7.2: *Pseudomonas xinjiangensis* strain 903_21 after 60 days

Fig 7.3: Brenneria goodwinii strain 903_22 showed signs of beads of black exudate approx. 20mm above the area of incision. (Area of red arrow)

Fig 7.4: *Brenneria goodwinii* strain 903_22 Staining/ necrotic tissue beyond the wound area is visible.

Tissue was isolated from the infected stem with *B. goodwinii* strain 903_22 from the area above the wound, the area below the wound, from the leaves and from the roots. The samples were placed in a 2 mL bead-beating tube with 1mL of Phosphate buffered saline (PBS). The tissue was homogenized at high speed (4m/s) for a total of 40 seconds. A dilution series was carried out using PBS as the diluent. 100µl of the pure sample was added to 900µl of PBS and mixed. 100µl of this solution (at 10^{-1}) was aspirated and diluted as before by adding to 900µl of PBS and mixed. This series was continued until a 10^{-6} suspension was prepared.

100µl of each dilution was then added to a plate of Luria-Bertani agar (LB) and spread to create an even lawn. The plates were incubated for between 3 and 5 days at 20°C. All isolates recovered from the area above the wound were identified through sequencing as the same *B. goodwinii* strain.

7.1.2 Field Inoculations 2013

(As of Chapter 2, section 2.7.2). Further tests were then carried out inoculating field grown trees approx. 5- 8 years old with a stem diameter of between 35mm and 60mm at 400mm from ground level. Two field trials in the University of Reading experimental grounds, were carried out in the Summer and Autumn of 2013 from July 2013 through to September 2013 and October 2013 through to December 2013.

16 trees were inoculated for each field trial. Cuts were made as previously described at a height of 400mm from ground level and 600mm.

2 trees were then inoculated in 2 areas with one of the following sets of inocula;

Pseudomonas flavescens strain 903_18, *Brenneria goodwinii* strain 903_22, *Pseudomonas xinjiangensis* strain 903_26, *Pseudomonas flavescens* strain 903_18 + *Brenneria goodwinii* strain 903_22, *Pseudomonas flavescens* strain 903_18 + *Pseudomonas xinjiangensis* strain 903_26, *Brenneria goodwinii* strain 903_22 + *Pseudomonas xinjiangensis* strain 903_26, *Pseudomonas flavescens* strain 903_18 + *Brenneria goodwinii* strain 903_22 + *Pseudomonas xinjiangensis* strain 903_26. As a control, Phosphate buffer saline was introduced within the remaining 2 trees.

30uL of inoculum was added to each bark incision, thus inoculations up to 90uL of total inoculum were introduced for the combination treatments. Staining was observed within the sapwood on harvesting, yet none of the inoculated sites exhibited any signs of exudate, the primary pathogenicity trait.

7.1.3 Green house Inoculations utilising BITE System

(As of Chapter 2, section 2.7.3). Nine trees were lifted from the field in October 2014 and placed under glasshouse conditions as previously described. Inoculations were made using the BITE infusion delivery system (Blade for infusion in Trees). This comprises of a hollow stainless-steel blade with a slide hammer which is used to hammer the blade into the tree. The principle is that the blade enters the tree by pushing the fibres of the wood apart (Dal Maso et al., 2014. Acimovic et al, 2016, Montecchio, 2013).

The previous method used had been to cut into the bark and wood material. This resulted in the tree developing wound or callous tissue in order to heal or close the wound. This wound tissue generally consists of smaller cells with a thicker wall structure, creating in effect a wall of defence against the spread of the pathogen (Shigo, 1989).

Whilst the BITE infusion system allows for a different method of introducing the inoculum, the system does not allow for quantities less than 2mL to be reliably introduced. Hence inoculum levels at 10^{9} CFU/mL were introduced with 2mL of inoculum. Three trees were initially inoculated in one area approx. 400mm from soil level. The inocula were as follows:

B. goodwinii strain 931_23 total 2mL inoculum, *G. quercinecans* strain 931_22 total 2mL inoculum, *B. goodwinii* strain 931_23 + *G. quercinecans* strain 931_22 total 2mL inoculum. Due to the limited availability of trees and the desire to test whether pathogenicity symptoms might first be observable, control inoculations were not included on this occasion.

The trees were reviewed every 5 to 10 days and the progress of the inoculations considered (Tables 7.1 and 7.2). These inoculations produced symptoms within the host trees consistent with those produced by symptomatic Oak trees, characterised as suffering from Acute Oak Decline. Exudates were seen on the host trees and gave positive results for *B. goodwinii* strain

931_23 (Fig. 7.7) and *Gibbsiella quercinecans* strain 931_22 (Fig. 7.8), as well as for a combined inoculum of both strains, (Fig. 7.9)

Table 7.1: Observations on inoculated trees with strains *Brenneria goodwinii* strain 931_23 + *Gibbsiella quercinecans* strain 931_22 after 20 days

Tree Ref	Inoculum and Ref	callous tissue	staining at wound	exudate at wound	bark rupture adjacent to wound	evidence of symptoms beyond wound area	Comments
792	*B.goodwinii* 931_23	no	yes	no	no	no	none
793	*B.goodwinii* 931_23	no	no	no	no	no	none
794	*B.goodwinii* 931_23	yes	no	no	no	no	none
795	*G. quercinecans* 931_22	yes	yes	yes - minor	yes	no	none
796	*G. quercinecans* 931_22	yes	yes	no	no	no	none
797	*G. quercinecans* 931_22	yes	no	no	no	no	none
798	*B.goodwinii* 931_23 + *G. quercinecans* 931_22	yes	yes	no	yes	no	none
799	*B.goodwinii* 931_23 + *G. quercinecans* 931_22	yes	yes	yes - heavy	yes	no	none
800	*B.goodwinii* 931_23 + *G. quercinecans* 931_22	yes	yes	yes - minor	yes	no	none

Table 7.2. Observations on inoculated trees with strains *Brenneria goodwinii* strain 931_23 + *Gibbsiella quercinecans* strain 931_22 after 45 days

Tree Ref	Inoculum and Ref	callous tissue	staining at wound	exudate at wound	bark rupture adjacent to wound	evidence of symptoms beyond	Comments
792	*B.goodwinii* 931_23	no	no	no	no	no	none
793	*B.goodwinii* 931_23	no	yes	yes	no	no	none
794	*B.goodwinii* 931_23	yes	no	no	yes	no	white fungal mat at wound area
795	*G. quercinecans* 931_22	yes	yes	yes - extensive	yes	no	callous tissue decayed
796	*G. quercinecans* 931_22	yes	yes	yes	no	no	none
797	*G. quercinecans* 931_22	yes	no	no	no	no	white fungal mat developing at wound area
798	*B.goodwinii* 931_23 + *G. quercinecans* 931_22	yes	yes minor	no	no	no	white fungal mat developing at wound area
799	*B.goodwinii* 931_23 + *G. quercinecans* 931_22	yes	yes	yes – extensive	yes	no	callous tissue decaying
800	*B.goodwinii* 931_23 + *G. quercinecans* 931_22	yes	yes	yes	yes	no	none

Tree 792

Tree 793

Tree 794

Fig 7.7: *Brenneria goodwinii* strain 931_23 after 45 days

Tree 795

Tree 796

Tree 797

Fig 7.8: *Gibbsiella quercinecans* strain 931_22 after 45 days

Tree 798	Tree 799	Tree 800

Fig 7.9: *Brenneria goodwinii* strain 931_23 and *Gibbsiella quercinecans* strain 931_22 after 45 days

7.1.4 Inoculated trees tested on drought stressed and watered trees in Glass house

Further tests were conducted within the glass house conditions to establish whether these results could be replicated and whether the bleed was affected by the amount of water that the tree received.

21 trees were lifted and placed into the Glasshouse in December 2015. This produced a relatively early flush of growth and trees were inoculated in Mid-April following complete flush of leaf. 18 trees were inoculated with bacteria and 3 were left as controls. The same bacterial strains were used, *B. goodwinii* 931_23 and *G. quercinecans* 931_22. Inoculation was via the BITE system using 2mL of inoculum at 10^9 CFU/mL introduced to each site. Each tree was inoculated 3 times at 150mm spacings. Once the inoculum was introduced the area was sealed with parafilm. Water control inoculations were also made.

Bleed was quantified and recorded over 65 days. 0 = No bleed, 1 = Minor bleed (Evident on lifting parafilm or less than 5% spread beneath parafilm). 2 = Normal Bleed. The area was wet

beneath parafilm within the immediate area of the inoculation site. An area between 5% to 35% of the tree girth appears wet from bleed beneath the parafilm. 3 = Major bleed. Wet beneath parafilm covering more than 40% of girth of the tree at area of inoculation. Other information recorded related to the development of callous or wound tissue at the area of inoculation. (Fig. 7.10).

Wood samples were recovered from the sites of inoculation. Bacterial colonies were isolated and sequenced as outlined within Chapter 2 section 2.7.8 (shown in Table 7.3). Morphological differences were identified between isolates and dominant colonies were chosen for sequencing. Samples were taken only from sites that exhibited bleed, except 2 sites on a drought stressed control tree.

There is a clear distinction showing a predominance of bleed with those trees which are watered within the 65day period. However, there is an increase in drought stressed trees with bleed toward the end of the monitoring period.

Sampling showed relatively good recovery of the bacteria with which the trees were inoculated. The exception to this was the watered trees inoculated with *Gibbsiella quercinecans.* In some instances, samples were also taken from inoculated areas on the tree which did not exhibit bleed symptoms but were adjacent to areas of bleed. Sequencing of the 16S rRNA gene of the bacterial isolates identified the bacterium inoculated at the distal site. Not all bacteria inoculated were recovered. Where trees were inoculated with both *B. goodwinii* and *G. quercinecans* sampling would in some instances only reveal one of these bacteria.

The trial was brought to conclusion in June due to the increased heated conditions in the glasshouse.

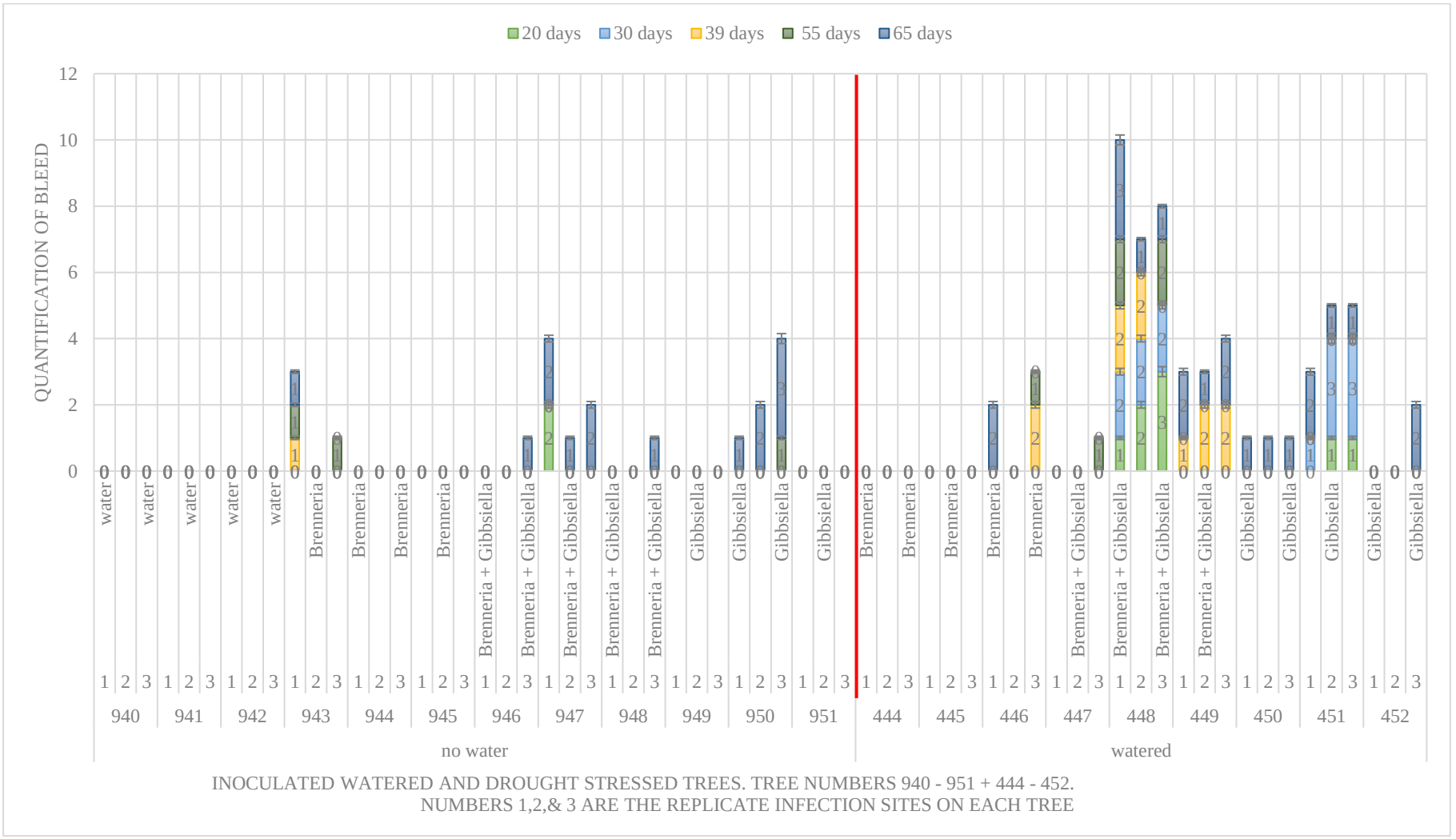


Fig 7.10: Observation of bleeds after the introduction of bacterial inoculations in *Q. robur* trees being watered and drought stressed. Error bars = Percentage error of 5%

Table 7.3: Identification by sequence analysis of the bacteria recovered from the tree inoculations.

Tree Number	**Position of inoculation**	**Bacteria recovered determined through sequencing**	**Inoculum/ inocula**
943	1	*Pseudomonas putida*	*Brenneria goodwinii*
943	2	*Brenneria goodwinii*	*Brenneria goodwinii*
946	3	*Gibbsiella quercinecans*	*Brenneria goodwinii* + *Gibbsiella quercinecans*
947	1	*Brenneria goodwinii* *Gibbsiella quercinecans*	*Brenneria goodwinii* + *Gibbsiella quercinecans*
947	3	*Gibbsiella quercinecans* *Stenotrophomonas rhizophila* *Brenneria goodwinii*	*Brenneria goodwinii* + *Gibbsiella quercinecans*
948	3	*Brenneria goodwinii*	*Brenneria goodwinii* + *Gibbsiella quercinecans*
950	1	*Gibbsiella quercinecans*	*Gibbsiella quercinecans*
950	3	*Gibbsiella quercinecans*	*Gibbsiella quercinecans*
446	1	*Brenneria goodwinii*	*Brenneria goodwinii*
446	2	*Rahnella sp*	*Brenneria goodwinii*
447	2	*Gibbsiella quercinecans*	*Brenneria goodwinii* + *Gibbsiella quercinecans*

Tree Number	**Position of inoculation**	**Bacteria recovered determined through sequencing**	**Inoculum/ inocula**
448	1	*Gibbsiella quercinecans* *Stenotrophomonas sp*	*Brenneria goodwinii* + *Gibbsiella quercinecans*
448	3	*Stenotrophomonas sp*	*Brenneria goodwinii* + *Gibbsiella quercinecans*
449	1	*Brenneria goodwinii* *Gibbsiella quercinecans*	*Brenneria goodwinii* + *Gibbsiella quercinecans*
449	3	*Gibbsiella quercinecans*	*Brenneria goodwinii* + *Gibbsiella quercinecans*
450	2	*Stenotrophomonas sp* *Bacillus sp*	*Gibbsiella quercinecans*
451	2	*Stenotrophomonas sp*	*Gibbsiella quercinecans*
451	3	*Pseudomonas sp*	*Gibbsiella quercinecans*
452	3	*Gibbsiella quercinecans*	*Gibbsiella quercinecans*

7.2 Controlled Environment (CE) Room Inoculation Tests

(As of Chapter 2, section 2.7.4). Given the variability in temperature being experienced in the glasshouses, it was decided to carry out further experiments in Controlled Environment (CE) rooms, which could control humidity, temperature and sunlight hours. Conditions were chosen that were considered to best represent growing conditions within a broad leaf woodland in late Spring. The humidity was set at 60%. 12 hours of daylight and 12 hours of dark were provided. The daylight hours temperature was set at 20°C, the non-light temperature was set at 16°C.

18 trees were lifted from the field and placed in 20 litre pots with an inert potting compost. Trees were lifted in dormancy for the experiments conducted from April to July 2017 and March to June 2018. Trees were lifted from the field whilst in full leaf for the experiment conducted between September and December 2017. The controlled environment rooms have a height restriction of approx. 2m and are limited in size. The trees were pruned whilst in leaf using natural target pruning techniques (Shigo, 1991), to a height of approximately 1.8m, with a crown diameter of approximately 1 metre. This represented approximately 50-75% canopy loss for each tree.

The effect of reducing the trees to this extent reduces the photosynthetic capabilities of the trees, as does the root loss consequent to the lifting of the tree reduce utilisable starch levels. (Shigo, 1990). All trees are thus subject to a degree of stress. This was not seen as counterproductive to the experiments because 1) all trees are similarly reduced, 2) all trees are of relatively similar age and vigour (initially established through Fluorimetry testing) and 3) that the ability to inoculate a functional plant within a controlled environment was the primary parameter for testing, rather than a tree that was in optimum health.

The bacteria for these experiments were selected from review of bacterial isolates from sampled symptomatic trees in Writtle Forest in 2016. This sampling regime had revealed

consistent isolation of two further bacterial species, *Rahnella* sp. and *Raoultella* sp., formerly designated *Klebsiella* (Drancourt et al, 2001). Sample strains selected for inoculation were taken from one tree from which all 4 species *Rahnella* sp., *Raoultella* sp., *B. goodwinii* and *G. quercinecans* were isolated (Tree tagged 1195).

All inoculations were delivered by creating an entry wound with the use of the BITE system. The area was bound with parafilm and the inoculum was introduced using a hypodermic syringe. Inoculum was prepared at 10^{9} CFU/mL in a Phosphate Buffer Solution (PBS). 1mL of inoculum was injected at three different areas at 150mm spacings on each tree.

Where combinations of bacteria were used as inoculum each bacterial suspension was prepared separately and added together at appropriate ratios to equate to 1mL of delivered inoculum.

7.2.1 CE inoculations April 2017 to July 2017

Prior to inoculations, samples were taken at a height of approx. 1.5m on the main stem (above all areas of inoculation), to establish the culturable bacterial species within the test trees (Table 7.4). The methods for sampling and culture of isolates were as previously described.

Delivery of inoculum was as of Chapter 2 section 2.7.7. Three trees were inoculated, in three places at 150mm spacings, 400mm from soil level with the following inocula: *Brenneria, Gibbsiella, Brenneria + Gibbsiella, Rahnella + Raoultella, Rahnella + Raoultella + Brenneria + Gibbsiella*. The control inocula used were 1mL of Phosphate Buffer solution and 1mL at 10^{9} CFU/mL *Escherichia coli* DH5α. This latter control was used as a non-pathogenic bacterial isolate control for non-specific symptoms.

Trees were monitored as of Chapter 2 section 2.7.8. The results of monitoring the effects of the inoculated trees over a period of 124 days are shown in Fig 7.11.

Of those wounds that exhibited signs of the bleed canker a sample (approximately 1 gram) was taken of the infected wood within the area of the wound after 124 days. This sample was

homogenised and plated out onto MacConkey's agar. Colonies were selected for isolation and for consequent sequencing based on morphological differences identified. As of Chapter 2 section 2.7.8. Results shown in Table 7.5.

The harvested stems were investigated to establish if there was any correlation between the amount of bleed and the consequent internal staining of the wood at the site of the inoculum (Fig. 7.12). The depth of the staining is not recorded within Fig. 7.12. This varied between 4 and 5mm dependent upon the depth at which the inoculum was inserted. There was no spread of staining further into the wood. The vertical spread/ length of stain was both upwards and downwards of the stem. The horizontal spread/ width of stain was directly measured from the area where the inoculation was made. These results are discussed within 7.3 Summary.

Table 7.4. Identification of bacterial isolates recovered from infected *Q. robur* trees before introduction of the inoculum for the experiment.

Tree number	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Inoculum**
175	*Erwinia sp*	*Pantoea agglomerans*	-	*Brenneria + Gibbsiella*
176	*Pantoea agglomerans*	*Pantoea agglomerans*	*Pantoea agglomerans*	*Brenneria + Gibbsiella*
177	*Pantoea agglomerans*	*Pantoea agglomerans*	-	*Brenneria + Gibbsiella*
890	*Pantoea agglomerans*	*Pantoea agglomerans*	-	PBS */ E. coli*

178	*Pantoea agglomerans*	*Pantoea agglomerans*	-	*Rahnella + Raoultella*
179	*Pantoea sp*	-	-	*Rahnella + Raoultella*
180	No isolates recovered			*Rahnella + Raoultella*
477	No isolates recovered			*Gibbsiella*
478	*Pantoea agglomerans*	-	-	*Gibbsiella*
479	*Pantoea agglomerans*	-	-	*Gibbsiella*
134	*Erwinia sp*	*Erwinia sp*	-	*Brenneria*
135	*Erwinia sp*	*Erwinia sp*	-	*Brenneria*
136	*Serratia/ Erwinia*	-	-	*Brenneria*
137	*Pantoea agglomerans*	*Erwinia sp*	-	*Rahnella + Raoultella + Brenneria + Gibbsiella*
138	No isolates recovered			*Rahnella + Raoultella + Brenneria + Gibbsiella*
139	*Pantoea sp*	-	-	*Rahnella + Raoultella + Brenneria + Gibbsiella*

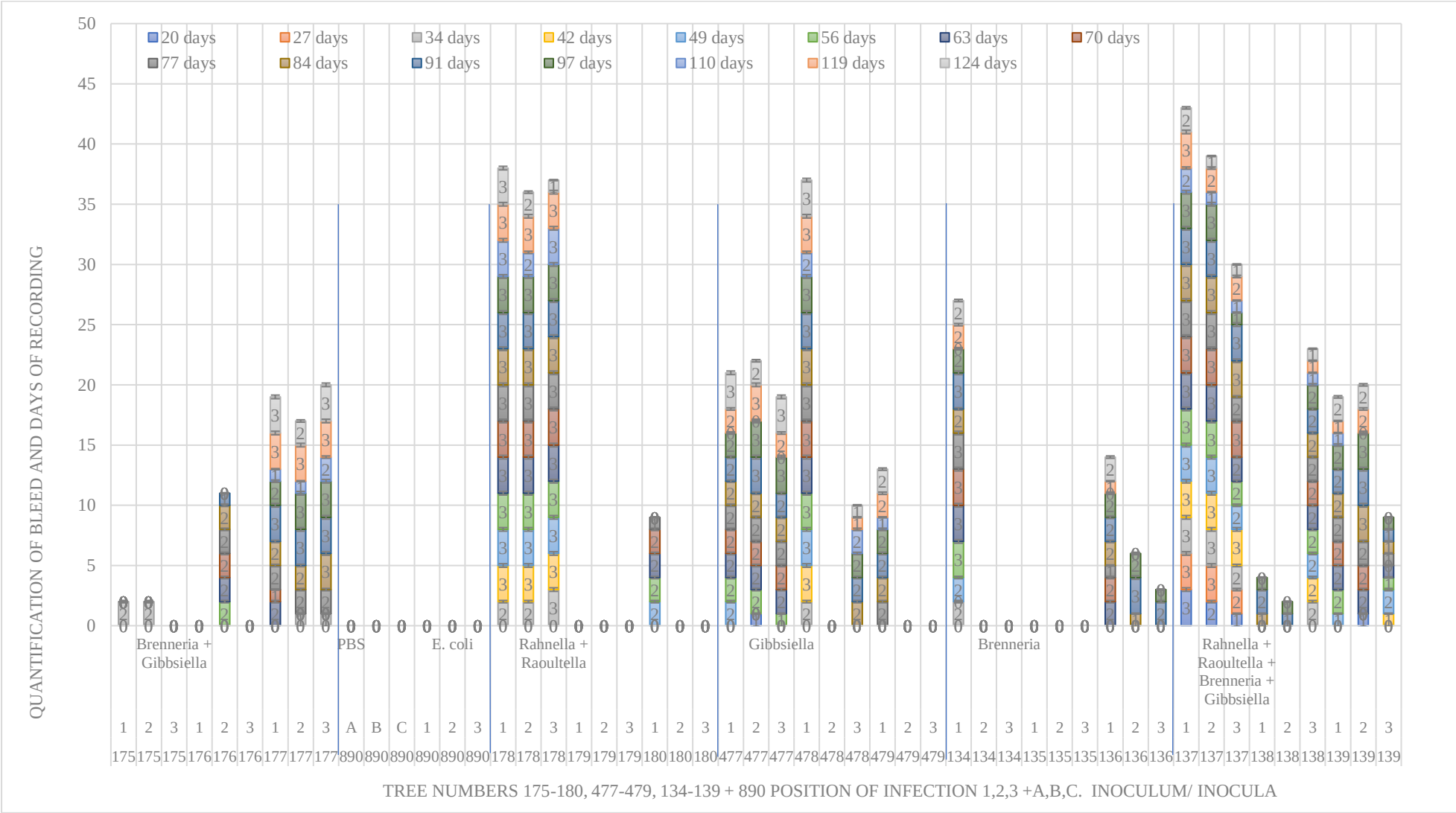


Fig 7.11: Observation of bleeds after the introduction of bacterial inoculations into *Q. robur* trees in April – July 2017. Error bars = Percentage error of 5%

Table 7.5: Identification of bacterial isolates recovered from infected *Q. robur* trees in the April - July 2017 experiment.

Tree number	**position**	**inoculum**	**isolate 1**	**isolate 2**	**isolate 3**	**isolate 4**	**isolate 5**
175	1	*Brenneria + Gibbsiella*	*B. goodwinii*	*G. quercinecans*	*Pseudomonas sp*		
175	2	*Brenneria + Gibbsiella*	*B. goodwinii*	*Gibbsiella sp*	*Erwinia sp*		
175	3	*Brenneria + Gibbsiella*	no bleed				
176	1	*Brenneria + Gibbsiella*	no bleed				
176	2	*Brenneria + Gibbsiella*	*B. goodwinii*	*G. quercinecans*			
176	3	*Brenneria + Gibbsiella*	no bleed				
177	1	*Brenneria + Gibbsiella*	*Stenotrophomonas sp*	*Pseudomonas sp*	*P. fluorescens*		
177	2	*Brenneria + Gibbsiella*	*Stenotrophomonas sp*	*G. quercinecans*			
177	3	*Brenneria + Gibbsiella*	*Stenotrophomonas sp*				
890	A	PBS	no bleed				
890	B	PBS	no bleed				
890	C	PBS	no bleed				
890	1	E. coli	no bleed				
890	2	E. coli	no bleed				
890	3	E. coli	no bleed				
178	1	*Rahnella + Raoultella*	*R. victoriana*	*Raoultella sp*			
178	2	*Rahnella + Raoultella*	*R. victoriana*	*Raoultella sp*			
178	3	*Rahnella + Raoultella*	*Stenotrophomonas sp*	*Raoultella sp*			
179	1	*Rahnella + Raoultella*	no bleed				
179	2	*Rahnella + Raoultella*	no bleed				

Tree number	position	inoculum	isolate 1	isolate 2	isolate 3	isolate 4	isolate 5
179	3	*Rahnella + Raoultella*	no bleed				
180	1	*Rahnella + Raoultella*	*R. victoriana*	*Raoultella sp*			
180	2	*Rahnella + Raoultella*	no bleed				
180	3	*Rahnella + Raoultella*	no bleed				
477	1	*Gibbsiella*	*G. quercinecans*				
477	2	*Gibbsiella*	*G. quercinecans*				
477	3	*Gibbsiella*	*G. quercinecans*				
478	1	*Gibbsiella*	*Gibbsiella sp*	*Pseudomonas sp*			
478	2	*Gibbsiella*	no bleed				
478	3	*Gibbsiella*	*G. quercinecans*				
479	1	*Gibbsiella*	*Pseudomonas sp*	*P. putida*			
479	2	*Gibbsiella*	no bleed				
479	3	*Gibbsiella*	no bleed				
134	1	*Brenneria*	*B. goodwinii*	*P. putida*			
134	2	*Brenneria*	no bleed				
134	3	*Brenneria*	no bleed				
135	1	*Brenneria*	no bleed				
135	2	*Brenneria*	no bleed				
135	3	*Brenneria*	no bleed				
136	1	*Brenneria*	*B. goodwinii*	*Pseudomonas sp*			
136	2	*Brenneria*	*P. fluorescens*	*P. putida*			
136	3	*Brenneria*	*Pseudomonas sp*				
137	1	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Raoultella sp*	*G. quercinecans*	*B. goodwinii*		

Tree number	position	inoculum	isolate 1	isolate 2	isolate 3	isolate 4	isolate 5
137	2	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*P. fluorescens*	*Gibbsiella sp*	*B. goodwinii*		
137	3	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Raoultella sp*	*Gibbsiella sp*	*B. goodwinii*	*R. victoriana*	
138	1	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Raoultella sp*	*G. quercinecans*	*B. goodwinii*	*Rahnella sp*	
138	2	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Raoultella sp*	*G. quercinecans*			
138	3	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Stenotrophomonas sp*	*Raoultella sp*	*R. victoriana*		
139	1	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Pseudomonas sp*	*Gibbsiella sp*	*Raoultella sp*	*R. victoriana*	*P. putida*
139	2	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Stenotrophomonas sp*	*Raoultella planticola*	*G. quercinecans*	*B. goodwinii*	
139	3	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Raoultella sp*	*G. quercinecans*	*B. goodwinii*	*R. victoriana*	

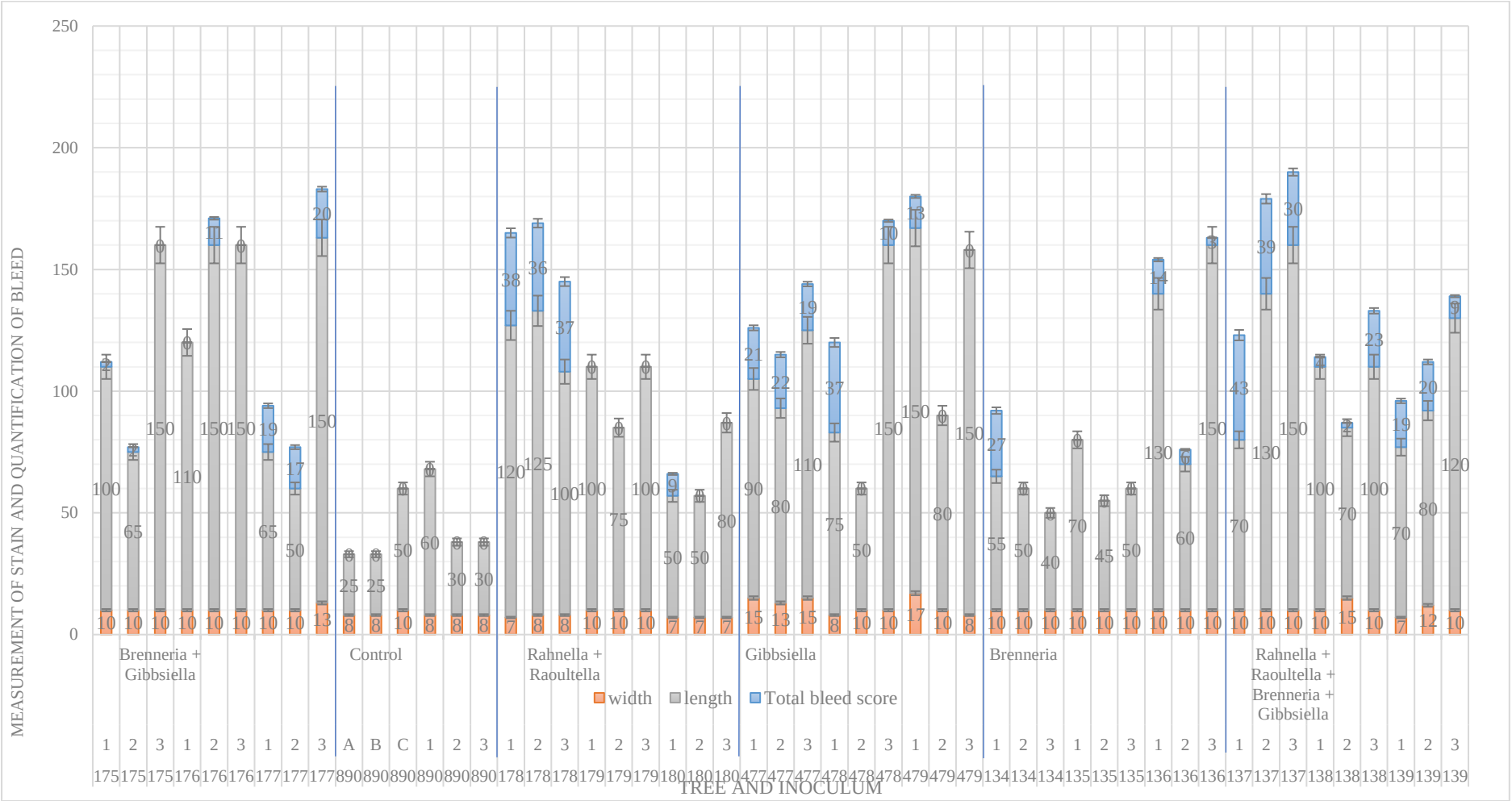


Fig. 7.12. Observation of staining within vascular tissue comparative to total quantification of bleed from April - July 2017 inoculations. Error bars = Percentage error of 5%

7.2.2 CE inoculations September 2017 to December 2017

18 trees were previously organised to fit within the CE room. As of the inoculations from September to December 2017 (and March to June 2018) 24 trees were fitted into the CE rooms. This allowed for consideration of more combinations of the bacteria.

24 trees were selected and lifted from the field plot in August/ early September. Bacterial samples were retrieved prior to inoculation. Colonies were cultured as of previous methodology. Morphologies were visual compared. As with the previous sampling of the trees prior to inoculation two main groups were identified. Sequencing of representative samples from these groups showed that the two dominant recoverable bacteria were as of the previous trees, pre- inoculation, *Pantoea agglomerans and Erwinia sp*. Fungal endophytes from the trees were also isolated prior to inoculation. The details of these findings are detailed and discussed within a consideration of Fungal endophytes as of Chapter 9.

As before three trees were inoculated, in three places at 150mm spacings, 400mm from soil level. In this instance the following inocula were used: *Raoultella, Rahnella, Rahnella + Brenneria, Rahnella + Gibbsiella, Rahnella + Brenneria + Gibbsiella, Raoultella + Brenneria + Gibbsiella, Rahnella + Raoultella + Brenneria + Gibbsiella*. The control inocula used was 1mL of Phosphate Buffer solution.

Trees were monitored as of Chapter 2 section 2.7.8. The results of monitoring the effects of the inoculated trees over a period of 124 days are shown in Fig 7.13.

Of those wounds that exhibited signs of the bleed canker a sample (approximately 1 gram) was taken of the infected wood within the area of the wound after 124 days. This sample was homogenised and plated out onto MacConkey's agar. Colonies were selected for isolation and for consequent sequencing based on morphological differences identified. As of Chapter 2 section 2.7.8. Results shown in Table 7.6.

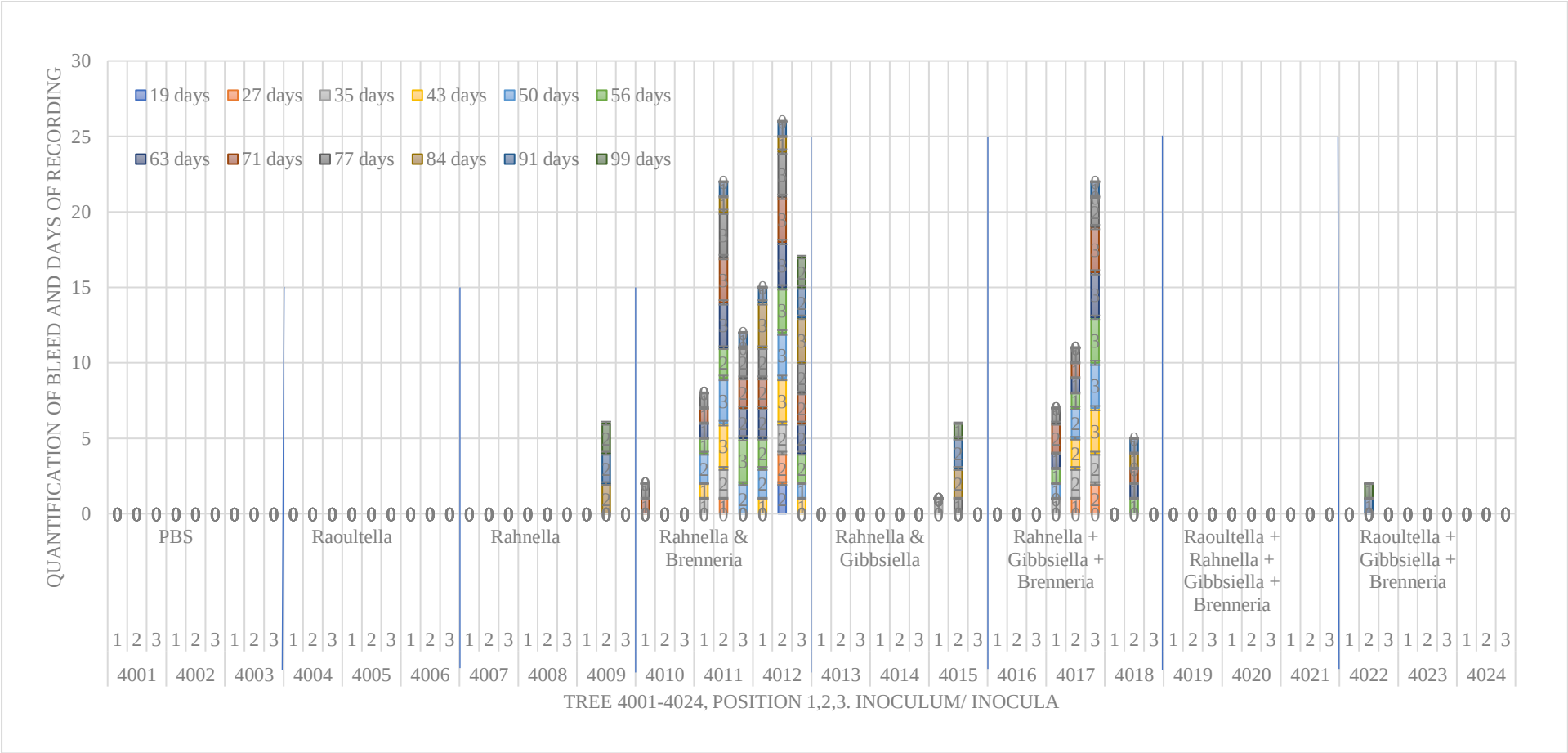


Fig. 7.13. Observation of bleeds after the introduction of bacterial inoculations into *Q. robur* trees in September – December 2017. Error bars = Percentage error of 5%

Table 7.6: Identification of bacterial isolates recovered from infected *Q. robur* trees in the September - December 2017 inoculations

Tree number	**position**	**inoculum**	**isolate 1**	**isolate 2**	**isolate 3**	**isolate 4**
4001	1	PBS	No bleed			
4001	2	PBS	No bleed			
4001	3	PBS	No bleed			
4002	1	PBS	No bleed			
4002	2	PBS	No bleed			
4002	3	PBS	No bleed			
4003	1	PBS	No bleed			
4003	2	PBS	No bleed			
4003	3	PBS	No bleed			
4004	1	*Raoultella*	No bleed			
4004	2	*Raoultella*	No bleed			
4004	3	*Raoultella*	No bleed			
4005	1	*Raoultella*	No bleed			
4005	2	*Raoultella*	No bleed			
4005	3	*Raoultella*	No bleed			
4006	1	*Raoultella*	No bleed			
4006	2	*Raoultella*	No bleed			
4006	3	*Raoultella*	No bleed			
4007	1	*Rahnella*	No bleed			
4007	2	*Rahnella*	No bleed			
4007	3	*Rahnella*	No bleed			
4008	1	*Rahnella*	No bleed			

Tree number	**position**	**inoculum**	**isolate 1**	**isolate 2**	**isolate 3**	**isolate 4**
4008	2	*Rahnella*	No bleed			
4008	3	*Rahnella*	No bleed			
4009	1	*Rahnella*	No bleed			
4009	2	*Rahnella*	*Erwinia sp*	*R. victoriana*		
4009	3	*Rahnella*	no bleed			
4010	1	*Rahnella + Brenneria*	*R. victoriana*	*Erwinia sp*	*Pseudomonas sp*	
4010	2	*Rahnella + Brenneria*	no bleed			
4010	3	*Rahnella + Brenneria*	no bleed			
4011	1	*Rahnella + Brenneria*	*R. victoriana*	*Raoultella planticola*	*Pseudomonas sp*	*Erwinia sp*
4011	2	*Rahnella + Brenneria*	*R. victoriana*	*Pseudomonas sp*	*Raoultella planticola*	
4011	3	*Rahnella + Brenneria*	*R. victoriana*			
4012	1	*Rahnella + Brenneria*	*R. victoriana*	*Erwinia sp*		
4012	2	*Rahnella + Brenneria*	*R. victoriana*	*Erwinia sp*		
4012	3	*Rahnella + Brenneria*	*R. victoriana*	*Erwinia sp*		
4013	1	*Rahnella + Gibbsiella*	No bleed			
4013	2	*Rahnella + Gibbsiella*	No bleed			
4013	3	*Rahnella + Gibbsiella*	No bleed			
4014	1	*Rahnella + Gibbsiella*	No bleed			
4014	2	*Rahnella + Gibbsiella*	No bleed			
4014	3	*Rahnella + Gibbsiella*	No bleed			
4015	1	*Rahnella + Gibbsiella*	*R. victoriana*	*Erwinia sp*	*Pseudomonas sp*	
4015	2	*Rahnella + Gibbsiella*	*Pseudomonas sp*			
4015	3	*Rahnella + Gibbsiella*	No bleed			
4016	1	*Rahnella + Brenneria + Gibbsiella*	No bleed			
4016	2	*Rahnella + Brenneria + Gibbsiella*	No bleed			
4016	3	*Rahnella + Brenneria + Gibbsiella*	No bleed			

Tree number	position	inoculum	isolate 1	isolate 2	isolate 3	isolate 4
4017	1	*Rahnella + Brenneria + Gibbsiella*	*Raoultella planticola*	*R. victoriana*		
4017	2	*Rahnella + Brenneria + Gibbsiella*	*Raoultella planticola*	*R. victoriana*	*Erwinia sp*	
4017	3	*Rahnella + Brenneria + Gibbsiella*	*Raoultella planticola*	*Stenotrophomonas sp.*		
4018	1	*Rahnella + Brenneria + Gibbsiella*	No bleed			
4018	2	*Rahnella + Brenneria + Gibbsiella*	*R. victoriana*			
4018	3	*Rahnella + Brenneria + Gibbsiella*	No bleed			
4019	1	*Rahnella + Raoultella + Brenneria + Gibbsiella*	No bleed			
4019	2	*Rahnella + Raoultella + Brenneria + Gibbsiella*	No bleed			
4019	3	*Rahnella + Raoultella + Brenneria + Gibbsiella*	No bleed			
4020	1	*Rahnella + Raoultella + Brenneria + Gibbsiella*	No bleed			
4020	2	*Rahnella + Raoultella + Brenneria + Gibbsiella*	No bleed			
4020	3	*Rahnella + Raoultella + Brenneria + Gibbsiella*	No bleed			
4021	1	*Rahnella + Raoultella + Brenneria + Gibbsiella*	No bleed			
4021	2	*Rahnella + Raoultella + Brenneria + Gibbsiella*	No bleed			
4021	3	*Rahnella + Raoultella + Brenneria + Gibbsiella*	No bleed			
4022	1	*Raoultella + Brenneria + Gibbsiella*	No bleed			
4022	2	*Raoultella + Brenneria + Gibbsiella*	*Raoultella planticola*			
4022	3	*Raoultella + Brenneria + Gibbsiella*	No bleed			
4023	1	*Raoultella + Brenneria + Gibbsiella*	No bleed			
4023	2	*Raoultella + Brenneria + Gibbsiella*	No bleed			
4023	3	*Raoultella + Brenneria + Gibbsiella*	No bleed			
4024	1	*Raoultella + Brenneria + Gibbsiella*	No bleed			
4024	2	*Raoultella + Brenneria + Gibbsiella*	No bleed			
4024	3	*Raoultella + Brenneria + Gibbsiella*	No bleed			

7.2.3 CE inoculations March 2018 to June 2018

In this instance no samples were taken prior to inoculating the trees to establish bacteria present before inoculation. This was due to financial constraints.

24 trees were selected and lifted from the field plot in February 2018. Bacterial samples in this instance were not retrieved prior to inoculation. The previous 2 isolations prior to infection had yielded similar results and there were financial constraints on materials.

As before three trees were inoculated, in three places at 150mm spacings, 400mm from soil level. In this instance the following inocula were used: *Raoultella, Rahnella, Rahnella + Brenneria, Rahnella + Gibbsiella, Rahnella + Brenneria + Gibbsiella, Raoultella + Brenneria + Gibbsiella, Rahnella + Raoultella + Brenneria + Gibbsiella.* The control inoculum used was 1mL of Phosphate Buffer solution.

Trees were monitored as of Chapter 2 section 2.7.8. The results of monitoring the effects of the inoculated trees over a period of 102 days are shown in Fig 7.14.

Of those wounds that exhibited signs of the bleed canker a sample (approximately 1 gram) was taken of the infected wood within the area of the wound after 102 days. This sample was homogenised and plated out onto MacConkey's agar. Colonies were selected for isolation and for consequent sequencing based on morphological differences identified. As of Chapter 2 section 2.7.8. Results shown in Table 7.7.

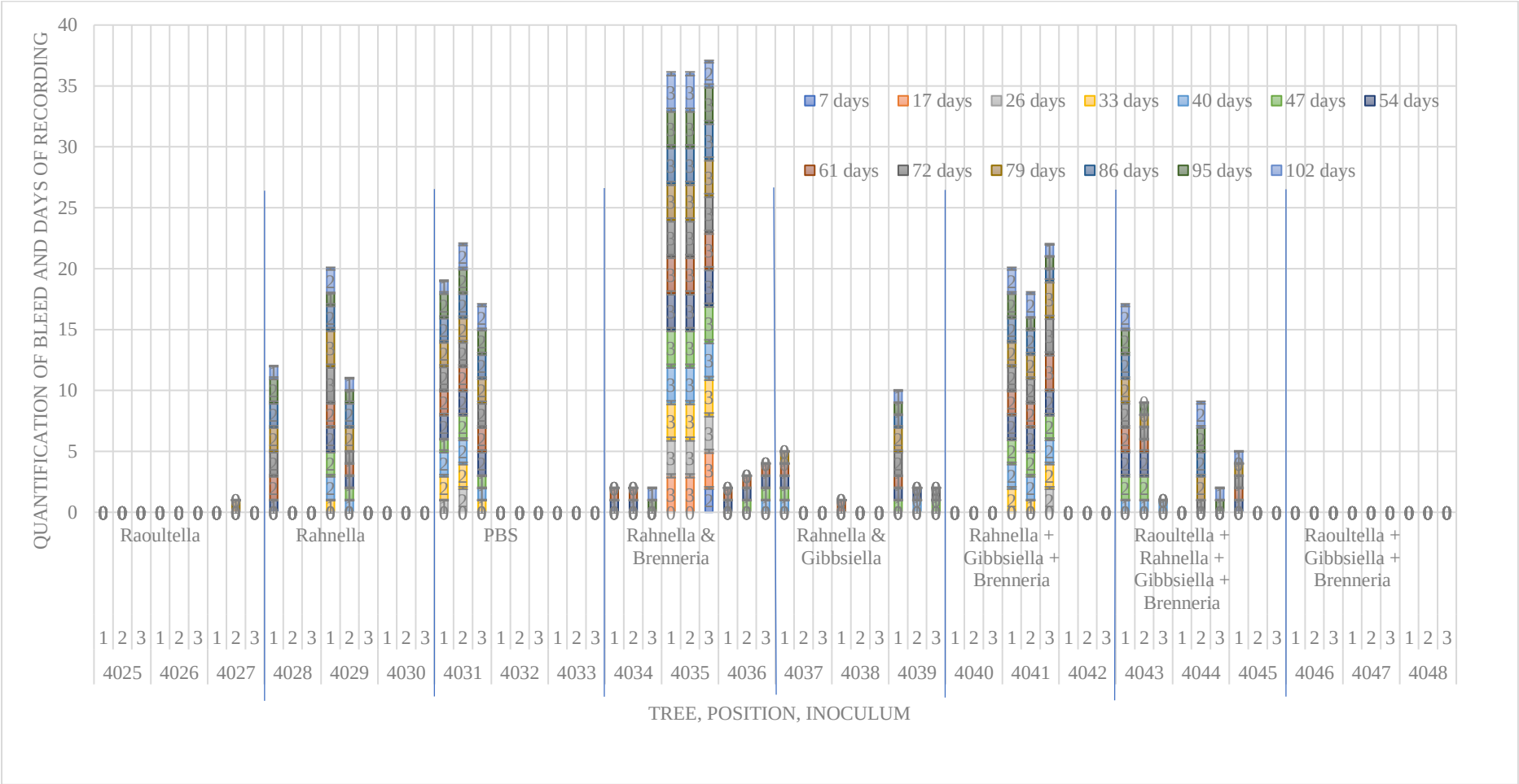


Fig. 7.14. Observation of bleeds after the introduction of bacterial inoculations into *Q. robur* trees in March – June 2018. Error bars = Percentage error of 5%

Table 7.7. Identification of bacterial isolates recovered from infected *Q. robur* trees in the March - June 2018 inoculations.

Tree number	**position**	**inoculum**	**isolate 1**	**isolate 2**	**isolate 3**	**isolate 4**
4025	1	*Raoultella*	no bleed			
4025	2	*Raoultella*	no bleed			
4025	3	*Raoultella*	no bleed			
4026	1	*Raoultella*	no bleed			
4026	2	*Raoultella*	no bleed			
4026	3	*Raoultella*	no bleed			
4027	1	*Raoultella*	no bleed			
4027	2	*Raoultella*	*Raoultella planticola*			
4027	3	*Raoultella*	no bleed			
4028	1	*Rahnella*	*R. victoriana*			
4028	2	*Rahnella*	no bleed			
4028	3	*Rahnella*	no bleed			
4029	1	*Rahnella*	*R. victoriana*			
4029	2	*Rahnella*	*R. victoriana*			
4029	3	*Rahnella*	no bleed			
4030	1	*Rahnella*	no bleed			

Tree number	**position**	**inoculum**	**isolate 1**	**isolate 2**	**isolate 3**	**isolate 4**
4030	2	*Rahnella*	no bleed			
4030	3	*Rahnella*	no bleed			
4031	1	PBS	*Erwinia sp.*			
4031	2	PBS	*G. quercinecans.*	*P. agglomerans*		
4031	3	PBS	*G. quercinecans.*			
4032	1	PBS	no bleed			
4032	2	PBS	no bleed			
4032	3	PBS	no bleed			
4033	1	PBS	no bleed			
4033	2	PBS	no bleed			
4033	3	PBS	no bleed			
4034	1	*Rahnella + Brenneria*	*R. victoriana*	*Erwinia sp.*		
4034	2	*Rahnella + Brenneria*	*R. victoriana*			
4034	3	*Rahnella + Brenneria*	*R. victoriana*			
4035	1	*Rahnella + Brenneria*	*R. victoriana*	*B. goodwinii*		
4035	2	*Rahnella + Brenneria*	*R. victoriana*	*Pseudomonas sp*		
4035	3	*Rahnella + Brenneria*	*R. victoriana*	*B. goodwinii*		
4036	1	*Rahnella + Brenneria*	*Pseudomonas sp*			
4036	2	*Rahnella + Brenneria*	*R. victoriana*	*B. goodwinii*		

Tree number	**position**	**inoculum**	**isolate 1**	**isolate 2**	**isolate 3**	**isolate 4**
4036	3	*Rahnella + Brenneria*	*R. victoriana*			
4037	1	*Rahnella + Gibbsiella*	*Gibbsiella sp.*			
4037	2	*Rahnella + Gibbsiella*	no bleed			
4037	3	*Rahnella + Gibbsiella*	no bleed			
4038	1	*Rahnella + Gibbsiella*	*G. quercinecans*			
4038	2	*Rahnella + Gibbsiella*	no bleed			
4038	3	*Rahnella + Gibbsiella*	no bleed			
4039	1	*Rahnella + Gibbsiella*	*G. quercinecans*			
4039	2	*Rahnella + Gibbsiella*	*Gibbsiella sp.*			
4039	3	*Rahnella + Gibbsiella*	*Stenotrophomonas sp.*			
4040	1	*Rahnella + Brenneria + Gibbsiella*	no bleed			
4040	2	*Rahnella + Brenneria + Gibbsiella*	no bleed			
4040	3	*Rahnella + Brenneria + Gibbsiella*	no bleed			
4041	1	*Rahnella + Brenneria + Gibbsiella*	*G. quercinecans.*	*R. victoriana*	*B. goodwinii*	
4041	2	*Rahnella + Brenneria + Gibbsiella*	*G. quercinecans*	*B. goodwinii*		
4041	3	*Rahnella + Brenneria + Gibbsiella*	*Gibbsiella sp.*	*R. victoriana*		
4042	1	*Rahnella + Brenneria + Gibbsiella*	no bleed			
4042	2	*Rahnella + Brenneria + Gibbsiella*	no bleed			
4042	3	*Rahnella + Brenneria + Gibbsiella*	no bleed			
4043	1	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Raoultella planticola*	*G. quercinecans.*	*B. goodwinii*	

Tree number	**position**	**inoculum**	**isolate 1**	**isolate 2**	**isolate 3**	**isolate 4**
4043	2	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Raoultella planticola*	*Gibbsiella sp*		
4043	3	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Raoultella planticola*	*Pseudomonas sp*		
4044	1	*Rahnella + Raoultella + Brenneria + Gibbsiella*	no bleed			
4044	2	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Raoultella planticola*	*G. quercinecans.*		
4044	3	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Raoultella planticola*			
4045	1	*Rahnella + Raoultella + Brenneria + Gibbsiella*	*Raoultella planticola*	*Pseudomonas sp*		
4045	2	*Rahnella + Raoultella + Brenneria + Gibbsiella*	no bleed			
4045	3	*Rahnella + Raoultella + Brenneria + Gibbsiella*	no bleed			
4046	1	*Raoultella + Brenneria + Gibbsiella*	no bleed			
4046	2	*Raoultella + Brenneria + Gibbsiella*	no bleed			
4046	3	*Raoultella + Brenneria + Gibbsiella*	no bleed			
4047	1	*Raoultella + Brenneria + Gibbsiella*	no bleed			
4047	2	*Raoultella + Brenneria + Gibbsiella*	no bleed			
4047	3	*Raoultella + Brenneria + Gibbsiella*	no bleed			
4048	1	*Raoultella + Brenneria + Gibbsiella*	no bleed			
4048	2	*Raoultella + Brenneria + Gibbsiella*	no bleed			
4048	3	*Raoultella + Brenneria + Gibbsiella*	no bleed			

7.3 Summary of Findings

7.3.1 Control tree 4031 - Anomaly

It was observed that tree 4031, which was inoculated only with Phosphate Buffer Solution, exhibited bleed symptoms. Bacteria were isolated from these bleed sites on this tree. These were identified as *P. agglomerans, Erwinia sp.* and *G. quercinecans*.

All initial sequencing was carried out using only 16S region (Table 7.7). Further sequencing was carried out on the *Gibbsiella* strain recovered from the bleed sites on 4031 to establish whether the strain was the same strain as had been used to inoculate the other trees. The *gyrB* region was sequenced in this instance and revealed that there was a 1% difference between the *G. quercinecans* strain isolated from this control inoculation and the *G. quercinecans* strain used to inoculate the trees. This difference suggests that the strain was not related to the strain used to inoculate the trees. It is probable that the bacterium was already present in the tree prior to inoculating with the control Phosphate Buffer Solution. That the bleed issued from the wound site would also suggest that there is a relationship between the wound created on the tree and the proliferation of bleed symptoms.

7.3.2 Combined results of all 3 CE room experiments

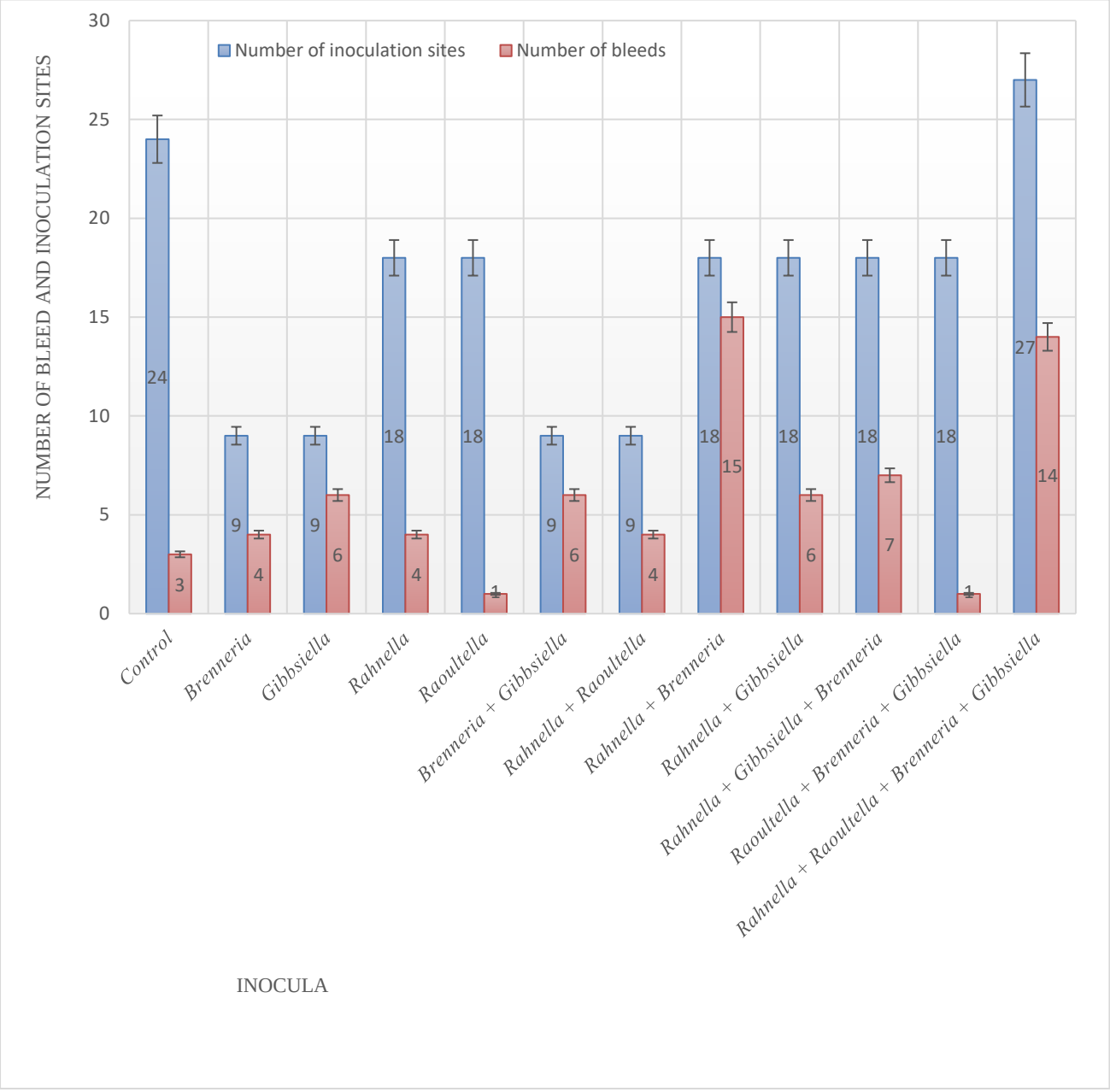


Fig.7.15: Combined results of all 3 experiments within the CE rooms considering the number of inoculated sites and the number of resultant bleeds. Error bars = Percentage error of 5%

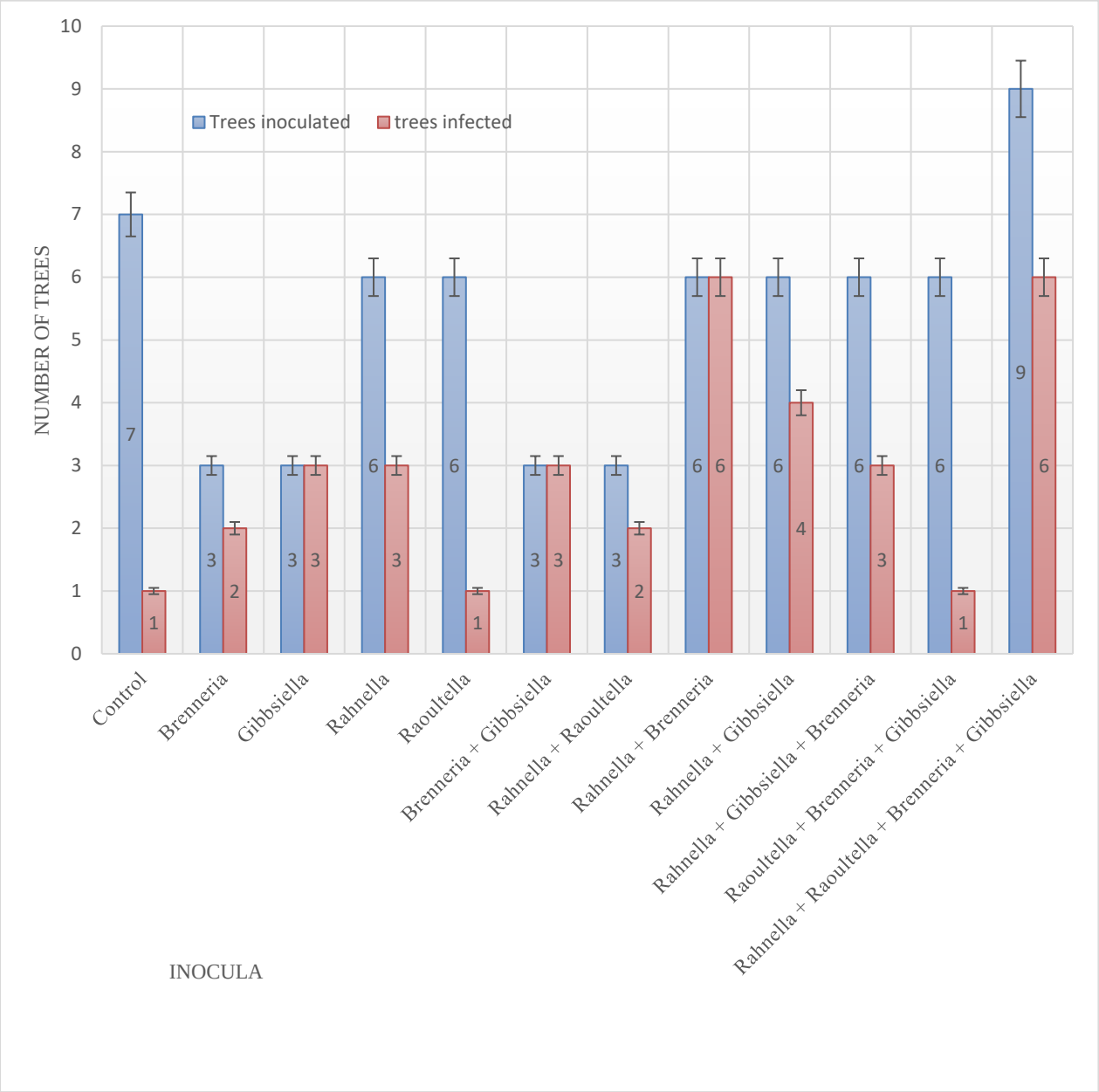


Fig.7.16: Combined results of all 3 experiments within the CE rooms considering the number of trees that were inoculated and the number of those trees that exhibited bleed, (regardless of the number of bleeds). Error bars = Percentage error of 5%

In the pathogenicity tests undertaken bleed exudate as of the exudate seen on mature Oak trees was produced as a consequence of introducing the inoculum of individual bacteria associated

with OBC. Bleeds were seen from individual bacteria *Brenneria goodwinii, Gibbsiella quercinecans* and *Rahnella victoriana.*

Bleeds were also seen from the inoculum of the following combined bacteria; B. goodwinii + G. quercinecans + R. victoriana + Raoultella sp.; R. victoriana + B. goodwinii + G. quercinecans; B. goodwinii + G. quercinecans + Raoultella sp.; B. goodwinii + G. quercinecans; R. victoriana + Raoultella sp.; R. victoriana + B. goodwinii; R. victoriana + G. quercinecans. Of all of these inoculations the combination of R. victoriana + B. goodwinii produce the most consistent bleeds. (Tables 7.15 & 7.16).

7.3.3 Koch's Postulates

From the experiments undertaken within this study Koch's postulates were not established. The first postulate that the suspected causal agent was present in every diseased organism was not established. The second postulate, that the suspected causal agent be able to be isolated from the tree and grown in culture was achieved. The third postulate was that pure cultures of the suspected causal agent/s produced bleed symptoms within the tree. This was achieved but was not consistent and varied from each bacterial inocula. The fourth postulate that the causal agent was recoverable from the host was achieved but again not consistently.

Chapter 8: Results – Bacterial Properties

8.1 Context of Research

The three principal pathogenic bacterial species associated with the Bleed symptoms on Oaks appeared, from the results presented in Chapter 7, to be *Brenneria goodwinii*, *Gibbsiella quercinecans* and *Rahnella victoriana*. All three of these are gram-negative, facultatively anaerobic bacteria within the family Enterobacteriaceae. (Brady et al, 2010. Denman et al, 2011. Brady et al, 2014. Denman et al, 2017. Brady et al, 2017). Tests were carried out to establish some of the physical properties, tolerance and reactions to gain further understanding of the bacteria, with a view to implementing possible mechanisms of control.

8.2 Gram staining and Microscopy of Bacteria

Preparation of slides, examination of bacterial cells and capture of images as detailed in Chapter 2 section 2.8.1.

All bacteria tested were Gram-negative short rods (Fig. 8.1-8.6). *Brenneria goodwinii* bacterial cells were approximately 1.5µm to 2µm long (average of 1.676 ± 0.096 standard error; Fig. 7.1-7.2), *Gibbsiella quercinecans* bacterial cells were approximately 1.1µm to 1.8µm long (average of 1.408 ± 0.118 standard error; Fig. 8.3-8.4), while *Rahnella victoriana* bacterial cells were approximately 0.8µm to 1.3µm long (average of 1.09 ± 0.109 standard error; Fig. 8.5-8.6).

The size of these cells is generally typical of Enterobacteriaceae. The higher surface to volume ratio of these cells will allow greater adaptability to changing environment as a consequence of mutation as well as adaptation to antibiotics (Madigan et al, 2009).

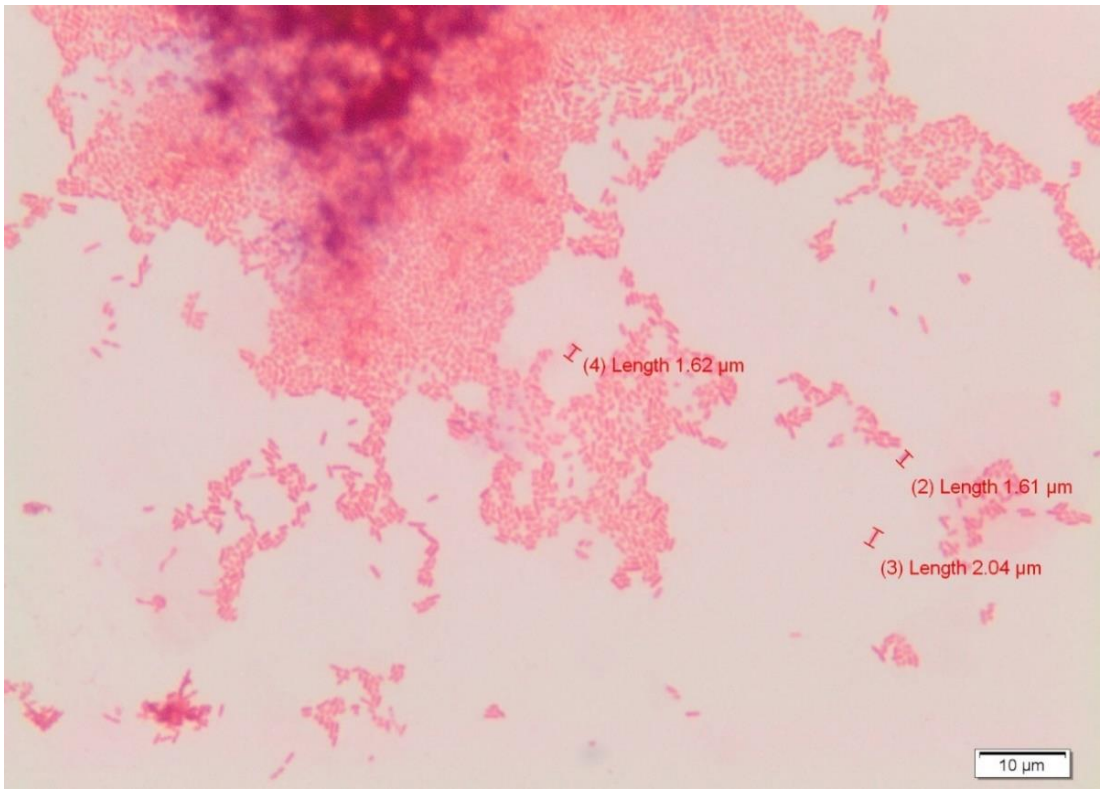


Fig. 8.1 Measurement of *Brenneria goodwinii* cells after Gram-staining using Cellsens Software.

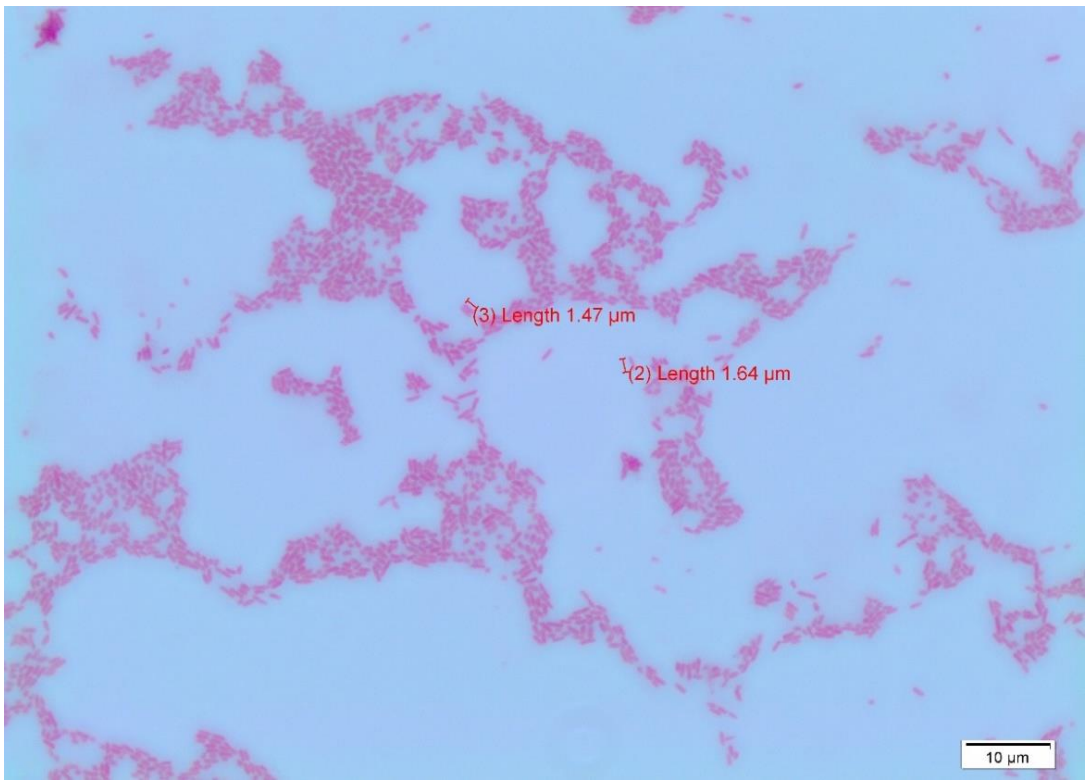


Fig. 8.2 Measurement of *Brenneria goodwinii* cells after Gram-staining using Cellsens Software.

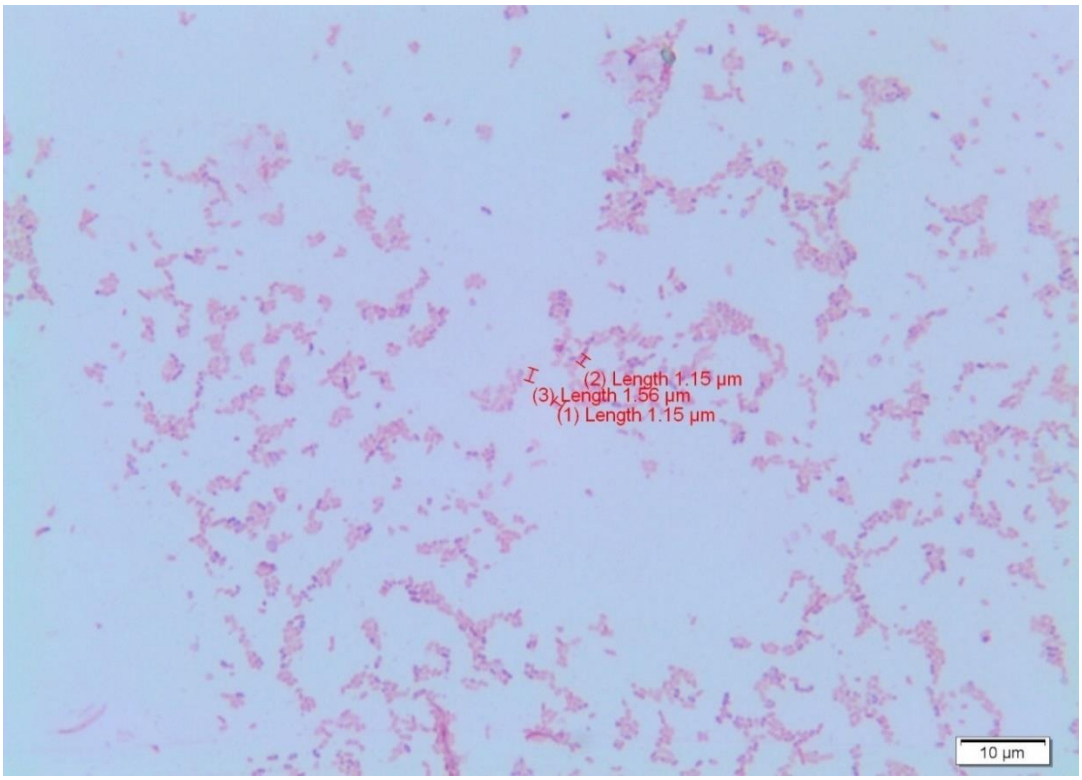


Fig. 8.3 Measurement of *Gibbsiella quercinecans* cells after Gram-staining using Cellsens Software.

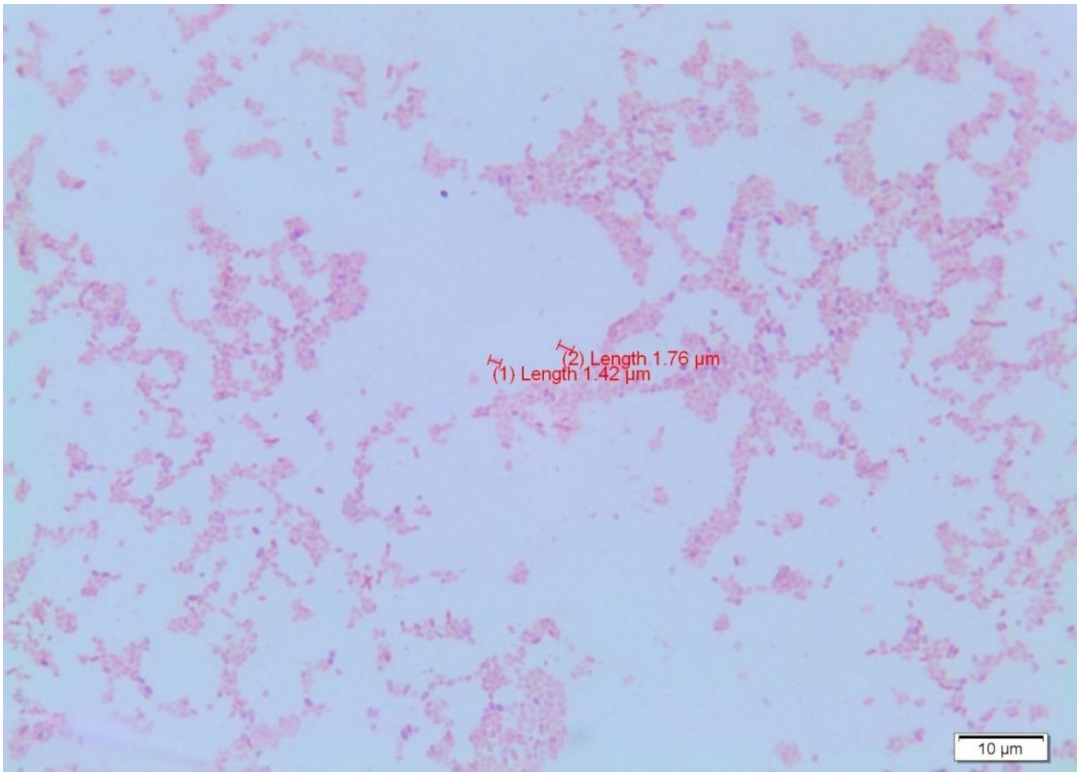


Fig. 8.4 Measurement of *Gibbsiella quercinecans* cells after Gram-staining using Cellsens Software.

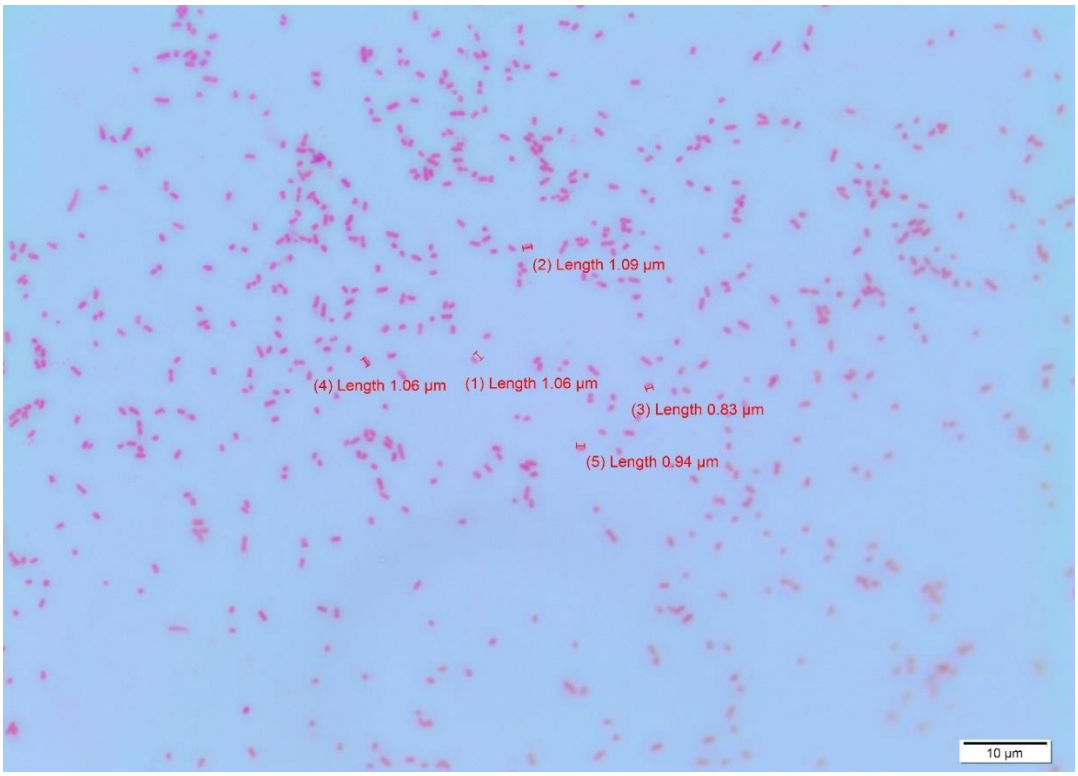


Fig. 8.5 Measurement of *Rahnella victoriana* cells after Gram-staining using Cellsens Software.

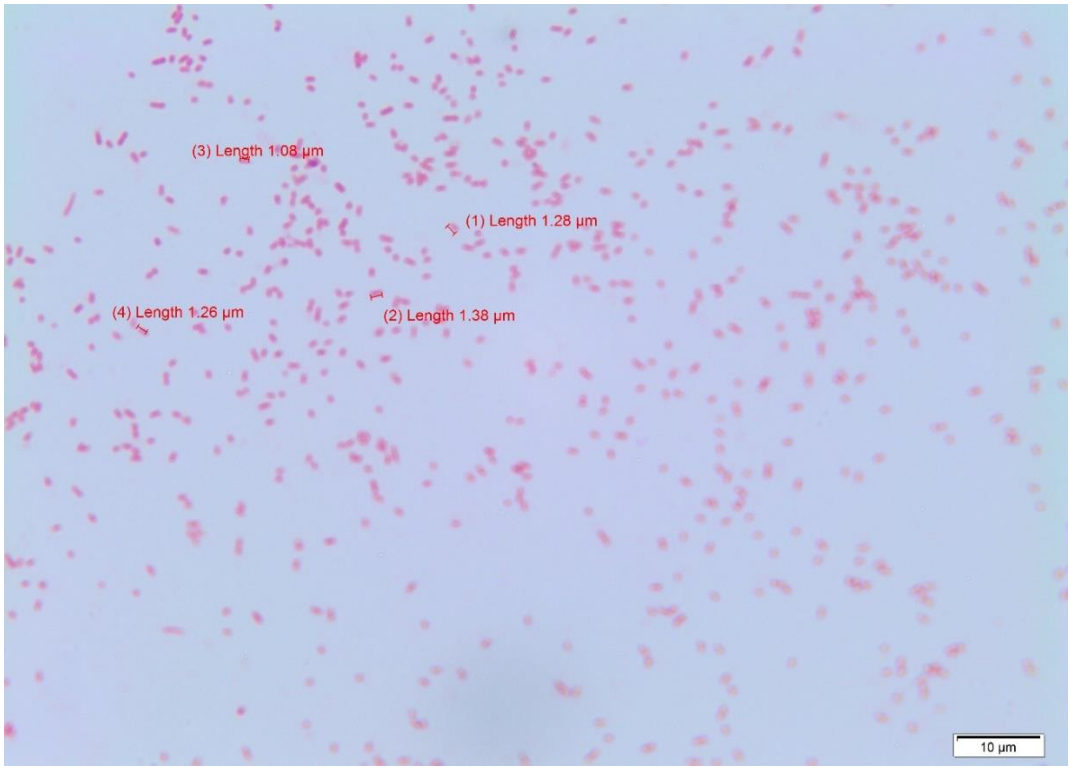


Fig. 8.6 Measurement of *Rahnella victoriana* cells after Gram-staining using Cellsens Software.

8.3 Growth Curves of Bacteria Using Turbidimetric Determination

To examine the growth characteristics of the bacteria, bacterial growth curves were established for *Brenneria goodwinii* strain 931_23, *Gibbsiella quercinecans* strain C2_5 and *Rahnella victoriana* strain 01195_20.

Growth curves of the bacteria were done using Luria-Bertani broth. A single colony was selected from an agar plate culture and incubated in 10mL of LB broth at 23ºC for 18hrs, with shaking at 225rpm. The resultant suspension was then equalized with fresh LB to reach an Optical density of 1, equivalent to 10^9 CFU/mL.

1mL of 10^9 CFU/mL was then added to 100mL of LB broth in a conical flask and each strain replicated four times. The optical density reading was checked at 600NM with an Eppendorf Biophotometer 6131 Spectrophotometer, every 30 minutes for 12 hours and then a final reading after 24 hours. Gibbsiella and Brenneria had similar growth curves although Gibbsiella grew slightly better (Fig. 8.7). Rahnella showed a distinctively higher growth rate.

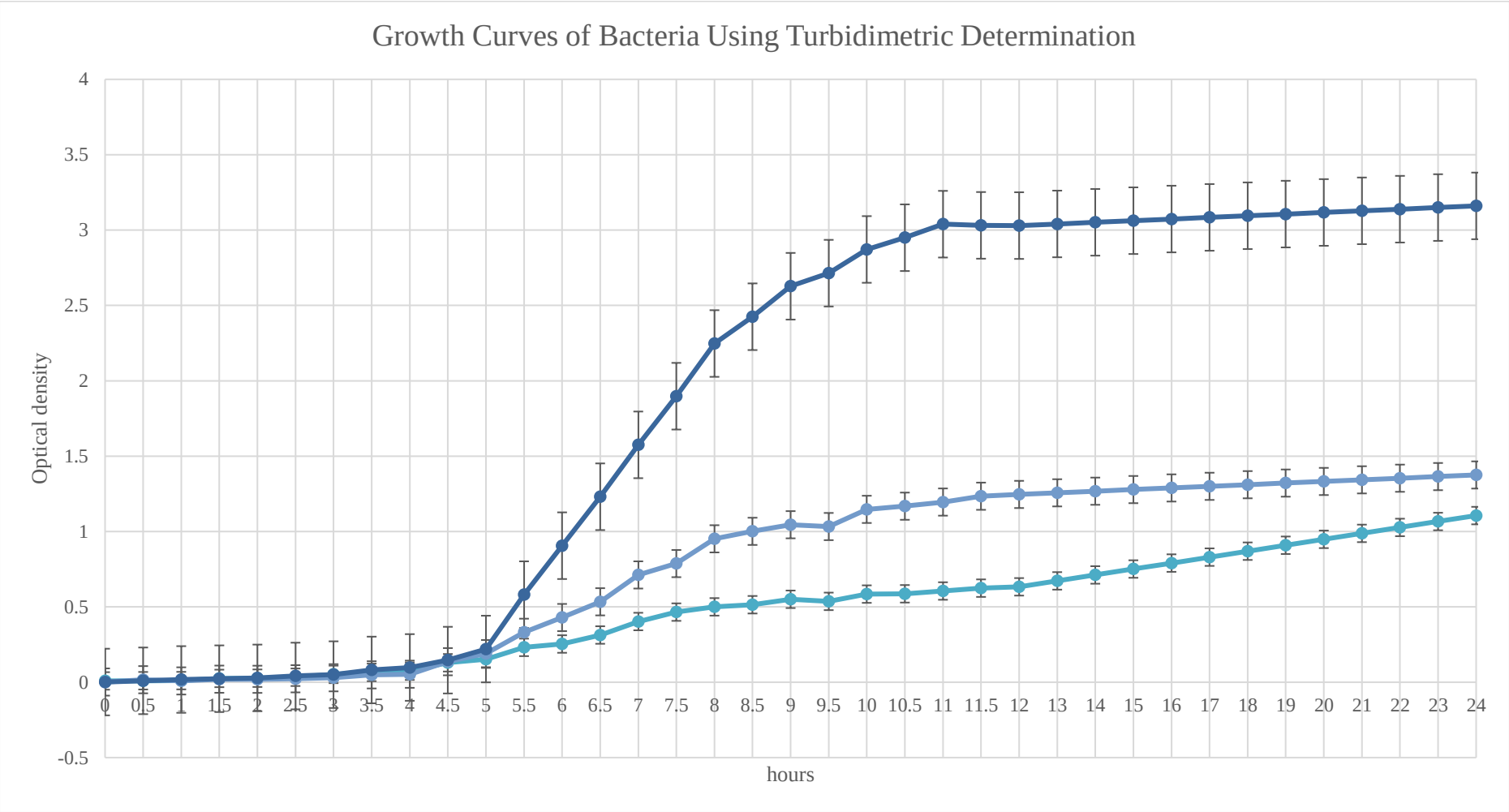


Fig. 8.7 Comparison of growth curves of *Brenneria goodwinii*, *Gibbsiella quercinecans* and *Rahnella victoriana*. Values are the mean of three replicates and error bars are the standard error of the mean.

8.4 Motility of Bacteria

Enteric bacteria are generally facultatively aerobic, Gram-negative non sporulating rods that are either non-motile or motile by peritrichous flagella (Madigan et al, 2009). The mechanisms by which bacteria move are either 1) Sliding, reliant on surfactants to reduce surface tension as the spread of growth is created by outward pressure of the cell growth; 2) Gliding, which involves the cell body moving as of a secretion contacting both the cell surface and the solid surface against which the cell moves; 3)Twitching, via extension and retraction of the pili to drag the cell over a surface; 4) Swimming, in liquid environments the cell is propelled by rotating flagella; 5) Swarming, is the rapid movement of cells across a surface as a consequence of coordinated movement powered by rotating helical flagella (Kearns, 2010; Madigan et al., 2009; Alberti & Harshey, 1990).

Understanding the movement of the bacteria will provide an understanding of how each species will spread within the localised environment, i.e. the wood tissues as well as the wider environment, such as the soils.

The Hanging Drop method can be used to test bacterial motility. However, Brownian movement and passive drift can produce false readings and the method does not identify the mode of movement. Measuring the motility of bacteria on semi – solid agar produces a visible halo as motile bacteria ‘swarm’ and give a diffuse spreading growth that is easily recognized by the naked eye (Kearns, 2010). Movement through the agar usually indicates swimming motility while movement over the agar is usually swarming motility or sliding motility, differentiated by the speed of movement. Petri dishes were prepared using Luria-Bertani agar at 25%. The dishes were air dried in a laminar flow cabinet for 24hrs prior to inoculation. Water content of the medium is a crucial factor: too little water will result in poor swarming while too much water may permit swimming motility.

A single colony was selected from a streaked agar plate culture and point inoculated onto the centre of the prepared plates. The plates were kept in dark room conditions at 24°C and the resultant halo forming around the inoculation recorded over a 10day period. Each strain was inoculated into 5 different plates and 5 control plates were also used. The average was taken from these 5 plates for each day when the measurements were taken. Also included in this experiment was another enteric strain obtained from disease lesions, *Raoultella* sp.

Both *Rahnella victoriana* and *Brenneria goodwinii* were seen to swarm on the plates (Figs 8.8, B&D) *Brenneria goodwinii* exhibited 2 concentric rings (Fig. 8.8, D). This is reported as characteristic of swarming. The cells at the outer edge of the colony are more rapidly motile than those in the centre of the colony. The outer cells move a short distance from the colony, then reduce in motility, divide again to form a new population of motile cells that again swarm. The concentric ring formation is a result of alternating higher and lower concentrations of cells (Madigan et al, 2009). Neither *Gibbsiella* nor *Raoultella* were observed to swim in the agar.

It has been considered that to demonstrate true swarming distinguished from swimming, the agar used must be greater than 30% to ensure sufficient viscosity (Kearns, 2010). Agar was used at 37% and the plates inoculated as before. As before both *Rahnella victoriana* and *Brenneria goodwinii* were seen to swarm on the plates (Figs 8.9, B & D). *Brenneria goodwinii again* exhibited concentric rings as part of the process of swarming. (Fig. 8.9, D). Neither *Gibbsiella* nor *Raoultella* were observed to swarm over the agar.

The consequent movement patterns of the different bacterial species effect the over all size of the primary colony growth of the bacterial species. As of Fig.8.10 it can be seen that the increase in the movement of *Rahnella victoriana* and *Brenneria goodwinii* has led to a decreasing size of the primary colony. Both the *Gibbsiella* and the *Raoultella* have progressively increased the primary colony size during the same time span.

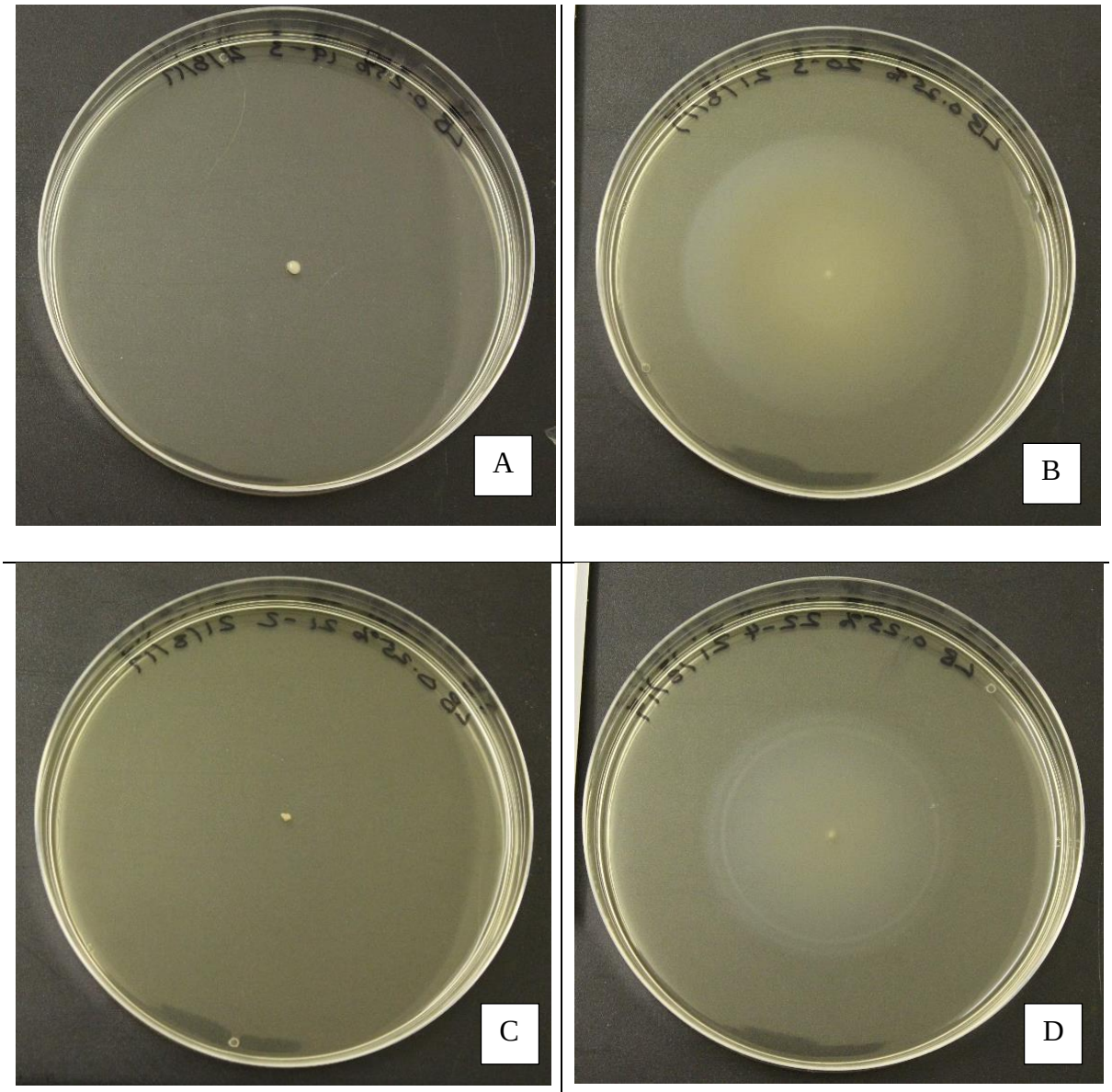


Fig 8.8: Swimming motility phenotypes for bacteria isolated from Oak Bleed Canker lesions. Shown are Raoultella strain 01195_19 (A), Rahnella strain 01195_20 (B), G. quercinecans strain 01195_21 (C) and Brenneria strain 01195_22 (D), photos taken after 4 days using 25% LB agar.

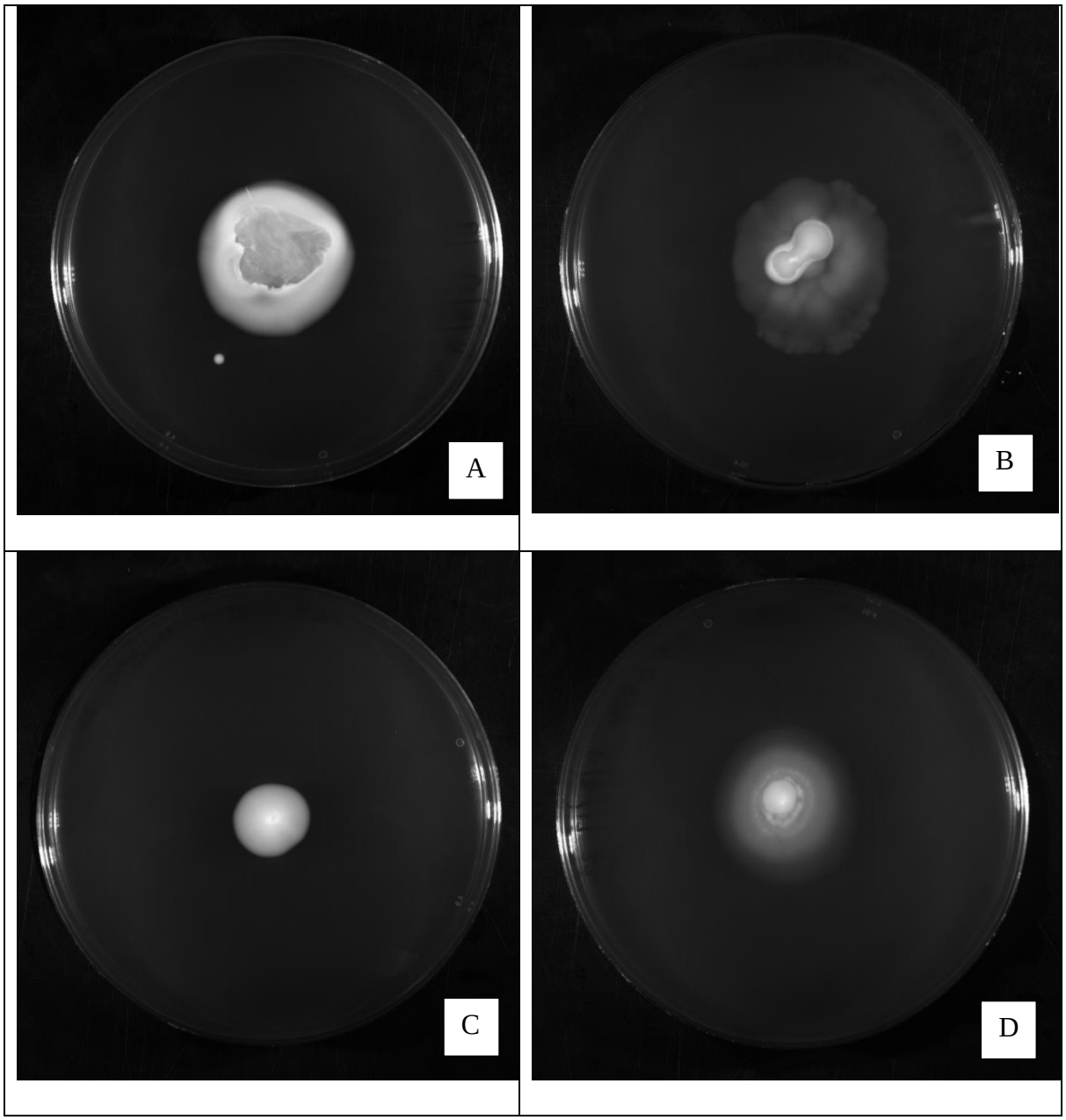
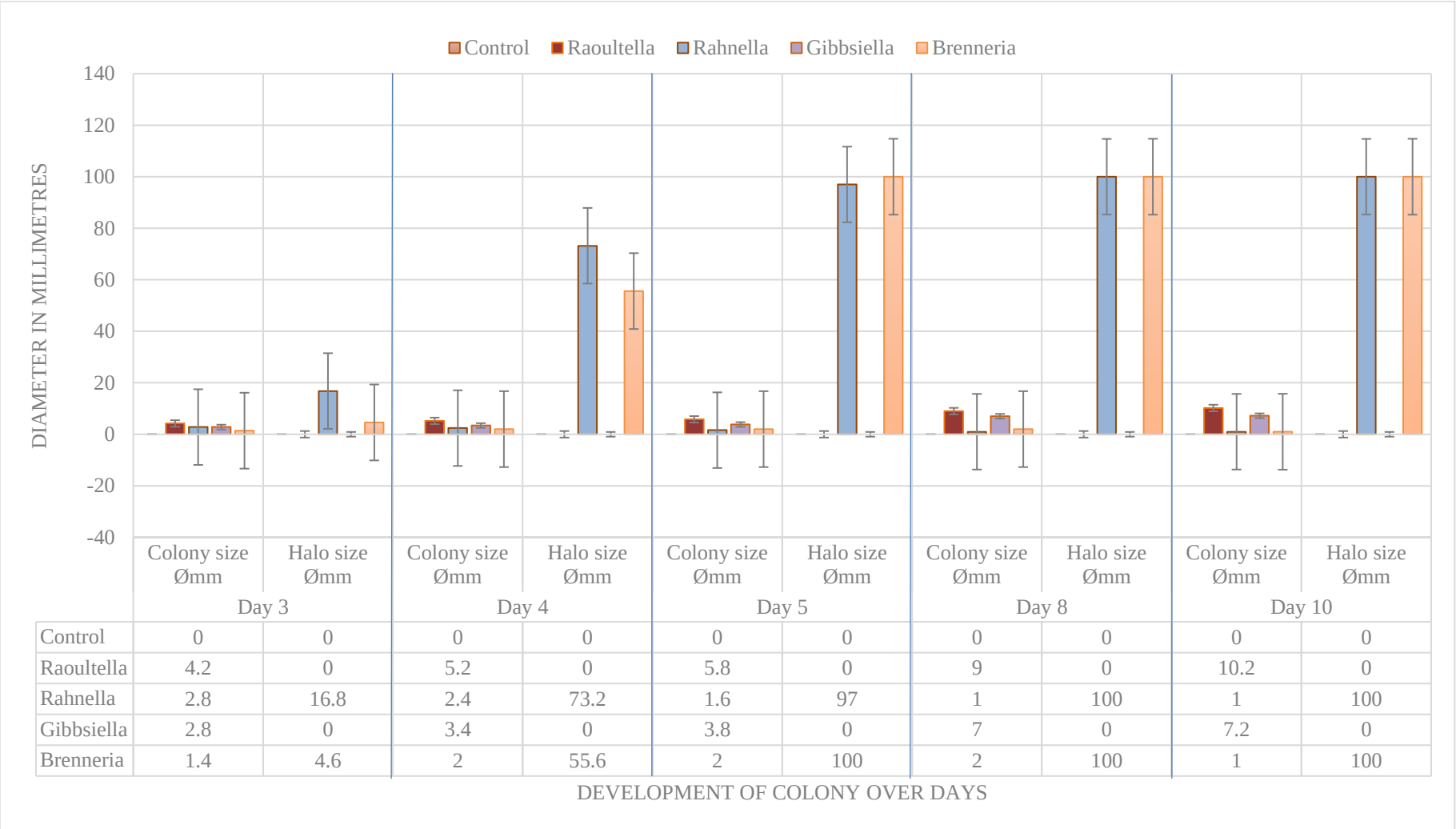


Fig 8.9: Swarming motility phenotypes for bacteria isolated from Oak Bleed Canker disease lesions. Shown are Raoultella strain 01195_19 (A), Rahnella strain 01195_20 (B), G. quercinecans strain 01195_21 (C) and Brenneria strain 01195_22 (D), photos taken after 6 days using 37% LB agar.



	Day 3		Day 4		Day 5		Day 8		Day 10	
	Colony size Ømm	Halo size Ømm	Colony size Ømm	Halo size Ømm	Colony size Ømm	Halo size Ømm	Colony size Ømm	Halo size Ømm	Colony size Ømm	Halo size Ømm
Control	0	0	0	0	0	0	0	0	0	0
Raoultella	4.2	0	5.2	0	5.8	0	9	0	10.2	0
Rahnella	2.8	16.8	2.4	73.2	1.6	97	1	100	1	100
Gibbsiella	2.8	0	3.4	0	3.8	0	7	0	7.2	0
Brenneria	1.4	4.6	2	55.6	2	100	2	100	1	100

Fig 8.10 Average colony sizes observed for the motility of *Raoultella* sp 01195_19, *R. victoriana* 01195_20, *G. quercinecans* 01195_21 and *B. goodwinii* 01195_22 on 25% agar. Values are the mean of five replicates and error bars are the standard error of the mean.

8.5 Reaction to Antibiotics

The resistance or otherwise to a range of anti- biotics provides a picture as to potential control of the bacterium associated with the bleed canker. It is unlikely that Antibiotics would be used to treat the bacterial bleed cankers on the Oak trees. Never-the-less the resistance or otherwise to a range of anti- biotics provides a picture as to potential control of the bacterium associated with the bleed canker.

As previously described, an individual colony was selected from a streaked culture and placed in 10ml of LB Broth. The culture was then agitated in a shake incubator for 12-18 hours at 27°C. 100µl was then added to a plate of LB agar and spread to create an even lawn. To the plates were added 3 different Mast rings (Fig 8.11, 8.12 and 8.13). These are pre-prepared paper rings with a number of tips which are impregnated with different antibiotics in different concentrations.

The plates were then incubated for 3 days at 27°C. Measurements were then recorded of the zone of inhibition in millimetres between the bacterial lawn and the antibiotic. Some of the antibiotic used is replicated on the Mast rings but at different concentrations. (Table 7.1). A graph is included that excludes replicates of the Antibiotics and shows the average area of inhibition from the 2 data sets. (Fig. 8.14).

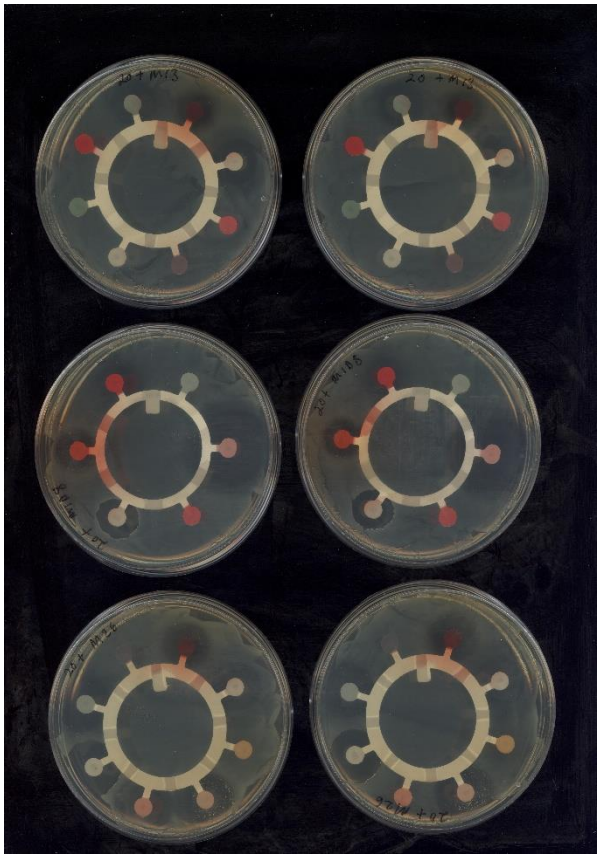


Fig 8.11 Antibiotic antagonism assay with *R. Victoriana*.

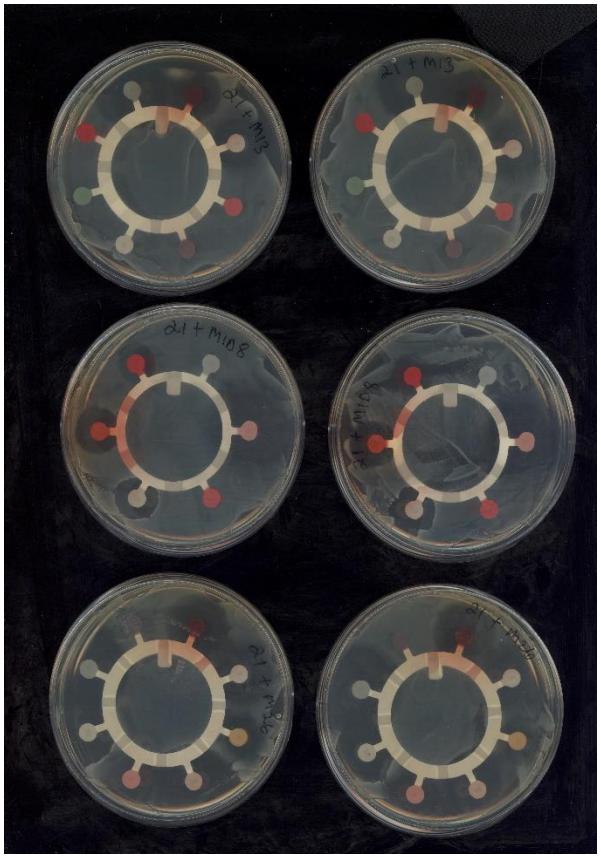


Fig 8.12 Antibiotic antagonism assay with *G. quercinecans*.

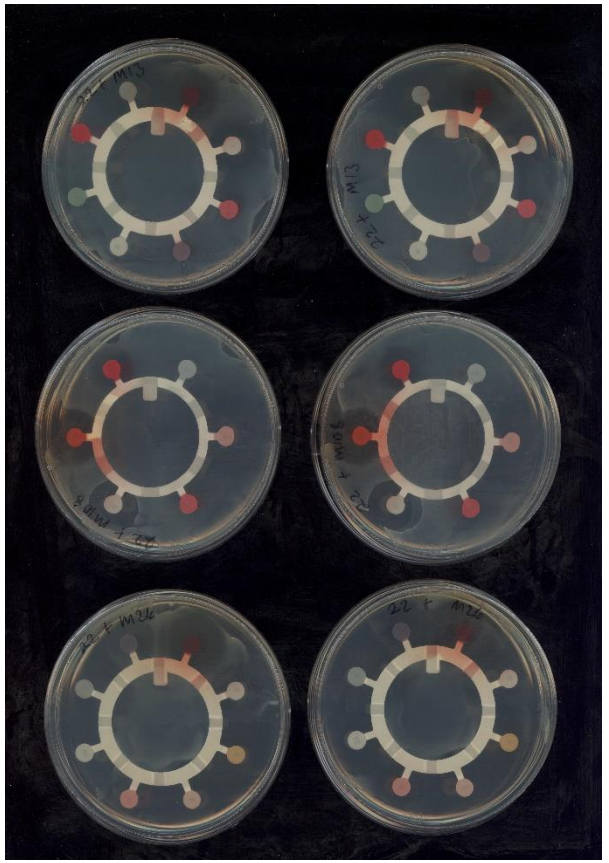


Fig 8.13 Antibiotic antagonism assay with *B. goodwinii*.

Table 8.1 Growth inhibition phenotypes for *R. victoriana*, *G. quercinecans* and *B. goodwinii* strains using an array of antibiotics.

Bacteria	Test No.	Mastring M26								Mastring MID8						Mastring M13							
		AP	C	CO	K	NA	NI	S	T	E	RP	CO	PG	K	VA	C	E	FC	OX	NO	PG	S	T
Rahnella	1	0	5	7	5	8	0	5	5	0	5	4	0	7	0	4	0	0	0	0	0	5	3
Rahnella	2	0	5	8	0	7	0	6	6	0	4	4	0	7	0	2	0	0	0	0	0	5	3
Gibbsiella	1	0	12	10	6	10	5	5	8	4	5	5	0	11	0	11	0	0	0	0	0	5	6
Gibbsiella	2	0	11	8	7	12	5	7	7	4	5	5	0	7	0	11	0	0	0	0	0	5	6
Brenneria	1	7	5	10	5	12	0	6	8	0	7	5	0	0	0	2	0	0	0	0	0	3	6
Brenneria	2	6	5	11	6	13	0	5	6	0	7	5	0	0	0	4	0	0	0	0	0	4	6

weak zone of inhibition

moderate zone of inhibition

strong zone of inhibition

measurement = zone of inhibition mm

AP	Ampicillin	25ug	E	Erythromycin	60ug	C	Chloramphenicol	25ug
C	Chloramphenicol	50ug	RP	Rifampicin	15ug	E	Erythromycin	5ug
CO	Colistin Sulphate	100ug	CO	Colistin Sulphate	10ug	FC	Fusidic Acid	10ug
K	Kanamycin	30ug	PG	Penicillin G	2 units	OX	Oxacillin	5ug
NA	Nalidixic Acid	30ug	K	Kanamycin	1000ug	NO	Novobiocin	5ug
NI	Nitrofurantoin	50ug	VA	Vancomycin	5ug	PG	Penicillin G	1 unit
S	Streptomycin	25ug				S	Streptomycin	10ug
T	Tetracycline	100ug				T	Tetracycline	25ug

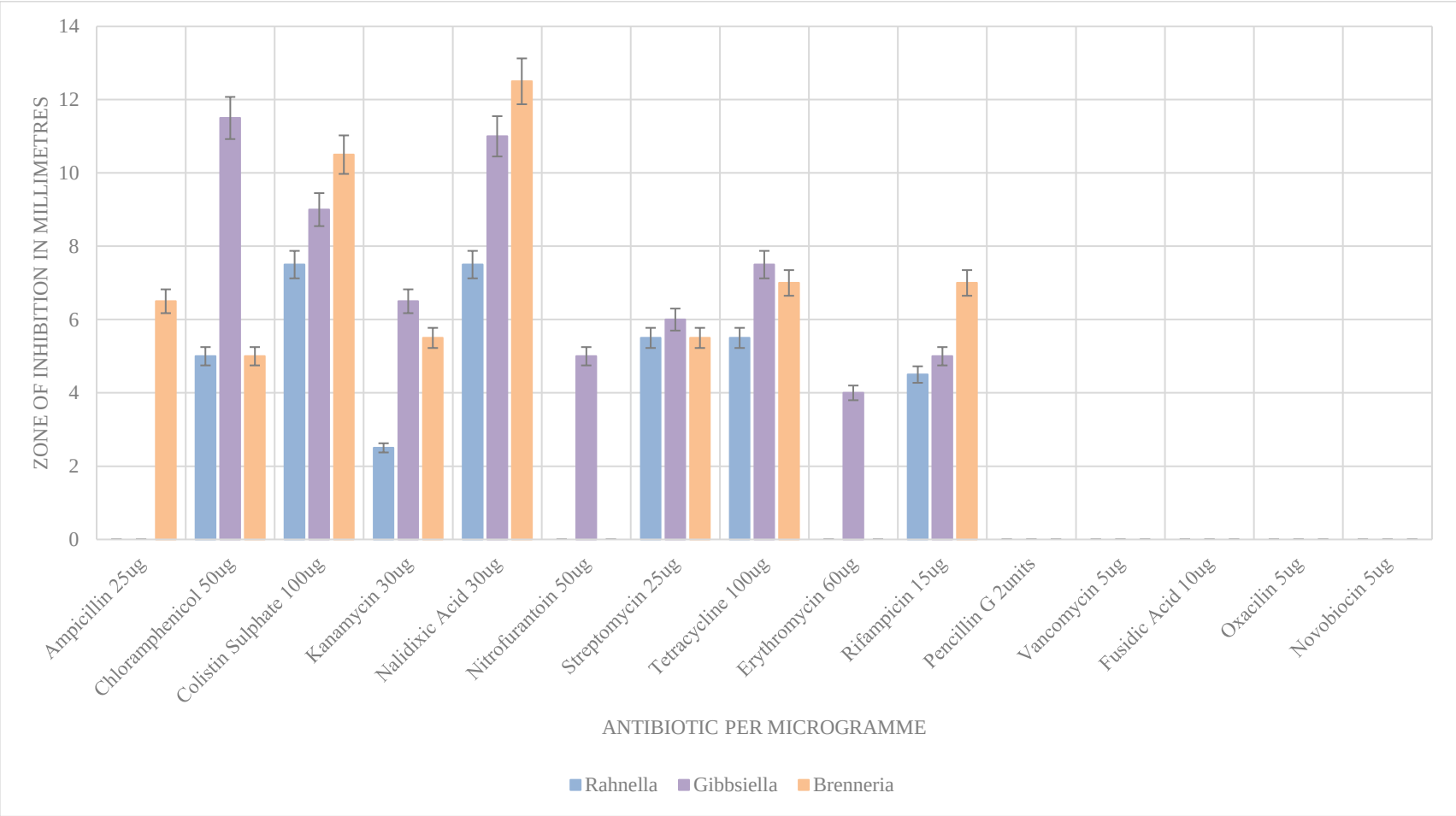


Fig 8.14 Comparative analysis of the amount of growth inhibition of *R. victoriana*, *G. quercinecans* and *B. goodwinii* to different antibiotics. Values represent the mean of two replicates and error bars are the standard error of the mean.

8.6 Inter-reactions between *R. victoriana, G. quercinecans* and *B. goodwinii*

MacConkey's agar was used on which to create a lawn of each bacterium. This allowed clearer visual identification of the interaction between the bacteria. To each lawn was added three infusion discs, one as a control with no addition and the other two discs with 10uL of each bacterium at 10^9 CFU/mL, added directly on to the disc. (Figs 8.15,8.16 and 8.17). This was carried out in triplicate for each bacterial species. The plates were left at 23°C for four days and the consequent interactions recorded (Table 8.2).

The interactive growth of the bacteria *in vitro* appears consistent. When infusion discs of *B. goodwinii* and *G. quercinecans* were grown on a lawn of *R. victoriana* both species did not grow, but a very small zone of inhibition could be seen to have developed around each disc. On lawns of *G. quercinecans* both *B. goodwinii* and *R. victoriana* were seen to grow by relatively similar amounts. On lawns of *B. goodwinii G. quercinecans* did not grow and a zone of inhibition was visible. However, *R. victoriana* was seen to grow much more successfully on the *B. goodwinii* lawn. It has not been established whether this is a commensal relationship. However, given the inhibition zone established by *B. goodwinii* on the *R. victoriana* lawn it is probable that *R. victoriana* is a well-adapted competitor (Hibbing, 2010).

Table 8.2: Interactive growth of *R. victoriana* 01195_20, *G. quercinecans* 01195_21 *and B. goodwinii* 01195_22 grown on bacterial lawns

100uL spread Bacterial Lawn	**Infusion disc with 10uL of *R. victoriana***	**Infusion disc with 10uL of *G. quercinecans***	**Infusion disc with 10uL of *B. goodwinii***	**Control**
R. victoriana - 1	n/a	2mm zone of inhibition	1mm zone of inhibition	No inhibition. No growth
R. victoriana - 2	n/a	1mm zone of inhibition	1mm zone of inhibition	No inhibition. No growth
R. victoriana - 3	n/a	2mm zone of inhibition	3mm zone of inhibition	No inhibition. No growth
G. quercinecans - 1	3mm growth on lawn beyond infusion disc	n/a	4mm growth on lawn beyond infusion disc	No inhibition. No growth
G. quercinecans - 2	2mm growth on lawn beyond infusion disc	n/a	4mm growth on lawn beyond infusion disc	No inhibition. No growth
G. quercinecans - 3	3mm growth on lawn beyond infusion disc	n/a	7mm growth on lawn beyond infusion disc	No inhibition. No growth
B. goodwinii - 1	20mm growth on lawn	3mm zone of inhibition	n/a	No inhibition. No growth

100uL spread Bacterial Lawn	**Infusion disc with 10uL of *R. victoriana***	**Infusion disc with 10uL of *G. quercinecans***	**Infusion disc with 10uL of *B. goodwinii***	**Control**
	beyond infusion disc			
B. goodwinii - 2	9mm growth on lawn beyond infusion disc	1mm zone of inhibition	n/a	No inhibition. No growth
B. goodwinii - 3	3mm growth on lawn beyond infusion disc	1mm zone of inhibition	n/a	No inhibition. No growth

Fig. 8.15: R. victoriana and B. goodwinii grown on a lawn of G. quercinecans

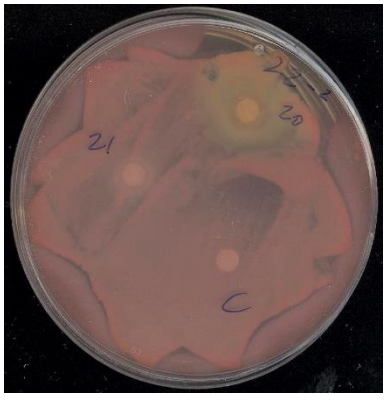


Fig. 8.16: R. victoriana and G. quercinecans grown on a lawn of B. goodwinii

Fig. 8.17: B. goodwinii and G. quercinecans grown on a lawn of R. victoriana

8.7 Reaction to Secondary Metabolite – Tannin

8.7.1 Secondary Metabolites - Introduction

The metabolism of a plant will direct most of the resultant energy to primary functioning cells such as lignin and cellulose known as primary metabolites. However, plants direct a significant proportion of energy to secondary metabolites, organic molecules that have no obvious role in cell functioning.

Secondary metabolites within trees have evolved to help the plant defense mechanisms develop against microbial pathogens (Taiz and Zeiger, 2010; Wink, 2003). Quercitannic acid is the main Tannin found within the leaves and bark of Oak trees. It is a secondary metabolite common to Oak trees and well documented for is anti-microbial properties (Hauben et al., 1998; Cowan, 1999; Akiyama et al., 2001). It is considered that Oak bark will contain 12-16% Tannin and that Tannin concentration at this level is generally found within younger trees (Hathway, 1958). Common to the current bleed canker on Oak trees is that it occurs in semi-mature trees (or trees over 50 years old). These trees are reported to have a lower tannin content within the bark (Hathway, 1958).

8.7.2 Reaction zones of bacterial lawns with Tannin

Tannic acid was used as a widely available substitute, to establish whether tannin would have an effect on the bacterium. This was prepared from powder as a 1 molar concentration.

An individual colony was selected from a streaked agar culture and placed in 10ml of LB Broth. The culture was then agitated in a shake incubator for 12 to 18 hours at 27°C. The bacterial suspension was equalised through spectrophotometry at 600NM at 10^9 CFU/mL. 100µl was then added to a plate of LB agar and spread to create an even lawn. A sterile infusion disc (without chemical infusion) was placed in the centre of the plate and 10µl of varying tannin

concentrations was added to the infusion disc on each plate. The cultures were then incubated at 27°C for 4 days. Measurements of the distance of the edge of the bacterial growth to the edge of the infusion disc were recorded. In this experiment, only the principle *Pseudomonas* and *Brenneria* species were tested.

8.7.3 Reaction zones of bacterial lawns with Tannin solution - Results

The zones that established around the disc infused with Tannin demonstrated the ability of the bacterium to grow within a Tannin enriched environment. Although the tests show that all bacterial species tested including *Brenneria goodwinii* were generally restricted in their growth by Tannic acid at relatively low concentrations.

Table 8.3: Reaction zones of bacterial lawns with 2mm infusion discs of 10µl of Tannin solution

Strain	**Bacterial species**	**10% Tannin**	**15% Tannin**	**20% Tannin**	**25% Tannin**	**50% Tannin**
903 - 18	*Pseudomonas flavescens*	1.5mm not distinct	1mm not distinct	1mm not distinct	1mm not distinct	4mm
903 - 21	*Pseudomonas sp.*	2mm	2mm	1.5mm	1.5mm	6mm
903 - 22	*Brenneria goodwinii*	2mm not distinct	1mm not distinct	2mm not distinct	2mm not distinct	4mm
903 - 25	*Brenneria goodwinii*	No zone visible	1.5mm not distinct	2mm not distinct	3mm not distinct	4mm

Strain	**Bacterial species**	**10% Tannin**	**15% Tannin**	**20% Tannin**	**25% Tannin**	**50% Tannin**
903 - 26	*Pseudomonas xinjiangensis*	2mm	2mm	4mm	3mm	8mm
903 - 27	*Brenneria goodwinii*	2mm not distinct	No zone visible	No zone visible	1mm not distinct	5mm

8.8 Summary of Findings

The primary bacterial species of interest *Brenneria goodwinii*, *Gibbsiella quercinecans* and *Rahnella victoriana* are all gram-negative bacteria within the family Enterobacteriaceae. The size of the bacterial cells are relatively similar, although the cells of *B. goodwinii & G. quercinecans* are slightly larger than *R. victoriana*.

The growth rates of *B. goodwinii & G. quercinecans* are nearly half the growth rate of *R. victoriana* in a 12hr period.

G. quercinecans is poorly, if at all motile through conventional propulsion, whereas both *B. goodwinii* & *R. victoriana* are motile. The consequent colony formation of *G. quercinecans* is larger than both *B. goodwinii & R. victoriana.*

B. goodwinii showed marginally more resistance to conventional antibiotics than *G. quercinecans* and *R. victoriana. G. quercinecans* and *R. victoriana* showed the same resistance and non -resistance to the array antibiotics tested, but with varying degrees of resilience.

In antagonistic growth assays the growth of *R. victoriana* was inhibited by G. quercinecans & *B. goodwinii*. The growth of *B. goodwinii* was inhibited by *G. quercinecans* but not *R. victoriana* and the growth of *G. quercinecans* was not inhibited by either *R. victoriana* or *B. goodwinii*.

Chapter 9: Culturable Fungal Endophytes within Quercus robur

9.1 Context of Research

Given that two mature trees can be growing within 5metres of each other and one exhibit the bleed symptoms associated with Acute Oak Decline whilst the other does not, would suggest that the cause of the symptoms (and associated decline) is not directly related to the immediate environment in which the trees are growing. They share the same soils, temperature and rainfall. Whilst one tree may develop in the shade of a more dominant canopy, the same daylight hours and light intensity are available to both.

It may be that resistance is conferred as a result of genetic divergence. Perhaps the resistance of one tree to the symptoms, rather than the other, is that the two originate from different provenance, with each tree having a differing genetic resistance to the bacteria associated with the bleed. (Ennos, 2015). This has not been evaluated as part of this study, for whilst planting from resistant stock will be an effective method by which to preserve the species in respect of future management, it cannot provide a solution to remediation of existing bleed symptoms. However, another possibility as to why one tree should appear resilient to the bleed canker whilst its neighbor is not, may lie within the endophytic microbial community that the tree hosts.

The use of endophytes as biocontrol agents has been well documented both for agricultural and forest crop production in the last twenty years. (Hodkinson et al (2019), Pirttilä and Frank (2011), Ryan et al (2008), Kloepper et al (1999)). This study considers only the viable,

culturable endophytes as potential bio-controls of the pathogenic bacteria 1) because the antagonism/inhibition can be tested *in vitro* and 2) because any potential bio-control must be easily accessible for production.

9.1 Definition and Role of Endophytes within Trees

Bacterial and fungal endophytes within land-based plants are generally considered ubiquitous. A definition of an endophyte is perhaps best simply expressed in terms of spatial relationship with the host. 'Endophytes colonise, symptomlessly, the living, internal tissues of their host, even though the endophyte may, after incubation or latency period, cause disease' (Petrini, 1991).

The interaction between the plant and the endophyte may change over time as the endophyte adopts different nutritional modes. Such relationships may range from commensal to pathogenic dependent upon the condition of the host. Deteriorating health or vitality of the host may induce the endophyte to switch mode from a biotrophic, mutualistic role within the tissue to an antagonistic or necrotrophic pathogen. (Parfitt et al, 2010, Song et al, 2017).

9.1.2 Introduction of the Endophyte within the tree

It has been suggested that endophytes can enter the host through either woody tissue or via infection of leaf surfaces. (Carroll, 1995). Though more commonly endophytes are believed to evolve within the plants themselves, often within the seed. In this way the endophytic population evolves with the development of the tree, developing varying strategies by which to survive and indeed refine the relationship within the plant. (Yu et al. 2010, Goyal et al. 2017).

9.1.3 The Role of the Endophyte

Endophytes have been attributed with diverse and varied ecological roles. (Stone et al, 2012). These include conferring protection in relation to insect herbivory (Saikkonen et al, 1996) and production of secondary metabolites (Peláez et al. 1998). Endophytes have also been attributed with antibacterial and antifungal properties (Strobel & Daisy, 2003; Yu et al, 2010), for conferring plant fitness as a consequence of plant – microbe symbiosis (Hardoim et al, 2015) and producing Induced Systemic Resistance (ISR), promoting plant defence reactions, leading to a higher tolerance of pathogens. (Robert-Seilaniantz et al, 2011).

9.1.4 Variance in Endophytic Microbial Communities

There is seen to be qualitatively similar assemblage of endophytes over the associated tree /host range. The location of the host tends to affect the abundance of endophytic species discoverable rather than the range of species. This is considered to be the case for trees over a wide spatial range but also amongst local communities of same species trees. (Wilson, 2000).

Variability of endophytic microbial populations has been shown to be influenced rather by genetic variation (Lamit et al, 2014). Further variability of the communities may also relate to environmental conditions such as physiology, phenology, seasonal temperature, water availability etc. (Pirttila & Frank, 2011; Bacon & White, 2000).

9.1.5 Competitiveness and equilibrium in Endophytic Microbial Communities

Inside the plant microecosystem, different microbial species, both bacterial and fungal, are able to interact and consequently establish an equilibrium. Some of these microorganisms can be considered to be dominant species and may be represented by those that are most frequently and in large numbers, isolated from the host plant (Fisher et al., 1992; Van Peer et al., 1990; Lodewyckx, 2002). It is considered that the microbial populations within the region of the main

stem of either *Q. robur* or *Q. petraea* will be specialised and restricted by an environment traditionally resilient to bacterial bleed cankers.

Outbreaks of pathogens (and supporting pathogens) are associated with shifts of the whole microbial endophytic community (Fürnkranz, 2012; Earlacher, 2014). Previously mutualistic endophytes maybe left in a competing environment as either the host is depleted of resources, or environmental changes affect the physiology of the host.

9.1.6 Discovery of Endophytes

Endophytes are generally revealed either histologically with the use of high-quality light microscopy or transmission electron microscopy. Sonification used to remove surface layers of plant tissue and the remaining tissue then used to define the endophyte microbiome. (Turner et al, 2013). Through cultivation from heavily surface sterilized tissue. It is the latter methodology that has been used within this study to determine fungal endophytic populations.

9.2 Endophytes in Symptomatic and Non- symptomatic trees

The endophytic community within the main stem of *Q.robur* will play a significant role in the determination of a bacterial infection that exhibits as a bleed exudate or bleed canker on the main stem of the tree. An understanding of the microbiome of symptomatic and non - symptomatic trees within the endosphere environment, may reveal differences within the culturable microbial communities. Such differences may then be exploited to consider bio-controls for remediation of the bleed cankers.

Previous sampling of the culturable bacterial populations of symptomatic and non-symptomatic trees revealed similar discoverable species between the trees with significant variation in the abundance of species. Hence sampling was undertaken to isolate the fungal

endophytes from both symptomatic and non -symptomatic trees, to consider significant shifts in species range. (Tables 9.1 and 9.2)

Sampling for fungal endophytes from the young Oak trees used for pathogenicity testing from those trees inoculated between September and December 2017. This was to establish whether presence of certain fungal endophytes conferred resistance to bleed. (Table 9.3)

Table 9.1: Details of the endophytic fungal isolates from Symptomatic trees with visible bleed canker within Writtle Forest

* signifies morphological identification rather than DNA identification

Tree No	No of samples isolated	Number of samples sequenced	Ident. 1	Ident. 2	Ident. 3	Ident. 4	Ident.5	Ident.6
902	3	2	*Penicillium olsonii*	*Eucasphaeria capensis*	*Cladosporium sp.**	-	-	-
905	5	3	*Phialocephala sp.*	*Ramularia endophylla*	*Botryosphaeria stevensii**	*Botryosphaeria stevensii**	-.	-
910	3	1	*Penicillium sp.*	*Penicillium sp.**	*Penicillium sp.**	-	-	-
1187	3	2	*Trametes versicolor*	*Phialemonium inflatum*	-	-	-	-
1191	4	3	*Penicillium sp.*	*Ramularia sp.*	*Penicillium sp.*	-	-	-
1194	3	0	*Botryosphaeria stevensii**	*Botryosphaeria stevensii**	-	-	-	-
1195	7	3	*Ophiognomonia melanostyla*	*Phialocephala compacta*	*Penicillium fellutanum*	*Botryosphaeria stevensii**	*Penicillium sp. **	-
1196	10	6	*Penicillium aeneum x2*	*Penicillium sp.*	*Penicillium roqueforti*	*Penicillium olsonii*	*Botryosphaeria stevensii**	*Penicillium sp.**
1197	5	2	*Clonostachys sp.*	*Penicillium roqueforti*	-	-	-	-
4021	3	1	*Penicillium carneum*	-	-	-	-	-

Table 9.2: Details of the endophytic fungal isolates from trees with no visible signs of bleed canker within Writtle Forest

Tree No	No of samples isolated	Number of samples sequenced	Ident. 1	Ident. 2	Ident. 3	Ident. 4	Ident.5
473	6	5	*Penicillium aeneum x3*	*Penicillium aurantiacobrunneum*	-	-	-
912	7	6	*Penicillium brevicompactum x2*	*Botryosphaeria stevensii x3*	*Cladosporium sp.*		
913	7	6	*Botryosphaeria stevensii x2*	*Cladosporium sp.*	*Phialocephala compacta*		-
474	6	5	*Botryosphaeria stevensii x3*	*Penicillium aurantiacobrunneum*	*Penicillium sp.*	-	-
915	8	6	*Penicillium aeneum x2*	*Phialocephala compacta*	*Penicillium aurantiacobrunneum*	*Penicillium manginii*	-
916	7	6	*Penicillium citrinum*	*Penicillium aeneum*	*Penicillium corylophilum*	*Talaromyces sp.*	*Aspergillus versicolor*
917	3	3	*Phialocephala sp.*	*Penicillium aeneum x2*	-	-	-
918	10	5	*Botryosphaeria stevensii x4*	*Trichoderma sp*	-	-	-
919	4	4	*Penicillium sp.*	*Phialocephala compacta*	-	-	-
920	7	4	*Botryosphaeria stevensii x2*	*Cladosporium sp.*	*Penicillium aurantiacobrunneum*	-	-

The number of samples sequenced was dependent on visual differences in morphology identified. Where one isolated cultured fungal endophyte appears the same as another only one is sequenced. Of the 51 DNA extractions 7 did not produce successful PCR product suitable to sequence.

Table 9.3: Endophytes taken from young oak trees prior to inoculating in September 2017

* signifies morphological identification rather than DNA identification

Tree Number	No. of samples isolated	Identification 1	Identification 2	Identification 3
4001	1	*Fusarium lateritium*	-	-
4002	2	*Botrytis cinerea*	*Botrytis cinerea**	-
4003	3	*Fusarium lateritium*	*Botrytis cinerea*	-
4004	3	*Botrytis cinerea*	*Botrytis cinerea**	-
4005	2	*Penicillium oxalicum*	*Penicillium oxalicum**	-
4006	1	-	-	-
4007	2	*Mucor racemosus*	-	-
4008	2	*Botrytis cinerea*	-	-
4009	2	*Epicoccum nigrum*	-	-
4010	1	*Not sequenced*	-	-
4011	2	*Fusarium lateritium*	-	-
4012	2	-	-	-
4013	1	-	-	-
4014	2	*Penicillium bialowiezense*	*Diaporthe sp.*	-
4015	1	*Fusarium acuminatum*	-	-
4016	1	-	-	-
4017	4	*Pestalotiopsis sp.*	*Penicillium commune*	*Cadophora sp*

Tree Number	No. of samples isolated	Identification 1	Identification 2	Identification 3
4018	4	*Fusarium sp.*	*Fusarium lateritium*	*Cladosporium cladosporioides*
4019	2	*Penicillium brevicompactum*	*Fusarium lateritium*	-
4020	1	*Fusarium sp.*	-	-
4021	1	*Truncatella sp.*	-	-
4022	3	*Fusarium sp.*	*Fusarium acuminatum**	-
4023	1	*Fusarium sp.**	-	-
4024	1	*Fusarium sp.**	-	-

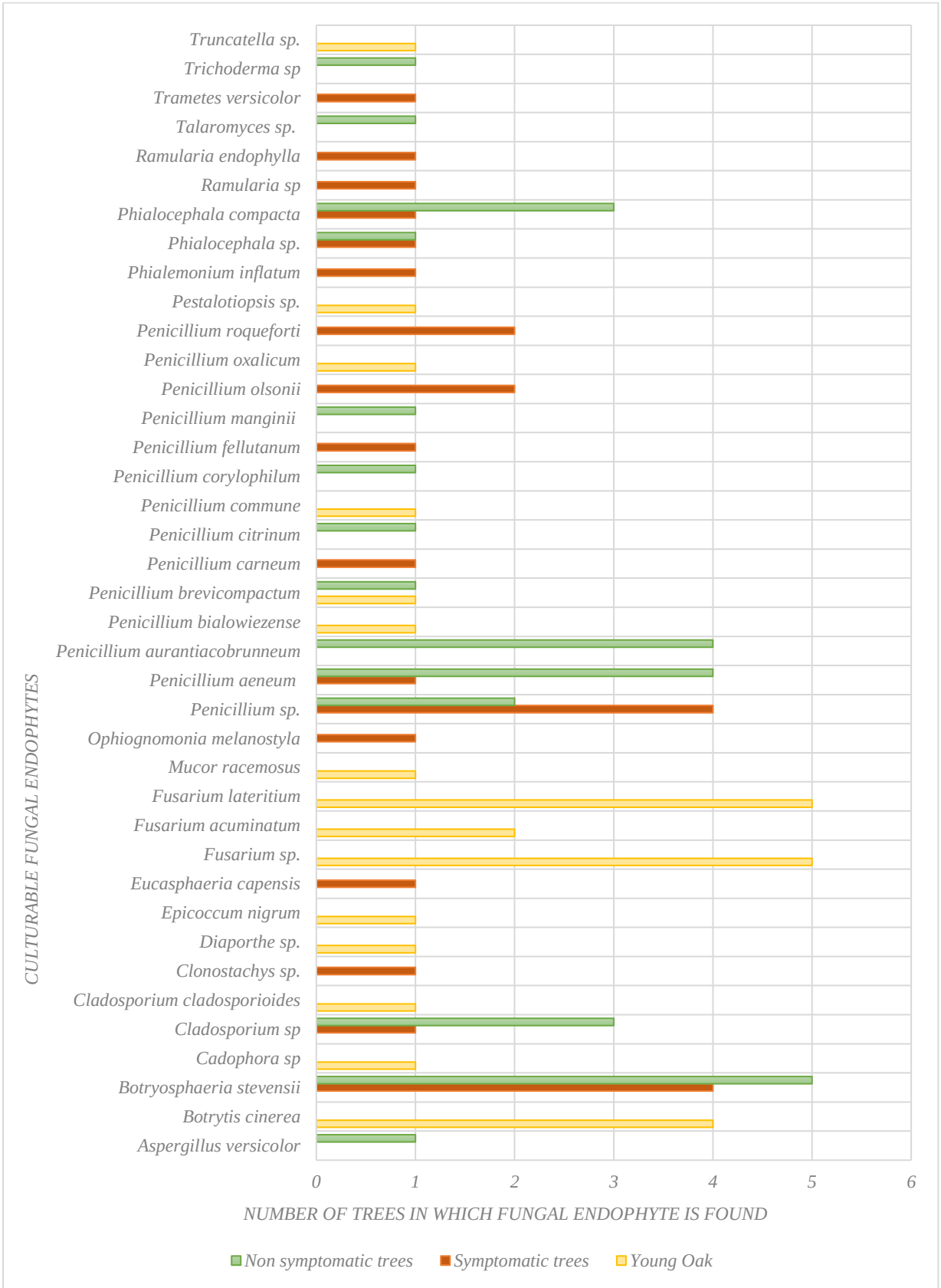


Fig. 9.1: Comparison of fungal endophytes identified in Symptomatic and Non symptomatic *Q.robur* aged 75 to 250 years old and Young *Q.robur* aged 10-15 years old

9.2.1 Differences between the Fungal endophyte communities of the Oak Cohorts

The young Oak group included sampling from 24 trees. The number of different fungal species identified was 15. From the 10 symptomatic Oak trees sampled 17 different species were identified. From the 10 non -symptomatic Oak trees sampled 14 different species were identified. Of the 15 species discoverable with the young Oak trees only 1 species was similarly found on the older symptomatic and non -symptomatic oak trees. This was *Penicillium brevicompactum* also found within 1 non symptomatic Oak.

There were 14 different species of *Penicillium or Penicillium sp*. identified. Of the 54 trees sampled *Penicillium sp.* was discoverable in 30. There was distinct clustering of *Penicillium* species within each group of trees. Within the group of trees that were non -symptomatic *P. aeneum* and *P. aurantiacobrunneum* were predominant. Of these two *P. aeneum* was discoverable in one of the symptomatic trees yet *P. aurantiacobrunneum* was not discoverable in any of the symptomatic trees. The absence of this endophyte may possibly be related to the incidence of bleed. It is perhaps that *P. aurantiacobrunneum* has been precluded from the symptomatic trees as a consequence of the bacterial strains associated with the bleed canker. This may be due to displacement related to spatial availability within the niche environment. However, when we consider the other endophytic fungal species that both the symptomatic and non- symptomatic trees share, such preclusion is unlikely. It may be possible that there is an antagonism between *P. aurantiacobrunneum* such that where this fungal endophyte does not hold dominance within the tree the pathogenic bacterial strains are able to gain a suitable foothold.

9.3 In vitro interaction between fungal endophytes and pathogenic Bacteria

9.3.1 Cladosporium aggregatocicatricatum strain 01185.

The possibility of antagonism between fungal endophytes and pathogenic bacterial strains was initially tested as a consequence of a predominance of fungal endophytes being isolated from non- symptomatic trees. Whilst the numbers and diversity of the microbial community may only be partially revealed by culturing on media it was considered a possible consideration for developing a therapeutic bio-control.

The fungal endophyte dominant from the initial sampling was *Cladosporium aggregatocicatricatum* strain 01185. The species was ascertained through sequencing and by considering the morphology of the conidia and spores with microscopy (Bensch et al., 2015)

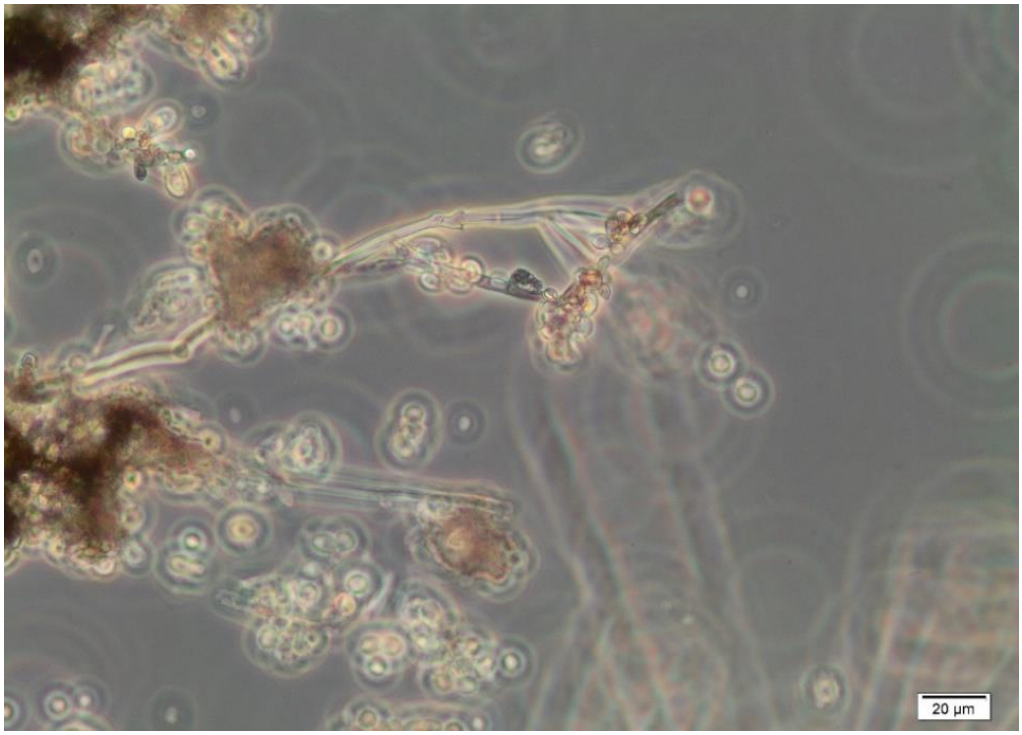


Fig 9.2: Cladosporium aggregatocicatricatum conidiophores Image captured with an Olympus SC100 camera on an Olympus CX41 microscope Cellsens Software.

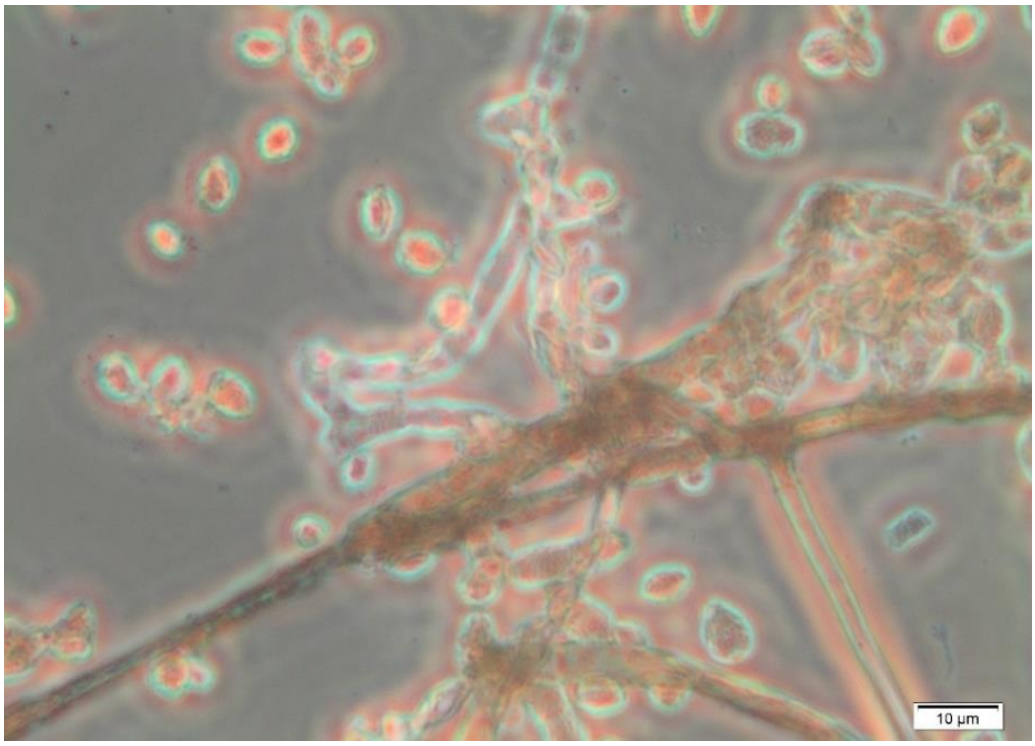


Fig 9.3: Cladosporium aggregatocicatricatum conidia. Image captured with an Olympus SC100 camera on an Olympus CX41 microscope Cellsens Software.

This was tested for antagonism with Brenneria goodwinii strain 931_23, Gibbsiella quercinecans strain 931_22 and Pseudomonas flavescens strain 903_18.

Cladosporium aggregatocicatricatum strain 01185 can be seen to have grown across the plate inhibited only by G. quercinecans strain 931_22. (Fig. 9.4).

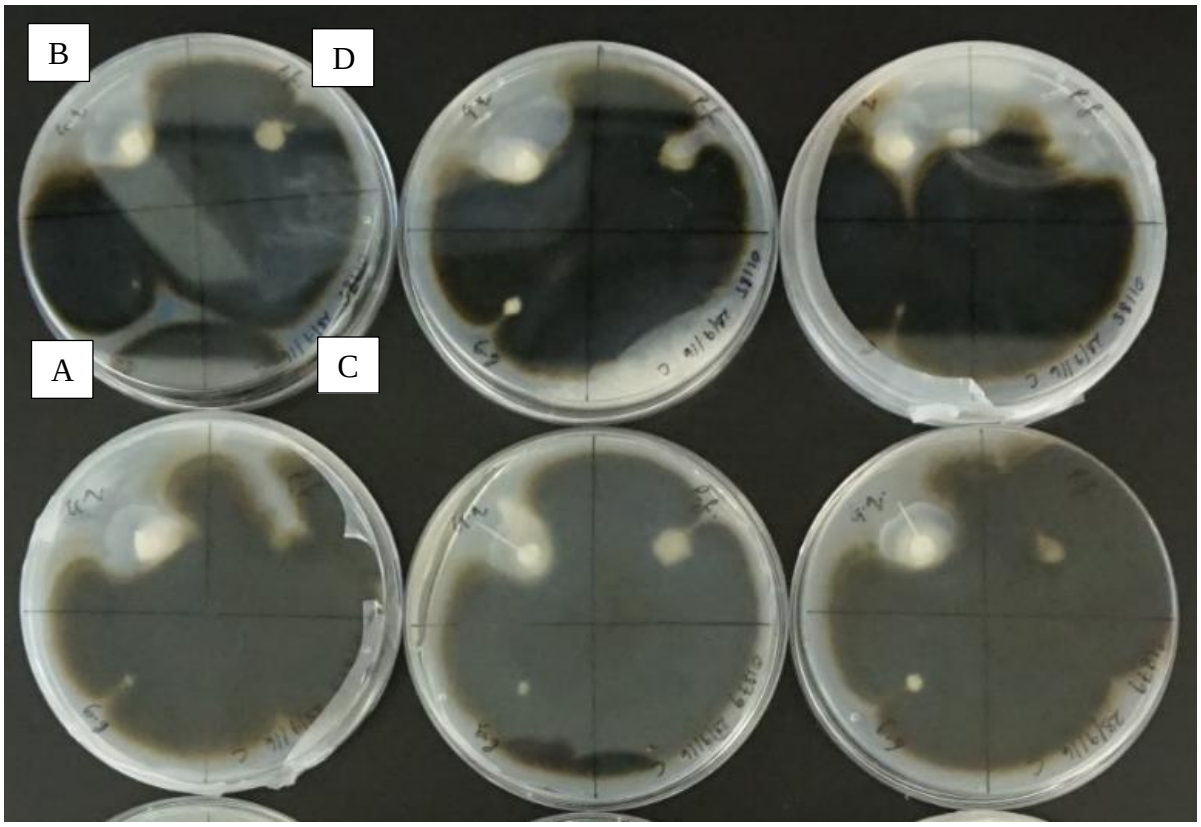


Fig. 9.4: *Brenneria goodwinii* strain 931_23 (A), *Gibbsiella quercinecans* strain 931_22 (B) and *Pseudomonas flavescens* strain 903_18 (D) antagonism with *Cladosporium aggregatocicatricatum* strain 01185. After 14 days grown at 20 ^{0}C. Control (C). Positioning is the same as marked for all plates.

9.3.2 Further Antagonistic Assays with Endophytic Fungi

Further testing of those endophytes discoverable and dominant within the non -symptomatic trees were tested against the most frequently recovered bacterial strains associated with the bleed canker.

Three replicates of each bacterial strain were grown as lawns to give control comparisons. Similarly, three replicates of each endophytic fungal strain were grown to give control comparisons. Each antagonistic assay was replicated three times and an average score was given over all the replicates. (Table 9.4 and Fig 9.5).

Table 9.4: Antagonistic assay results comparing inhibitory growth between endophytic fungal strains and bacterial strains isolated from Acute Oak decline bleed cankers.

Endophyte Strain reference	Identification of Endophyte	Antagonism with Raoultella sp. strain 01195_19	Antagonism with R. victoriana strain 01195_20	Antagonism with G. quercinecans strain 01195_21	Antagonism with B. goodwinii strain 01195_22	Total score
473_EN1	*Penicillium aeneum*	1	3	1	4	9
912_EN8	*Botryosphaeria stevensii*	0	1	1	2	4
913_EN14	*Phialocephala compacta*	1	3	1	3	8
915_EN24	*Penicillium aurantiacobrunneum*	1	4	1	4	10
917_EN37	*Penicillium aeneum*	2	3	1	4	10
920_EN47	*Cladosporium sp.*	1	3	1	3	8

Scoring is based as of following:

0= Bacterial lawn impedes fungal growth. Bacteria growing over fungi

1= Limited fungal growth, fungal colonies outside of spread bacterial lawn

2= Partial fungal growth on plate, poor development relative to control

3= Fungal growth on plate, fair development relative to control

4= Fungal growth on plate, good development relative to control

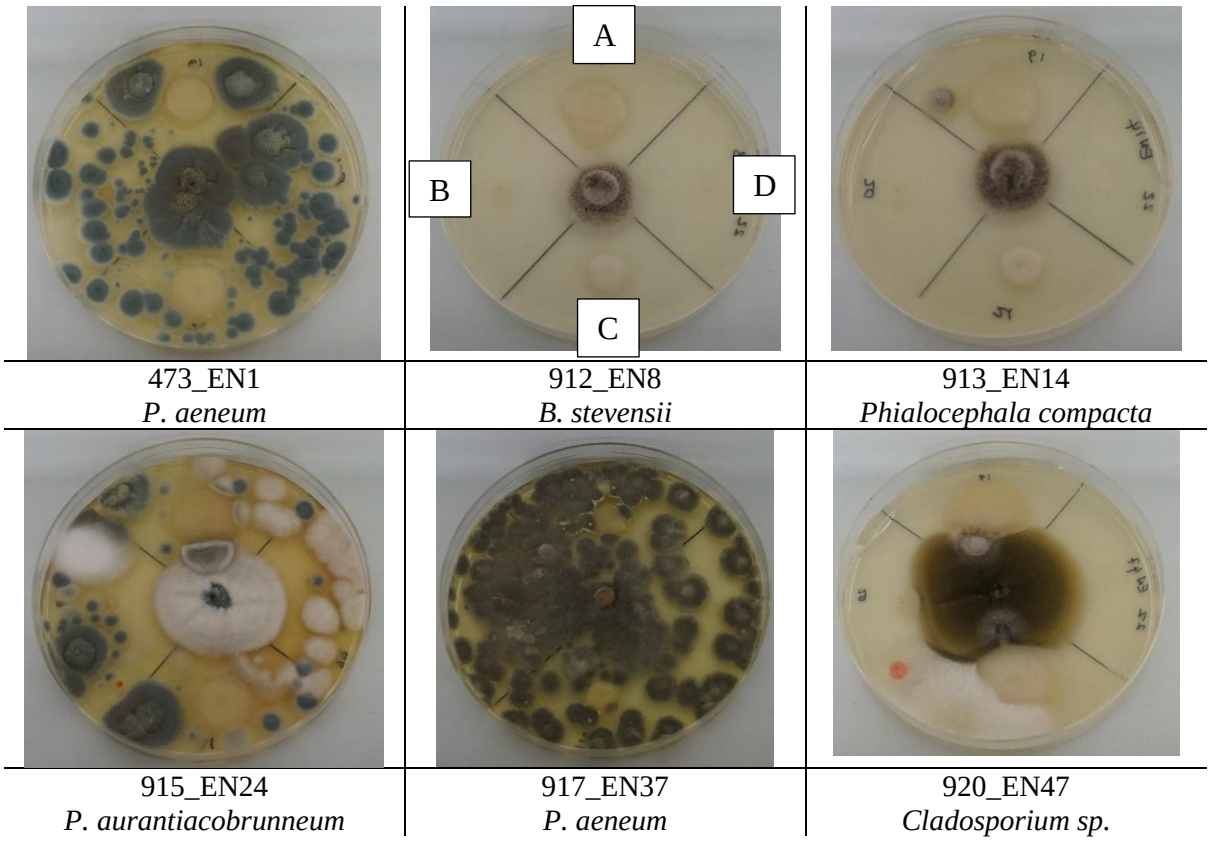


Fig. 9.5: *Raoultella sp.* strain 1195_19 (A), *Rahnella victoriana* strain 1195_20 (B), *Gibbsiella quercinecans* strain 1195_21 (C) *Brenneria goodwinii* strain 1195_22 (D), Positioning is the same as marked for all plates. Antagonism after 14 days grown at 20 ^{0}C.

P. aeneum and *P. aurantiacobrunneum* proved to be the more antagonistic within the tests than the other fungal endophytes. It is notable that both of these fungi are dominant within the non-symptomatic trees and that whilst *P. aeneum* is noted on one symptomatic tree *P. aurantiacobrunneum* was not isolated from any of the non -symptomatic trees.

9.4 Summary of Findings

From sampling and culturing endophytic fungi from both symptomatic and non -symptomatic trees differences were detected in the fungal endophytic populations. *In vitro* antagonistic testing between pathogenic bacterial strains associated with OBC and culturable endophytic fungi from non -symptomatic trees revealed competitive and dominant *Penicillium sp.* strains. The differences between the *Penicillium sp* strains found within the symptomatic and non-symptomatic trees provide an example of microbiome shifts within the endophytic populations of the trees. This microbiome shift may be related to 1) Abiotic changes such as changes in phenological patterns or nutrient availability in soils. 2) The introduction of a biotic pathogenic agent.

Such culturable endophytic fungi may present a means of biocontrol of the Oak Bleed Canker.

Chapter 10: Discussion

10.1 The Context of this research

The primary focus of this study was to establish 1) whether there are any differences between symptomatic trees and non -symptomatic trees that may indicate a pre-disposing factor facilitating the onset of the bleed canker, 2) what the associated /causal bacterial agents are relating to the Oak Bleed Canker and 3) to identify possible methods by which to disrupt or halt the bleed canker.

10.1.1 The Definition of Acute Oak Decline

In the original definition of Acute Oak Decline in 2009, (Denman and Webber, 2009) the decline was likened to a disease event in 1920s. In this instant the damage then was associated with defoliation of trees in early summer by caterpillars of the oak Tortrix moth (Tortrix viridana). This was compounded by an instance of heavy powdery mildew fungus infestation (Erysiphe alphitoides), leading to reports of the acute decline of Oak trees.

It is clear that two factors were contributory to the acute decline in the 1920s. The two events attributed to the same acute decline today are the presence of the Agrilus beetle and the bleed canker. However, common parlance and use of the phrase ‘Acute Oak Decline’ has become (or is becoming) synonymous solely with the indicative bleed canker. (https://en.wikipedia.org/wiki/Acute_oak_decline, accessed 27.03.2021). The bleed canker has perhaps not been attributed its own name, because the causative pathogen/s are undetermined. None- the- less, the bleed or exudate should be named separately from the phenomena of Acute Oak Decline. (Just as the Tortrix moth and powdery mildew are

distinguishable in name and as events from the episode of decline). It is believed that this is important for the following reasons:

1) If Acute Oak Decline is a syndrome and has been identified as an event that occurred previously with two distinct biological agents, then presumably this could occur again with biological agents that were neither a beetle nor a bleed canker. Hence Acute Oak Decline is not synonymous with the bleed canker.

2) If the bleed is part of a decline syndrome then it must be possible for the associated bleed to occur independently of the decline. Hence every tree that has the bleed symptoms is not necessarily suffering from Acute Oak Decline.

10.1.2 Distinguishing Oak Bleed Canker

The target of this study is not to present a complete understanding of the decline event named Acute Oak Decline within the forest complex. Acute Oak Decline by its primary definition requires the presence of the Agrilus beetle as well as the bleed canker. This study is simply to elucidate one aspect of the decline, the bleed canker.

A canker has been defined as ‘a visible dead area, usually of limited extent, in the cortex or bark of a plant’ (Tainter & Baker, 1996). It became apparent from the study that the bleed canker could occur independently of the Agrilus beetle. The bleed canker did not of its own necessarily result in the accelerated demise of the tree or indeed in a decline that was significant or irreversible. The bleed canker is an independent event from Acute Oak Decline, as of this study named independently as Oak Bleed Canker (OBC).

10.2 The Decline Spiral & The Disease Triangle

Manion's Spiral Theory of Decline identifies three factors namely predisposing, inciting and contributing factors, leading to a spiral of decline. This differs from the more traditional disease triangle which considers the conducive environment, susceptible host and damaging agents as the primary factors which when united will lead to disease.

10.2.1 Decline is not a distinct category of disease

It has been argued that decline is not a distinct category of disease and that the overarching term to describe unhealthy trees is 'nonetiologically discriminant'. (Skelly, 1992. Ostry et al., 2011). The reasons given for this include (1) tree decline is a natural progression within forests, (2) that many diseases have decline symptoms, (3) a decline diagnosis does not forecast the fate of the tree, (4) that the decline disease diagnosis does not improve tree management, (5) the disease triangle adequately describes the expression of decline symptoms.

It has been considered that a specific cause of a disease or disease event is rare and more often may involve a complex of diseases involving fungi, insects and weather events. In this sense, and with such complexity of etiology, the disease event is more related to an ecological problem rather than a pathological issue. (Wallace, 1978. Franklin et al, 1987. Oliva et al, 2020).

10.2.2 Modification to the Disease Triangle

The pre -disposing factors attributable within the decline model fall suitably into the disease triangle model. These predominantly fit within the section of the susceptible host (e.g. a weakened condition due to stress) (Ostry et al, 2011), but also cross over into the section of the conducive environment (e.g. historic weather conditions) and the damaging agents (e.g. favourable breeding conditions for the pathogen).

The Oak Bleed Canker (OBC) fits adequately within the disease triangle. Yet there are some notable modifications to consider.

10.2.3 Time and the Disease Triangle

A more accurate Disease triangle model would include Time. Time is relevant to the pathogen lifecycle. This will form a seasonal linear time line, cross correlating with the host. (e.g. tannin production in Oak leaves to offset insect predation (Harborne, 1993)). Disruption to such phenological relationships will bring about significant change. This is clearly seen with changes in climatic conditions in relation to woodlands (Ray et al, 2010).

The timescale in which the disease event is considered will contribute not only to the characterisation of the disease but to the understanding of the ecology and lifecycles of the biotic elements associated. The timing of the event where environment, host and pathogenic meet is crucial to the defining of a disease event.

10.2.4 Observable Anomaly and the Disease Triangle

OBC is a relatively recently recorded disease, first records dating back to 2006 within the Writtle Forest woodland complex. Yet this is not to say that the disease was not present prior to this date. The literature records Oak bleed cankers as occurring on the main stems of the trees for the last 100 years but these are presumed noteworthy once they increase in abundance or appear to be associated with the demise of the tree. In light of this consideration a fifth aspect to the model is proposed, that there must be an observable anomaly. For example, a mutualistic relationship between a potentially pathogenic microbe and the host may well exist prior to the coalescence of changes in the environment over time that may then produce a disease event. Yet our definition of this event as disease is reflective of the view point that we take. This

viewpoint is that an observable (or measurable) anomaly has taken place, classified within the context of interaction between the environment, host pathogen with a set time frame.

10.2.5 Measurable differences to understand the Disease event

To understand the factors resulting in the disease event the adopted methodology was to compare between those trees that exhibit signs of the anomaly or disease (symptomatic) and those that do not (non-symptomatic). At the outset of a disease event these are the only discriminatory subsets by which to ascertain reasons as to the susceptibility to the disease.

This is not necessarily the synoptic approach of ecology whereby the use multiple regression analysis is used by which to consider the determinants. The bleed canker is experienced by Oak trees in forest and urban situations where the ecology and determinants of the disease event are highly variable (as of the varying management strategies toward Oak Processionary Moth. (Williams & Jonusas, 2019)). Hence the methodology of study to ascertain possible suitable methods to mitigate for the Oak Bleed Canker were adopted to allow management of the disease in both a landscape setting, as well as for individual trees in an urbanized environment.

10.3 The Progression of the Oak Bleed Canker over a 6year period

10.3.1 Movement and Persistence of OBC

It was observed that the number of trees with Oak bleed canker increased over the study period within the wider area of the Writtle Forest woodland complex by up to three times. However, within the area of woodland with dense Oak clustering and an existent OBC population exceeding a third of the trees at the beginning of the survey, the increase in OBC amounted to no more than 1.8% over a 6 year period.

Most notable was the movement of the Oak Bleed Canker through the Oak population within the Beta study area. The variance between those trees that had bleed canker in 2013 and those that had the bleed canker in 2019 was 27.3%. Yet given that the overall increase in the number of trees with OBC only increased by 1.79% this meant that those trees that then entered remission and those that contracted the OBC were relatively balanced.

This increase in new OBC (22%) was not reflected in the increase of Agrilus activity (3%). Furthermore, the topographical pattern presented suggests localised movement through the soils, either by inter- connectivity of the root systems, water or possibly shared mycorrhizal relationships.

10.3.2 Mortality of trees with OBC

Of the trees that were to die during the course of the study of 10 symptomatic trees throughout the Writtle Forest Woodland Complex four died during the period between 2012 and 2018. All four trees exhibited signs of Agrilus and bleed in 2012.

The number of dead trees within Stoneymore wood (the 1 Hectare area of study), increased by 8 over the six-year period. 12 had been logged as standing dead in 2013. Of a cohort of 180 live trees this represents an increase in dead trees of 4.44% over the six-year period, (or an annual mortality rate of 0.74%).

Of the 8 trees that died within the six-year period one tree showed no signs of canker, four trees showed signs of 5 or less cankers and three trees showed signs of 10 or more cankers in 2013. Once the trees had died their deteriorated state made identification of the amount of bleed canker present in-determinable. This was due to shedding bark as well as three of the 8 trees having been windblown.

There was no direct correlate established between the death of the tree and the bleed canker. If we were to assume that all seven of the dead trees of the 62 showing signs of infection in 2019,

died due to the bleed canker this would represent 11.29% mortality rate due to infection. However, it is unlikely that those trees which exhibited less than 5 bleed cankers in 2013 died as a consequence of the bleed canker. The demise of 1 of these seven trees is most certainly due to storm damage, a further two trees recorded with less than 5 bleed cankers both exhibited advanced signs of Armillaria sp. root decay. This would give a revised mortality rate for those trees with bleed canker alone of 4.84%. As an annual rate of mortality this would equate to 0.806%, only 0.066% greater than the mortality rate for the Oak cohort within the 1 Hectare area.

The number of trees that were recorded as having the bleed canker and the Agrilus in 2013 was 12, three of these were recorded as dead in 2013. Of these 9 trees 2 were dead in 2019. On this basis the mortality rate is 22.2% for trees that have both the Agrilus and bleed symptoms (although the data set from which this percentage is extrapolated is limited).

The focus of the study was the bleed canker with details recorded as to visible activity of the *Agrilus* beetle. Details were considered as to the presence of *Armillaria sp*. However, the presence or absence of this root decay pathogen is harder to determine. It is only clearly visible after the demise of the tree. Of the 8 trees that died over the six- year period five had visible *Armillaria sp.* infection. *Armillaria mellea* is known to be a secondary pathogen, resulting in the demise of weakened or stressed trees (Wargo, 1996). It cannot be stated with accuracy the presence of *Armillaria sp* root decay within the Oak cohorts in 2013 to provide a true consideration of the effect of the pathogen on the trees, comparative to the bleed canker or *Agrilus*. Suffice to say that of the two dead trees that were recorded with both symptoms of *Agrilus* and bleed canker one was recorded in 2019 as having *Armillaria sp.* root decay.

10.4 Pre-disposition of trees to Oak Bleed Canker

Those factors considered to be pre-disposing to the disease event as part of this study concerned comparative studies between;

1) the immediate soils around symptomatic and non-symptomatic trees

2) the vitality/ condition based on the incremental growth of trees exhibiting new OBC, trees with no OBC, trees with consistent OBC and trees in remission of OBC, all considered over a 6year period.

Clearly the factors that affect the pre-disposition of the trees to possible disease that would enable comparison of symptomatic and non -symptomatic trees are numerable. Such considerations as microtopography (Barsoum et al, 2021) , microbial rhizosphere relationships (Barsoum et al, 2021.Pinho et al, 2020) have been studied in relation to AOD. Other aspects such as historic management processes, water management and cation exchange as well as related vegetation and ecological traits were not the focus of this study.

10.4.1 Comparison of soil nutrient levels

The soil nutrient levels were considered because possible discrepancy would allow for relative ease of remediation both in an urban and landscape setting. Recent work has identified that acidic soils contribute to the level of Oak Bleed Canker observed (Pinho et al, 2020).

The early testing within this study, of calcium amelioration with symptomatic and non-symptomatic trees was undertaken not with the intention to raise pH levels. The intention was to replace calcium to the soils, a nutrient known to effect the biological functioning of trees. It is notable however, that the Calcifert product was more successful than the Root-Gyp product.

The later product claims to improve usable Calcium and Sulphur concentrations within the soil, whilst the Calcifert emphasizes its proven ability to neutralize soil acidity.

This testing was limited due to the size of the trees to ameliorate and the consequent prohibitive cost. It was hoped to have scaled- up the investigation with field testing of young trees, but this did not prove feasible due to the variability of the pathogenicity of the bacteria.

10.4.2 Comparative Tree ring analysis

From rainfall and temperature details from the National Meteorological Library & Archive from the area of Writtle, Essex it can be seen that in the last 33 years temperatures have risen during March to November on average by 1^0C. Over the same time period, but considered over all months, rainfall has decreased on average by 25mm. These factors, as well as any extremes of weather patterns and phenological events will have been generally experienced by all the trees within Writtle Forest.

From the analysis of the tree rings it was expected to be found that the vitality of those trees showing symptoms of the Oak Bleed Canker would appear weaker and that this would be exhibited in the reduced development of the annual rings. However, trees that had continuing signs of OBC had annual rings comparable to those trees that had not shown signs of OBC over the 6 -year period. Hence a predisposing factor that reduces the vitality of the trees was not evident. It was discovered however, that trees that had recovered consequential to the bleed symptoms, exhibited narrower annual rings over the entire 37 years considered.

One reason that the annual ring growth of those trees that are more resilient to the Oak Bleed Canker are smaller than those where the OBC is present, may relate to a trade -off of resources. The tree with smaller annual rings maybe expending more resource on secondary metabolites and defence systems rather than growth (Hirons and Thomas, 2018). This potential difference

in trade off of resources within the Oak population, may relate to genetic traits or to differences in microbial relationships that each tree has established within the woodland.

Traditionally incremental growth has been a means by which to measure the success or otherwise of trees within a woodland. Forest mensuration has been a method by which to predict wood yield and guide management of tree stock. Hence tree ring growth is generally considered an appropriate measure of the condition of a tree (Helama et al., 2009). From the tree ring analysis there appears to be progressive incremental growth of those trees with OBC, in line with all other Oak trees which were non-symptomatic. This would counter the consideration that these trees were in decline or that the presence of OBC alone was indicative of decline.

10.5 Causal Bacterial agents of Oak Bleed Canker

10.5.1 Koch's postulates to establish causative agents of OBC

From the experiments undertaken within this study Koch's postulates were not established. It has been considered that lesion formation as a result of decline-diseases of trees cannot be fully addressed by the use of Koch's postulates due to the polymicrobial nature of the disease syndrome (Denman et al., 2018). The more problematic of issues is that not all potential causative agents are culturable. It is considered that postulates two and four can be overcome using molecular methods looking to characterise microbial communities (Singh et al., 2016).

Koch's postulates are not necessarily a requirement to establish the causative agent of disease. However, they may well serve as a useful process of elimination in determining whether a suspected causative agent is saprophytic or pathogenic. (Agrios, 2005).

10.5.2 Bacteria causing OBC

In the pathogenicity tests undertaken bleed exudate as of the exudate seen on mature Oak trees was produced as a consequence of introducing the inoculum of individual bacteria associated with OBC. Bleeds were seen from individual bacteria *Brenneria goodwinii, Gibbsiella quercinecans and Rahnella victoriana.*

Bleeds were also seen from the inoculum of the following combined bacteria; *B. goodwinii* + *G. quercinecans* + *R. victoriana* + *Raoultella* sp.; *R. victoriana* + *B. goodwinii* + *G. quercinecans; B. goodwinii* + *G. quercinecans* + *Raoultella sp*.; *B. goodwinii* + *G. quercinecans*; *R. victoriana* + *Raoultella sp.; R. victoriana* + *B. goodwinii; R. victoriana* + *G. quercinecans*. Of all of these inoculations the combination of *R. victoriana* + *B. goodwinii* produce the most consistent bleeds.

The OBC can be caused by individual and multiple bacteria. Other species not considered within the pathogenicity tests but that were also regularly (if not consistently) present in samples were *Erwinia spp*, *Pantoea spp* as well as a fungal endophyte, *Botryosphariea stevensii* (anamorph: *Diplodia mutila*), a fungi known to produce bleed cankers on Oak (Alves et al., 2004; Luque et al., 2002; Slippers & Wingfield, 2007). The pathogenicity of these microbials was not ascertained as part of this study. The *Erwinia spp,* and *Pantoea spp.* were not isolated from samples as regularly as other discoverable bacterial species. *B. stevensii* was only isolated when considering the endophytic population within the symptomatic and non-symptomatic trees.

10.5.3 Introduction of the Bacteria into the tree

The entry of the bacteria into the tree has not yet been established. Given the association with A. biguttatus (Brown et al., 2017) it is reasonable to assume that the beetle is a vector of at least one of the pathogenic bacteria. Recent studies show that A. biguttatus harboured various

bacterial genera including Serratia spp., Rahnella spp. Erwinia spp. and Pantoea spp. as well as undifferentiated species of Enterobacteriaceae which may include Brenneria spp. and Gibbsiella spp. (Lewis et al., accessed June 2019).

It can be seen from the epidemiology study that Oak Bleed Canker can occur without the presence of the Agrilus beetle. The spread of OBC between the trees was relatively high with 27% of the trees within the Oak cohort of Stoneymore wood showing new symptoms. The clustering of trees exhibiting signs of new OBC closely related to those trees with recorded OBC in 2013. This would suggest that the mode of transference was related to movement of bacteria through either root connectivity or the soils environment.

10.5.4 Pervasiveness of OBC

Beneath the exudate the excavated bark reveals an area of far more extensive deteriorated wood in varying degrees of degradation. The rates by which the bacteria decay the wood is not known. From observation the decay tends to persist purely within the vascular conducting tissue. Generally, it is the ray cells, perpendicular to the annual growth rings, which provide the main source of nutrients in the form of carbohydrates to invading bacteria (Greaves, 1971). Yet sugars provided by disrupted phloem vessels may also provide nutrition. It is possible that the nutrients required by the bacteria is sufficient to be found within the outer sapwood, without requiring the pathogen to break down wood tissues at depth. Physical barriers in the form of latewood cells may also contribute to halting the spread of the decay further into the tree. Yet as the spread of decay develops without the tree creating suitable barriers to the movement and decay mechanism, so the risk of the tree stem being girdled increases. This disruption of the vascular tissue reduces the biological functioning of the tree.

The process will be accelerated with the presence of A. biguttatus larvae often moving horizontally through the sapwood and spreading the bacteria at an increased rate.

10.5.5 Mode of decay

It is evident on excavation from bleed sites that the wood directly beneath the area of exudation is heavily decayed and that this area of decayed wood spreads beneath the bark through the sapwood tissue both laterally and longitudinally on the stem. When sampling the amount of water that is released as a consequence of cutting into the wood is dependent upon the time of year. In April the release of exudate is relatively passive consequent to excising the bleed. In late June/ July the release of fluid is at pressure often with a sustained flow of discoloured water. This would indicate that the area observed on the bark as bleed is consequent to decayed sapwood and blocked xylem vessels behind the bark. The build -up of pressure is what causes the bleed to flow, either from weakened wood tissue or possibly from adjacent wounding.

Microscopy was not used in this study to determine the mode of wood biodegradation. The penetration of wood by bacteria is limited. Lack of mobility restricts the bacterial mode of attack, prolonging the process of wood cell wall deterioration (Clausen, 1996). Consequent mobility of the bacteria inside the wood environment will rely on motility, transport by an aqueous environment, or synergistic effects of multiple enzymes upon wood cell wall penetration (Greaves, 1971).

From study of the inoculated stems the staining revealed that the horizontal spread was generally limited to the area of the inoculation. It was only with inoculations with G. quercinecans that the stain within the sapwood spread horizontally, beyond the inoculation site if only minimally. This would seem to link with the apparent lack of motility that G. quercinecans exhibited in the motility tests. It would also link with the reported mode of decay from bacteria being most effective when residing alongside the wood cells for longer allowed degradation of the cell wall.

Most staining witnessed was vertical. The more motile B. goodwinii and R. victoriana will be able to spread within the water transporting xylem vessels. Even once these vessels become dysfunctional, as the water conducting vessels are blocked by the G. quercinecans, both B. goodwinii and R. victoriana are still able to colonise through swarming. There appears to be minimal antagonism between B. goodwinii and R. victoriana. This may account for the increased bleed exhibited from inoculated trees as the two bacteria are able colonise more rapidly.

The fact that the bleeds tend to occur and coalesce on the main stem of the tree at heights of around 2m may be due to the movement of the bacteria from the roots through the xylem tissue. Generally, bacteria cannot progress downwards through the phloem cells due to their construction as of a sieve (Bove and Garnier, 2002). Yet the size of the phloem cells of Oak produced at the beginning of the growing season may allow the smaller rod shaped Enterobacteriaceae to flow back down the root systems and into the soil environment (Gričar, 2010). Another means by which the bacteria can enter the soil environment is from heavy bleeds. It was observed that where the exudate was heavy, enough inoculum can reach the ground to permeate the soils.

10.6 Management of Oak Bleed Canker

Whilst the bleed canker itself does not appear to cause mortality of the Oak trees it is none-the-less contributory to the general dysfunction of the tree. The consequent disruption to the vascular tissue will affect the overall biological functioning of the tree. Integrated management of disease provides a holistic approach to reducing the inoculum, either within focal areas within the landscape or for individual trees of merit.

10.6.1 Management of Stand density

The spread of the disease appears to be soil borne. Reduction of the number of same species cohorts within the forest stands will reduce the movement and levels of the inoculum within the Writtle Forest woodland. General encouragement of diverse species planting spread over larger areas of the woodland will reduce such inoculum sinks. Given that the predominance of Oak Bleed Canker occurs on early mature to mature trees, adoption of Continuous Cover Forestry principles are also recommended. This would allow for a more heterogenous tree stock.

10.6.2 Management of *Agrilus* population

It would appear that trees which exhibit symptoms of both the bacterial pathogen and the *Agrilus* beetle are more likely to decline acutely and have reduced mortality. To which end the reduction of the Agrilus population within the woodland would be favourable. This may be achieved using suitable pheromone traps. (Imrei et al, 2020).

10.6.3 Soil remediation

From general testing of soils the visible bleed cankers were reduced on trees when applying calcium products. It was noticeable that of the 2 products tested that the product that focused on neutralisation of acidic soils rather than replenishment of calcium levels was more successful. Hence soil ameliorants which restore pH levels to those recorded previously (minimum pH 5.5 as of Rackham, 2003) will improve the general soil conditions for the Oak trees. More research will be required to ascertain optimum pH levels to be achieved through appropriate application rates, timings and application methods for applying the amendments to the soils.

10.6.4 Canker treatment with Tannin

Remediation methods of treating canker on trees have included the use of soil drenches of Paclobutrazol (Weiland et al, 2009) and Phosphites (Percival & Banks, 2015) as well as soil ameliorants such as Biochar (Zwart & Kim, 2012). More recent experimental work has looked at evolved bacteriophage to reduce inoculum levels of Horse Chestnut Bleed Canker, applied directly to the infection. (James et al, 2020).

The presence of OBC appears mainly on early mature to mature Oak trees. This age of tree has reduced levels of Tannin compared to younger trees whose bark will contain 12-16% Tannin. (Hathway, 1958). The differences in Tannin levels between young and old trees and the differences in Tannin levels between symptomatic and non -symptomatic trees has not been established as part of this study. However, in vitro testing did show that there was antagonism between Tannin and pathogenic bacteria.

Tannin production plays a large role in protecting the Oak tree against insect herbivoration. Tannin is released to the leaf at a specific time in the season relative to insect/ caterpillar lifecycles, to reduce defoliation (Harborne, 1993). Recent changes in phenological events due

to climatic change (Chmielewski & Rötzer, 2001: Cleland et al., 2007) may have altered the Tannin production cycle rendering the tree more vulnerable to bacterial pathogens that would otherwise be deterred by the anti-microbial effect of Tannin production. Tannin maybe applied in a suitable concentration directly to the bleed canker as method of control, utilising defence compounds ordinarily produced by the tree. Secondary metabolites such as Tannin may be produced, not only by the plant but also by fungal endophytes. It has been shown that *Penicillium spp.* can produce Tannin (Bhat et al., 1998; Murugana et al., 2007; Govindarajan et al., 2016).

10.6.5 Endophytic Bio controls

Antagonistic endophytes as biocontrols are being used more extensively within both Agriculture and Forestry. (De Silva et al, 2019). *Trichoderma spp.* has been well known as a biocontrol agent for the last twenty years. (Benítez et al, 2004. Ghazanfar et al, 2018).

In vitro antagonistic testing between pathogenic bacterial strains associated with OBC and culturable endophytic fungi from non -symptomatic trees revealed competitive and dominant *Penicillium sp.* strains. Such culturable endophytic fungi may present a means of biocontrol of the Oak Bleed Canker.

Penicillium aeneum or *Penicillium aurantiacobrunneum,* the two *Penicillium sp* strains found predominantly within the non- symptomatic Oak trees, were shown to be antagonistic to all 3 pathogenic bacteria associated with the Oak Bleed Canker in *in vitro* testing. There is very sparse literature on either of these fungi and none that correlates with Oak trees from my research. That both fungi are naturally occurring and culturable from within non symptomatic Oak tissue would suggest that if the fungi is generally safe to work with, then its use as a naturally occurring biocontrol is ideal.

Endophytes are generally specific to aspects of the tree; e.g. in forest soils, the dominant fungal group is Basidiomycota, whereas Ascomycota is the most prevalent group within plant tissues. (Terhonen et al, 2019). Thus to ensure the success of the application of the endophytes this should be made within favourable plant tissue, as preventative of spread of the bacterial pathogen.

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Appendix

Appendix 1: 2013 Data relating to Oak Cohorts within Stoneymore

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
701	SM	460	22	8	Normal	N	0	0	0	0	0	0	none
702	EM	540	22	10	Above normal	N	0	0	0	0	0	0	Ivy clad stem. Next to road
703	EM	640	24	12	Above normal	N	0	0	0	0	0	0	Exposed buttress roots. Minor exit holes - pinhole borer

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
704	SM	470	20	9	Normal	N	0	0	0	0	0	0	Epicormic. Next to road
705	EM	520	22	12	Normal	N	0	0	0	0	0	0	none
706	EM	520	22	8	Excessive	N	10	4	6	4	6	S	none
707	EM	530	22	12	Excessive	N	5	2	3	4	3	S	none
708	SM	490	22	12	Normal	N	2	1	1	3	0	N	Frost crack at base
709	EM	540	20	9	Excessive	N	35	25	10	8	2	N,S,E+W	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
710	M	840	24	14	Above normal	N	9	4	5	3	5	S	Ivy . Possible Armillaria
711	SM	350	20	5	Above normal	N	9	0	9	2	9	N,S	none
712	EM	590	22	9	Above normal	N	1	0	1	1.5	1	N,S	Heavy burring
713	EM	540	22	12	Above normal	N	5	0	5	3	9	S	none
714	EM	555	22	10	Excessive	Y	5	0	5	3	3	N,S,E +W	Storm damage, Armillaria. Hypoxylon

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
715	EM	510	22	14	Normal	N	3	0	3	5	2	S	none
716	EM	670	24	14	Above normal	N	0	0	0	0	0	0	Minor cracking at base. Possible Armillaria
717	EM	520	22	8	Normal	N	0	0	0	0	0	0	Possible Armillaria
718	SM	430	24	6	Above normal	N	7	4	3	2.5	4	S	none
719	SM	495	24	6	Above normal	N	10	4	6	4	6	S	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
720	Y	290	18	3	Above normal	Y	10	5	5	2.5	7	S	none
721	EM	630	24	12	Normal	N	2	0	2	2	2	S	Possible Armillaria
722	SM	360	22	5	Above normal	N	5	0	5	2.5	5	N,S,E +W	none
723	SM	475	24	6	Above normal	N	4	0	4	3	4	S	none
724	SM	495	24	15	Above normal	N	10	3	7	12	5	S	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
725	EM	530	22	7	Above normal	N	0	0	0	0	0	0	none
726	EM	630	25	16	Above normal	N	0	0	0	0	0	0	none
727	SM	410	22	8	Above normal	N	5	1	4	3	5	S	Burrowing to base of tree
728	Y	295	18	4	Above normal	N	1	0	1	2	1	S	Minor wounding at base of tree
729	SM	400	20	5	Above normal	Y	11	5	6	3	9	S	Damage at base ot tree to north

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
730	EM	590	25	14	Normal	N	2	0	2	2	2	N	none
731	SM	460	24	11	Normal	N	0	0	0	0	0	0	none
732	SM	310	16	10	Normal	N	0	0	0	0	0	0	none
733	SM	430	20	12	Normal	N	5	3	2	3	4	S	none
734	SM	390	20	4	Normal	N	3	0	3	1.5	3	N	none
735	SM	445	20	7	Above normal	N	1	0	1	2	1	S	none
736	SM	440	20	6	Above normal	N	0	0	0	0	0	0	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
737	EM	540	20	9	Normal	N	3	0	3	2.5	3	N	none
738	SM	490	20	8	Above normal	Y	5	3	2	2	5	S	Hypoxolyn canker
739	SM	415	20	9	Normal	N	4	2	2	2	4	S	none
740	EM	550	22	9	Above normal	N	6	2	4	3	6	S	none
741	EM	600	24	11	Normal	N	0	0	0	0	0	0	none
742	SM	380	18	6	Above normal	N	0	0	0	0	0	0	Storm damaged central leader

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
743	SM	440	22	7	Normal	N	0	0	0	0	0	0	none
744	EM	680	26	15	Normal	N	0	0	0	0	0	0	none
745	SM	475	18	10	Above normal	N	1	0	1	1.5	1	E	none
746	SM	495	20	10	Normal	N	0	0	0	0	0	0	none
747	SM	330	20	5	Above normal	N	0	0	0	0	0	0	none
748	M	725	26	14	Normal	N	0	0	0	0	0	0	Large storm damaged lateral

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
749	SM	440	20	7	Excessive	Y	5	0	5	1.5	5	S	Major dieback of central lead at 4m
750	EM	680	26	12	Normal	N	1	0	1	1	1	E	Large storm damaged lateral
751	SM	325	18	8	Normal	N	0	0	0	0	0	0	none
752	EM	665	24	12	Above normal	N	4	0	4	1.5	4	E	none
753	EM	675	24	14	Normal	N	0	0	0	0	0	0	none
754	EM	570	24	14	Normal	N	0	0	0	0	0	0	none
755	EM	670	25	10	Normal	N	0	0	0	0	0	0	Limb lost +damage to main stem

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
756	EM	535	20	9	Normal	N	0	0	0	0	0	0	none
757	EM	575	20	15	Normal	N	0	0	0	0	0	0	none
758	EM	675	22	15	Normal	N	0	0	0	0	0	0	Major crack to base of tree
759	SM	385	10	3	DEAD	N	0	0	0	0	0	0	Possible Armillaria Exit holes - not Agrilus
760	SM	400	20	7	Normal	N	0	0	0	0	0	0	none
761	SM	430	20	7	Normal	N	0	0	0	0	0	0	none
762	SM	470	18	9	Normal	N	0	0	0	0	0	0	Minor decay to buttress. Adjacent to ditch line

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
763	SM	285	10	1	DEAD	Y	0	0	0	0	0	0	Hypoxolyn canker
764	EM	575	20	12	Normal	N	0	0	0	0	0	0	none
765	SM	435	20	8	Normal	N	0	0	0	0	0	0	none
766	SM	400	20	7	Normal	N	0	0	0	0	0	0	none
767	SM	380	20	7	Normal	N	0	0	0	0	0	0	none
768	EM	665	24	10	Normal	N	0	0	0	0	0	0	none
769	SM	460	20	7	Normal	N	0	0	0	0	0	0	none
770	EM	580	22	10	Normal	N	0	0	0	0	0	0	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
771	SM	400	20	6	Normal	N	6	3	3	3	3	E	none
772	EM	535	22	10	Above normal	Y	0	0	0	0	0	0	Armillaria + Hypoxylon
773	EM	580	22	8	Normal	N	0	0	0	0	0	0	Minor basal decay
774	SM	340	18	5	Normal	N	0	0	0	0	0	0	Minor basal decay
775	EM	500	22	7	Above normal	N	6	1	5	3	6	S	none
776	SM	480	22	8	Normal	N	0	0	0	0	0	0	none
777	SM	425	20	7	Normal	N	0	0	0	0	0	0	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
778	SM	365	16	6	Above normal	N	5	1	4	3	5	S	none
779	SM	450	20	6	Normal	N	2	0	2	2	2	S	none
780	SM	360	18	5	Normal	N	0	0	0	0	0	0	none
781	SM	390	18	8	Normal	N	0	0	0	0	0	0	none
782	SM	435	18	9	Normal	N	0	0	0	0	0	0	none
783	SM	480	22	8	Normal	N	0	0	0	0	0	0	none
784	SM	485	22	8	Normal	N	0	0	0	0	0	0	Minor basal damage

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
785	SM	460	20	8	Above normal	N	2	0	2	1	2	S	Basal decay
786	Y	210	14	4	Above normal	N	0	0	0	0	0	0	Lost central leader
787	Y	265	16	2	DEAD	N	0	0	0	0	0	0	Insect exit holes, Hypoxylon canker
788	SM	295	18	6	Normal	N	2	0	2	2	2	N,S	Damage to main stem
789	SM	455	20	8	Normal	N	0	0	0	0	0	0	none
790	EM	540	22	9	Normal	N	0	0	0	0	0	0	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
791	SM	370	20	8	Normal	N	0	0	0	0	0	0	none
792	SM	405	22	8	Normal	N	0	0	0	0	0	0	none
851	EM	565	23	10	Above normal	N	0	0	0	0	0	0	none
852	SM	420	23	7	Above normal	N	0	0	0	0	0	0	Woodpecker hole in main stem at 5m
853	EM	530	22	7	Normal	N	0	0	0	0	0	0	none
854	EM	615	24	6	Above normal	N	0	0	0	0	0	0	Waterlogged track 4m to N

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
855	SM	410	24	4	Normal	N	8	5	3	3	0	S, W	Pinhole exit wounds present + drainage ditch 2m to E
856	SM	340	24	4	Above normal	N	1	0	1	2	0	S	none
857	EM	515	24	5	Normal	N	6	4	2	3	0	N, S	Ivy covered, drainage ditch 1m to E
858	SM	490	22	7	Normal	N	1	0	1	2	1	W	none
859	SM	465	23	7	normal	N	0	0	0	0	0	0	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
860	SM	395	22	5	DEAD	N	22	12	10	10	10	N, S, E, W	DEAD
861	EM	680	24	9	Normal	N	0	0	0	0	0	0	Wounding to lower stem + buttress. Possible Armillaria
862	EM	640	23	10	Normal	N	25	15	10	8	11	N, S, E, W	Possible Armillaria
863	SM	460	18	4	DEAD	Y	1	0	1	1	1	N	DEAD
864	SM	345	23	4	normal	N	0	0	0	0	0	0	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
865	SM	470	24	5	Above normal	N	0	0	0	0	0	0	Possible Armillaria Drainage ditch 1m to E
866	EM	550	22	6	Above normal	N	0	0	0	0	0	0	none
867	EM	535	23	4	Normal	N	4	1	3	3	4	W	Wounding on Buttress roots
868	SM	480	24	6	Above normal	N	0	0	0	0	0	0	none
870	SM	420	23	3	Normal	N	0	0	0	0	0	0	Possible Armillaria
871	SM	350	23	4	Normal	N	0	0	0	0	0	0	Possible Armillaria

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
872	SM	450	23	7	Above normal	N	0	0	0	0	0	0	Possible Armillaria
873	SM	430	23	4	Normal	N	5	0	5	3	5	N, W	Lost central leader. Wounding on Buttress roots
874	SM	410	24	4	Above normal	N	1	1	0	3	0	W	Growing on bank
875	EM	520	24	6	Above normal	N	17	7	10	10	6	S, E, W	Wounding to lower stem. Possible Armillaria
876	EM	520	22	4	DEAD	Y	25	0	25	10	0	S	DEAD. Possible Armillaria

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
877	SM	320	23	3	Above normal	N	10	0	25	3	7	S, W	Possible Armillaria
878	EM	525	24	6	Above normal	N	15	9	6	5	6	N, W	Ditch 2m to W
879	SM	390	24	4	Above normal	N	0	0	0	0	0	0	none
880	SM	405	23	5	Normal	N	5	0	5	8	5	W	none
881	SM	310	23	3	Excessive	N	4	0	4	4	4	E	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
882	EM	540	23	6	Above normal	N	11	1	10	5	11	E	Possible Armillaria
883	SM	380	24	4	Excessive	N	5	0	5	4	4	S, W	none
884	SM	440	24	4	Normal	N	0	0	0	0	0	0	none
885	EM	630	26	10	Normal	N	0	0	0	0	0	0	none
886	EM	550	25	5	Above normal	N	2	2	0	3	0	S	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
887	SM	270	14	7	Above normal	N	0	0	0	0	0	0	none
888	EM	505	25	7	Above normal	N	0	0	0	0	0	0	Ditch 4m to W
889	SM	340	21	4	DEAD	N	0	0	0	0	0	0	DEAD + multiple exit holes
890	EM	635	25	9	Above normal	N	0	0	0	0	0	0	Basal decay
891	SM	485	23	10	Normal	N	0	0	0	0	0	0	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
892	SM	365	15	8	Above normal	N	10	0	10	4	8	N, S, E, W	none
893	SM	345	13	3	DEAD	N	0	0	0	0	0	0	Ditch 4m to SW + Pin exit holes
894	SM	495	23	7	Excessive	N	10	6	4	4	9	N, S, W	Armillaria. Decay at base + Ditch 4m to W
895	SM	490	18	3	DEAD	Y	3	0	3	2	0	N, S, W	DEAD
896	Y	195	12	0	DEAD	N	0	0	0	0	0	0	DEAD + exit holes

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
897	EM	555	24	8	Above normal	N	1	1	0	2	0	S	none
898	EM	605	26	8	Normal	N	0	0	0	0	0	0	Decay at base. Possible Armillaria
899	SM	465	24	4	Above normal	N	0	0	0	0	0	0	Ditch 2m to E
900	SM	370	18	4	Above normal	N	4	2	2	3	1	S,W	Top blown out + Ditch 3m to E
901	EM	505	24	8	Above normal	N	0	0	0	0	0	0	Ditch 3m to W

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
902	EM	510	24	10	Above normal	N	0	0	0	0	0	0	N/A
903	EM	555	25	4	Normal	N	0	0	0	0	0	0	N/A
904	EM	500	23	11	Above normal	N	0	0	0	0	0	0	N/A
905	SM	460	18	6	Above normal	N	0	0	0	0	0	0	Top blown out + Exit holes. Possible Armillaria
906	M	720	24	10	Above normal	N	0	0	0	0	0	0	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
907	SM	380	23	7	Normal	N	0	0	0	0	0	0	Half in ditch
908	SM	350	22	7	Normal	N	4	2	2	2	0	SW	Ditch 2m to E
909	EM	650	25	10	Above normal	N	0	0	0	0	0	0	Ditch 1m to E
910	SM	410	23	6	Normal	N	0	0	0	0	0	0	Decay at base. Possible Armillaria
911	SM	405	23	6	Normal	N	0	0	0	0	0	0	none
912	EM	655	24	10	Above normal	N	0	0	0	0	0	0	Ditch 3m to NW + Basal decay + Exit hole
913	EM	610	23	9	Normal	N	0	0	0	0	0	0	Armillaria.

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
914	EM	660	24	7	Above normal	N	0	0	0	0	0	0	none
915	EM	610	24	7	Above normal	Y	0	0	0	0	0	0	Exit holes
916	SM	330	13	3	Normal	Y	7	0	7	5	1	W	none
917	EM	510	21	7	Normal	N	1	1	0	5	1	E	Ditch 1m to E
918	EM	505	22	6	Normal	N	2	0	2	4	2	W	none
919	EM	535	22	8	Normal	N	0	0	0	0	0	0	none
920	SM	380	23	4	Normal	N	0	0	0	0	0	0	Ditch 1m to E

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
921	SM	415	18	3	DEAD	N	0	0	0	0	0	0	DEAD. Armillaria + Ditch 1m to E
922	SM	370	22	3	Above normal	N	0	0	0	0	0	0	none
923	SM	490	21	9	Above normal	N	0	0	0	0	0	0	Armillaria
924	M	720	25	8	Normal	N	0	0	0	0	0	0	none
925	EM	540	22	8	Excessive	Y	25	15	10	9	7	N,S,E +W	Possible Armillaria

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
926	SM	370	22	9	Normal	N	4	2	2	4	4	W,E,N	none
927	SM	440	22	7	Normal	N	0	0	0	0	0	0	Damage to base of tree
928	SM	445	22	4	Normal	N	0	0	0	0	0	0	none
929	SM	320	12	4	Above normal	N	5	2	3	2	4	W	Central leader failed
930	EM	570	22	7	Above normal	N	1	1	0	2	0	W	none
931	SM	335	18	5	Excessive	N	0	0	0	0	0	0	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
932	SM	365	23	3	Normal	N	0	0	0	0	0	0	Basal decay
933	SM	445	23	6	Above Normal	N	0	0	0	0	0	0	none
934	EM	510	23	5	Above Normal	N	0	0	0	0	0	0	none
935	SM	355	19	2	DEAD	Y	0	0	0	0	0	0	Dead
936	EM	620	23	12	Normal	N	0	0	0	0	0	0	none
937	EM	660	23	8	Above Normal	N	0	0	0	0	0	0	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
938	SM	400	20	6	Above Normal	Y	10	4	6	3	5	S,W	none
939	EM	600	22	6	Above Normal	N	4	2	2	2	3	W	Ditch 2m to E, basal decay
940	SM	445	22	3	Normal	N	0	0	0	0	0	0	none
941	M	815	25	12	Normal	N	0	0	0	0	0	0	none
942	EM	525	22	7	Above normal	N	0	0	0	0	0	0	none

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
943	SM	470	22	6	Above normal	N	1	0	1	2	2	W	Basal decay
944	EM	515	22	6	Above normal	N	1	1	0	2	0	S	Ditch 2m to S
945	EM	630	24	10	Normal	N	0	0	0	0	0	0	Ditch 2m to E
946	SM	350	24	4	Normal	N	0	0	0	0	0	0	Ditch 1m to E
947	EM	630	24	10	Normal	N	0	0	0	0	0	0	Ditch 3m to E
948	SM	470	22	6	Above normal	Y	2	2	0	2	0	N,W	By ditch

Tag no.	Age	DBH 1.5m (mm)	Height (metres)	Crown Spread (metres)	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
949	EM	515	22	7	Normal	N	0	0	0	0	0	0	none
950	SM	455	22	8	Normal	N	0	0	0	0	0	0	none
951	EM	555	21	8	Above normal	N	6	1	5	2	0	S	Basal decay. Possible Honey Fungus

Appendix 2: 2016 Data relating to Oak Cohorts within Stoneymore

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
701	Normal	N	0	0	0	0	0	0	none
702	Above normal	N	0	0	0	0	0	0	Ivy
703	Above normal	N	0	0	0	0	0	0	Exposed buttress roots
704	Normal	N	0	0	0	0	0	0	none
705	Normal	N	0	0	0	0	0	0	very minor bleed symptoms
706	Excessive	N	8	3	5	4	6	S,N	none
707	Above Normal	N	4	1	3	4	3	S, N	none
708	Normal	N	4	2	2	3	0	E,S	Damage to base of tree
709	Dead	N	35	15	20	8	25	N,E,S,W	none
710	Above Normal	N	4	2	2	2	1	W,S	Ivy
711	Above Normal	N	5	4	1	2	3	N,S	none
712	Normal	N	0	0	0	0	0	0	Heavy burring
713	Above Normal	N	5	0	5	3	8	S,N,W	none
714	Excessive	Y	3	0	3	2	3	S,N,W	Extensive decay to SE at base due to Armillaria
715	Normal	N	1	0	1	3	1	S	none
716	Above normal	N	0	0	0	0	0	0	none
717	Normal	N	3	2	1	3	2	N,S	none
718	Above normal	N	6	3	3	3	4	N,W,S	Pinhole borer exit holes to base to S
719	Normal	N	6	2	4	4	5	S,N,E	none
720	Above normal	Y	5	3	2	3	6	N,S,E+ W	none
721	Normal	N	2	0	2	2	2	S	none
722	Normal	N	4	1	3	3	5	N,S,E+ W	Exit holes - not Agrilus
723	Normal	N	3	0	3	3	4	N,S	none
724	Normal	N	5	2	3	8	5	S	none
725	Above normal	N	1	1	0	2	0	W	none

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
726	Normal	N	0	0	0	0	0	0	none
727	Above normal	N	3	1	2	0	2	S	none
728	Normal	N	0	0	0	0	0	0	none
729	Above normal	N	11	4	7	3	6	S+W	Damage at base ot tree to north
730	Normal	N	2	1	1	2	2	N,W	none
731	Normal	N	1	1	0	3	0	W	none
732	Normal	N	2	2	0	2	0	S	none
733	Normal	N	3	1	2	3	2	S	none
734	Normal	N	3	1	2	1	3	N	none
735	Above normal	N	0	0	0	0	0	0	none
736	Above normal	N	1	1	0	2	0	N	none
737	Normal	N	2	1	1	3	3	N,S	none
738	DEAD	Y	2	0	2	2	2	S	Standing Dead, Agrilus and Armillaria
739	Normal	N	6	2	4	4	3	N,S,W	none
740	Above normal	N	12	6	6	3	6	N,S,E+W	Extensive Bleeds
741	Normal	N	1	1	0	2	0	S	none
742	Above normal	N	0	0	0	0	0	0	Storm damaged central leader
743	Normal	N	0	0	0	0	0	0	none
744	Normal	N	0	0	0	0	0	0	Perfect Timber tree
745	Excessive	N	0	0	0	0	0	0	Possible Armillaria
746	Above normal	N	2	1	1	3	0	S,W	none
747	Above normal	N	0	0	0	0	0	0	none
748	Normal	N	0	0	0	0	0	0	Storm damage
749	DEAD	Y	5	1	4	1	4	S,E	Standing Dead
750	Normal	N	2	1	1	2	1	E	none
751	Above Normal	N	3	2	1	2	0	S	none
752	Above normal	N	3	2	1	4	2	E	none
753	Normal	N	0	0	0	0	0	0	none
754	Normal	N	0	0	0	0	0	0	none

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
755	Normal	N	0	0	0	0	0	0	storm damage limb 3m to E
756	Above normal	N	0	0	0	0	0	0	none
757	Normal	N	0	0	0	0	0	0	none
758	Normal	N	0	0	0	0	0	0	Historic damage to base of tree
759	DEAD	N	0	0	0	0	0	0	Wind blown dead tree, Armillaria
760	Normal	N	0	0	0	0	1	0	none
761	Normal	N	0	0	0	0	0	0	none
762	Normal	N	0	0	0	0	0	0	none
763	DEAD	Y	0	0	0	0	0	0	Wind blown dead tree
764	Normal	N	0	0	0	0	0	0	none
765	Normal	N	0	0	0	0	0	0	none
766	Normal	N	0	0	0	0	0	0	none
767	Normal	N	0	0	0	0	0	0	none
768	Normal	N	0	0	0	0	0	0	none
769	Normal	N	0	0	0	0	0	0	none
770	Normal	N	0	0	0	0	0	0	none
771	Normal	N	4	1	3	3	0	N,E	none
772	DEAD	Y	0	0	0	0	0	0	Wind blown dead tree Armillaria evident
773	Normal	N	0	0	0	0	0	0	Storm damaged crown
774	Normal	N	1	1	0	1	0	S	none
775	Above normal	N	3	0	3	3	3	S	none
776	Normal	N	0	0	0	0	0	0	none
777	Normal	N	0	0	0	0	0	0	none
778	Normal	N	2	1	1	2	0	W	none
779	Normal	N	1	0	1	2	2	W	none
780	Above normal	N	0	0	0	0	0	0	none
781	Normal	N	0	0	0	0	0	0	none
782	Normal	N	0	0	0	0	0	0	none
783	Normal	N	0	0	0	0	0	0	none
784	Above normal	N	0	0	0	0	0	0	Bleed at base
785	Excessive	N	1	0	1	2	1	S	Extensive basal decay believed to be related to Armillaria

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
786	Excessive	N	1	1	0	2	0	W	Lost central leader
787	DEAD	N	0	0	0	0	0	0	Wind blown dead tree
788	DEAD	N	0	0	0	0	0	0	Standing stem to height of 5m
789	Normal	N	0	0	0	0	0	0	none
790	Above normal	Y	1	0	1	2	0	N	none
791	Normal	N	0	0	0	0	0	0	none
792	Normal	N	0	0	0	0	0	0	none
851	Above normal	N	0	0	0	0	0	0	none
852	Above normal	N	0	0	0	0	0	0	Woodpecker hole in main stem at 5m
853	Normal	N	0	0	0	0	0	0	none
854	Normal	N	0	0	0	0	0	0	none
855	Above normal	N	8	3	5	3	0	S, W	Pinhole exit wounds present
856	Normal	N	1	0	1	2	1	S	none
857	Normal	N	4	1	3	3	3	N, S	Ivy
858	Normal	N	0	0	0	0	1	0	none
859	Normal	N	0	0	0	0	0	0	none
860	DEAD	Y	20	6	14	10	10	N, S, E, W	Standing dead tree
861	Normal	N	0	0	0	0	0	0	Storm damaged limbs to west
862	Above normal	N	25	10	15	10	10	N, S, E, W	none
863	DEAD	Y	0	0	0	0	0	0	Wind blown dead tree Armillaria evident
864	Normal	N	0	0	0	0	0	0	none
865	Above normal	N	0	0	0	0	0	0	none
866	Above normal	N	0	0	0	0	0	0	none
867	Normal	N	2	0	2	3	4	W	none
868	Above normal	N	0	0	0	0	2	0	none
870	Normal	N	0	0	0	0	0	0	none
871	Normal	N	0	0	0	0	0	0	none

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
872	Above normal	N	0	0	0	0	0	0	none
873	Normal	N	8	2	6	3	5	N,S,W	Lost central leader. Wounding on Buttress roots
874	Above normal	N	0	0	0	0	1	0	none
875	Above normal	N	5	2	3	8	6	S,E,W	none
876	DEAD	Y	0	0	0	0	0	0	Wind blown dead tree - Armillaria
877	Above normal	N	6	2	4	2	4	S, W	none
878	Normal	N	10	5	5	5	3	S,E,W	none
879	Above normal	N	0	0	0	0	1	0	none
880	Normal	N	3	0	3	6	3	W	none
881	Normal	N	2	1	1	4	2	E, W	none
882	Above normal	N	5	1	4	4	7	E	none
883	Excessive	N	5	2	3	4	5	S,E,W	none
884	Normal	N	0	0	0	0	0	0	none
885	Normal	N	0	0	0	0	0	0	Perfect Timber tree
886	Above normal	N	0	0	0	0	1	0	none
887	Above normal	N	0	0	0	0	0	0	none
888	Above normal	N	0	0	0	0	0	0	none
889	DEAD	N	0	0	0	0	0	0	Wind blown dead tree
890	Above normal	N	1	1	0	2	0	S	Minor bleed
891	Normal	N	1	1	0	2	0	W	none
892	DEAD	N	0	0	0	0	0	0	Wind blown dead tree, Armillaria
893	DEAD	N	0	0	0	0	0	0	Standing dead with Agrilus holes
894	Excessive	N	4	1	3	3	5	N,S	none
895	DEAD	Y	0	0	0	0	0	0	Standing dead with Agrilus holes
896	DEAD	N	0	0	0	0	0	0	Wind blown dead tree

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
897	Above normal	N	2	1	1	2	0	N,S	none
898	Normal	N	0	0	0	0	0	0	none
899	Above normal	N	0	0	0	0	0	0	none
900	Above normal	N	6	2	4	3	3	N,S,W	none
901	Normal	N	0	0	0	0	0	0	none
902	Normal	N	0	0	0	0	0	0	none
903	Normal	N	0	0	0	0	0	0	none
904	Normal	N	0	0	0	0	0	0	none
905	Above normal	N	0	0	0	0	0	0	none
906	Above normal	N	0	0	0	0	0	0	none
907	Normal	N	0	0	0	0	0	0	none
908	Normal	N	2	1	1	1	1	S	none
909	Normal	N	0	0	0	0	0	0	none
910	Normal	N	0	0	0	0	0	0	none
911	Above normal	N	0	0	0	0	0	0	none
912	Normal	N	0	0	0	0	0	0	none
913	Normal	N	1	1	0	1	0	S	Large storm damaged limb to west
914	Normal	N	0	0	0	0	0	0	none
915	Above normal	Y	5	2	3	3	0	S,W	Excessive storm damage limbs
916	Normal	Y	4	2	2	4	0	S,W	none
917	Normal	N	1	0	1	5	1	E	none
918	Above normal	N	2	1	1	4	2	W	Exit holes not Agrilus
919	Normal	N	0	0	0	0	0	0	none
920	Normal	N	0	0	0	0	0	0	none
921	DEAD	Y	0	0	0	0	0	0	Standing dead with Agrilus and Pinhole exit
922	Normal	N	0	0	0	0	0	0	none
923	Normal	N	0	0	0	0	0	0	none
924	Normal	N	0	0	0	0	0	0	none
925	Excessive	Y	25	15	10	9	6	N,S,E+ W	none

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
926	Normal	N	1	0	1	0	0	0	none
927	Above normal	N	0	0	0	0	0	0	Damage to base of tree
928	Normal	N	0	0	0	0	0	0	none
929	Excessive	N	3	1	2	2	2	W	central leader lost
930	Above normal	N	0	0	0	0	0	0	none
931	Above normal	N	0	0	0	0	0	0	none
932	Normal	N	0	0	0	0	0	0	none
933	Normal	N	0	0	0	0	0	0	none
934	Normal	N	0	0	0	0	0	0	none
935	DEAD	Y	0	0	0	0	0	0	Wind- blown, dead tree
936	Normal	N	0	0	0	0	0	0	none
937	Normal	N	1	1	0	3	1	S	none
938	Normal	Y	7	2	5	3	5	S,W	none
939	Normal	N	4	1	3	2	4	S,W	none
940	Normal	N	2	2	0	3	0	S	none
941	Normal	N	0	0	0	0	0	0	none
942	Normal	N	0	0	0	0	0	0	none
943	Excessive	N	0	0	0	0	2	0	Basal decay
944	Above normal	N	6	3	3	3	1	S	none
945	Normal	N	0	0	0	0	0	0	none
946	Normal	N	0	0	0	0	0	0	none
947	Normal	N	0	0	0	0	0	0	none
948	Normal	Y	1	1	0	3	1	W	none
949	Normal	N	0	0	0	0	0	0	none
950	Normal	N	0	0	0	0	0	0	none
951	Normal	N	3	1	2	2	2	S	Basal decay. Possible Honey Fungus

Appendix 3: 2019 Data relating to Oak Cohorts within Stoneymore

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
701	Above normal	N	0	0	0	0	0	0	none
702	Normal	N	0	0	0	0	0	0	Ivy
703	Above normal	Y	0	0	0	0	0	0	Exposed buttress roots
704	Normal	N	0	0	0	0	0	0	none
705	Normal	N	2	1	1	1	1	S W	very minor bleed symptoms
706	Excessive	N	8	2	6	4	6	S,N,W	none
707	Normal	N	4	0	1	4	3	S, N	none
708	Normal	N	5	4	1	2	1	E+S	Damage to base of tree
709	DEAD	N	35	0	35	8	25	E,S	Standing dead
710	Normal	N	5	2	3	2	1	W,S	Ivy
711	Normal	N	2	2	0	2	2	N,S	none
712	Normal	N	0	0	0	0	0	0	Heavy burring
713	Normal	N	5	0	5	4	6	S,N,W	none
714	Excessive	Y	0	0	0	0	3	0	Extensive decay to SE at base due to Armillaria
715	Normal	N	0	0	0	0	0	0	none
716	Above normal	N	0	0	0	0	0	0	none
717	Normal	N	3	0	3	3	2	N,S	none
718	Above normal	N	6	2	4	3	2	N,W,S	Pinhole borer exit holes to base to S
719	Normal	N	4	0	4	4	5	S,N,E	none
720	Above normal	Y	3	1	2	3	4	N,S,E+W	none
721	Normal	N	0	0	0	0	3	0	none
722	Normal	N	1	0	1	3	3	N,S	Exit holes - not Agrilus
723	Normal	N	1	0	1	2	2	N	none
724	Normal	N	1	1	0	2	5	S	none
725	Above normal	N	2	0	2	3	2	N,W	none
726	Normal	N	0	0	0	0	0	0	none
727	Normal	N	0	0	0	0	2	S	none
728	Normal	N	0	0	0	0	0	0	none
729	Above normal	N	6	5	1	3	4	S+W	Damage at base ot tree to north

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
730	Normal	N	3	3	0	2	2	W	none
731	Normal	N	1	0	1	3	0	W	none
732	Normal	N	5	3	2	2	0	S	none
733	Normal	N	0	0	0	0	1	0	none
734	Normal	N	1	0	1	1	3	N	none
735	Above normal	N	0	0	0	0	0	0	none
736	Above normal	N	1	0	1	2	1	N	none
737	Normal	N	5	0	5	3	3	W,S	none
738	DEAD	Y	0	0	0	0	0	0	Standing Dead, Agrilus and Armillaria
739	Normal	N	12	11	1	8	4	N,S,E+W	Extensive
740	Above normal	N	30	25	5	6	7	N,S,E+W	Extensive Bleeds
741	Normal	N	2	0	2	2	0	S,W	none
742	Above normal	N	1	0	1	2	3	N	Storm damaged central leader
743	Normal	N	1	1	0	2	2	W	none
744	Normal	N	0	0	0	0	0	0	Perfect Timber tree
745	DEAD	N	0	0	0	0	0	0	Fallen - Root decay - Probable Armillaria
746	Above normal	N	6	2	4	3	1	S,E,W	none
747	Above normal	N	3	0	3	1	0	W	none
748	Normal	N	2	1	1	1	0	S,W	Storm damage
749	DEAD	Y	3	3	0	1	4	E	Standing Dead
750	Normal	N	6	4	2	2	1	S,E	none
751	Excessive	N	22	18	4	4	0	N,S,E+W	none
752	Above normal	N	4	2	2	4	4	E	none
753	Normal	N	0	0	0	0	0	0	none
754	Normal	N	0	0	0	0	0	0	none
755	Normal	N	1	1	0	2	0	E	storm damage limb 3m to E
756	Above normal	N	0	0	0	0	0	0	none
757	Normal	N	0	0	0	0	0	0	none
758	Normal	N	0	0	0	0	0	0	Historic damage to base of tree

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
759	DEAD	N	0	0	0	0	0	0	Wind- blown dead tree, Armillaria
760	Normal	N	1	0	1	2	1	S	none
761	Normal	N	0	0	0	0	0	0	none
762	Normal	N	0	0	0	0	0	0	none
763	DEAD	Y	0	0	0	0	0	0	Wind- blown dead tree
764	Normal	N	0	0	0	0	0	0	none
765	Normal	N	0	0	0	0	0	0	none
766	Normal	N	0	0	0	0	0	0	none
767	Normal	N	0	0	0	0	0	0	none
768	Normal	N	0	0	0	0	0	0	none
769	Normal	N	0	0	0	0	0	0	none
770	Normal	N	0	0	0	0	0	0	none
771	Normal	N	6	0	6	3	0	N,E	none
772	DEAD	Y	0	0	0	0	0	0	Wind- blown dead tree Armillaria evident
773	Normal	N	0	0	0	0	0	0	Storm damaged crown
774	Normal	N	1	0	1	1	0	S	none
775	Above normal	N	2	0	2	2	0	S	none
776	Normal	N	0	0	0	0	0	0	none
777	Normal	N	0	0	0	0	0	0	none
778	Normal	N	2	1	1	2	0	W	none
779	Normal	N	1	0	1	2	0	W	none
780	Above normal	N	0	0	0	0	0	0	none
781	Normal	N	0	0	0	0	0	0	none
782	Normal	N	0	0	0	0	0	0	none
783	Normal	N	0	0	0	0	0	0	none
784	Above normal	N	0	0	0	0	0	0	Bleed at base but considered to be related to Armillaria
785	Excessive	N	2	1	1	2	1	S	Extensive basal decay believed to be related to Armillaria
786	Excessive	N	2	0	2	2	0	W	Lost central leader
787	DEAD	N	0	0	0	0	0	0	Wind-blown dead tree
788	DEAD	N	0	0	0	0	0	0	Standing stem to height of 5m
789	Normal	N	0	0	0	0	0	0	none

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
790	Above normal	Y	3	0	3	2	0	N	none
791	Normal	N	0	0	0	0	0	0	none
792	Normal	N	0	0	0	0	0	0	none
851	Above normal	N	0	0	0	0	0	0	none
852	Above normal	N	0	0	0	0	0	0	Woodpecker hole in main stem at 5m
853	Normal	N	0	0	0	0	0	0	none
854	Normal	N	0	0	0	0	0	0	none
855	Above normal	N	7	3	4	3	0	S, W	Pinhole exit wounds present
856	Normal	N	2	0	2	2	1	S, W	none
857	Normal	N	9	0	9	3	9	N, S	Ivy
858	Normal	N	0	0	0	0	1	0	none
859	Normal	N	0	0	0	0	0	0	none
860	DEAD	Y	20	0	20	10	10	N, S, E, W	Standing dead tree - Agrilus
861	Normal	N	4	2	2	4	1	S	Storm damaged limbs to west
862	Above normal	N	30	8	22	8	11	N, S, E, W	none
863	DEAD	Y	0	0	0	0	0	0	Wind blown dead tree Armillaria evident
864	Normal	N	0	0	0	0	0	0	none
865	Above normal	N	0	0	0	0	0	0	none
866	Above normal	N	0	0	0	0	0	0	none
867	Normal	N	1	0	1	2	1	W	none
868	Above normal	N	0	0	0	0	2	0	none
870	Normal	N	0	0	0	0	0	0	none
871	Normal	N	1	1	0	1	0	S	none
872	Above normal	N	0	0	0	0	0	0	none
873	Normal	N	10	1	9	3	5	N,S,E+W	Lost central leader. Wounding on Buttress roots
874	Above normal	N	0	0	0	0	1	0	none

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
875	Above normal	N	0	0	0	0	6	0	none
876	DEAD	Y	0	0	0	0	0	0	Wind blown dead tree - Armillaria
877	Above normal	N	2	1	1	1	2	S, W	none
878	Normal	N	4	3	1	2	0	S,E	none
879	Above normal	N	0	0	0	0	1	0	none
880	Normal	N	0	0	0	0	1	0	none
881	Normal	N	2	0	2	4	2	E, W	none
882	Above normal	N	0	0	0	3	3	E	none
883	Excessive	N	11	3	8	4	5	S,E,W	none
884	Normal	N	1	1	0	1	0	S	none
885	Normal	N	0	0	0	0	0	0	Perfect Timber tree
886	Above normal	N	0	0	0	0	1	0	none
887	Above normal	N	0	0	0	0	0	0	none
888	Above normal	N	0	0	0	0	0	0	none
889	DEAD	N	0	0	0	0	0	0	Wind blown dead tree
890	Above normal	N	2	1	1	2	0	S	Minor bleed
891	Normal	N	3	0	3	2	0	W	none
892	DEAD	N	0	0	0	0	0	0	Wind blown dead tree - Armillaria
893	DEAD	N	0	0	0	0	0	0	Standing dead with Agrilus holes
894	DEAD	N	0	0	0	0	4	0	Standing dead - Armillaria
895	DEAD	Y	0	0	0	0	0	0	Standing dead with Agrilus holes - Armillaria
896	DEAD	N	0	0	0	0	0	0	Wind blown dead tree - Armillaria
897	Above normal	N	6	3	3	2	0	N,S	none
898	Normal	N	0	0	0	0	0	0	none
899	Above normal	N	0	0	0	0	0	0	none

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
900	Above normal	N	6	0	6	3	3	N,S,W	none
901	Normal	N	0	0	0	0	0	0	none
902	Normal	N	1	1	0	2	0	N	none
903	Normal	N	0	0	0	0	0	0	none
904	Normal	N	0	0	0	0	0	0	none
905	Above normal	N	0	0	0	0	0	0	none
906	Above normal	N	0	0	0	0	0	0	none
907	Normal	N	0	0	0	0	0	0	none
908	Normal	N	1	0	1	1	3	S	none
909	Normal	N	0	0	0	0	0	0	none
910	Normal	N	0	0	0	0	0	0	none
911	Above normal	N	0	0	0	0	0	0	none
912	Normal	N	0	0	0	0	0	0	none
913	Normal	N	2	0	2	1	0	S	Large storm damaged limb to west
914	Normal	N	0	0	0	0	0	0	none
915	Above normal	Y	30	5	25	4	3	S,E,W	Excessive storm damage limbs
916	Normal	Y	5	2	3	4	2	S,W	none
917	Normal	N	2	0	2	4	1	E	none
918	Above normal	N	4	1	3	4	1	W	Exit holes not Agrilus
919	Normal	N	0	0	0	0	0	0	none
920	Normal	N	0	0	0	0	0	0	none
921	DEAD	Y	0	0	0	0	0	0	Standing dead with Agrilus and Pinhole exit
922	Normal	N	0	0	0	0	0	0	none
923	Normal	N	0	0	0	0	0	0	none
924	Normal	Y	0	0	0	0	0	0	none
925	Excessive	Y	25	7	18	9	6	N,S,E+W	none
926	Normal	N	0	0	0	0	0	0	none
927	Above normal	N	0	0	0	0	0	0	Damage to base of tree
928	Normal	N	0	0	0	0	0	0	none
929	Excessive	N	1	0	1	2	1	W	central leader lost

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
930	Above normal	N	0	0	0	0	0	0	none
931	Above normal	N	0	0	0	0	0	0	none
932	Normal	N	0	0	0	0	0	0	none
933	Normal	N	0	0	0	0	0	0	none
934	Normal	N	0	0	0	0	0	0	none
935	DEAD	Y	0	0	0	0	0	0	Wind blown dead tree
936	Normal	N	1	1	0	2	0	S	none
937	Normal	N	1	0	1	3	1	S	none
938	Normal	Y	8	2	6	3	5	S,W	none
939	Normal	N	4	1	3	2	3	S,W	none
940	Normal	N	3	0	3	3	1	S,E	none
941	Normal	N	0	0	0	0	0	0	none
942	Normal	N	0	0	0	0	0	0	none
943	Excessive	N	0	0	0	0	2	0	Basal decay
944	Above normal	N	11	0	11	3	1	S	none
945	Normal	N	0	0	0	0	0	0	none
946	Normal	N	0	0	0	0	0	0	none
947	Normal	N	0	0	0	0	0	0	none
948	Normal	Y	1	1	0	3	1	W	none
949	Normal	N	0	0	0	0	0	0	none
950	Normal	N	0	0	0	0	0	0	none
951	Normal	N	0	0	0	0	2	0	Basal decay. Possible Honey Fungus

Appendix 4: 2012 Data relating to Oak trees throughout Writtle Forest

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
901	OM	950	22	18	Above Normal	Y	22	8	14	7	2	S, W	Adjacent to historic woodland extraction track. Minor adjacent rutting to south. Dense bramble to base of tree	Overstored Hornbeam coppice with Oak standards	12m to 15m	Possible development of Hypoxylon canker within deep fissures of previously infected areas
902	EM	690	22	16	Normal	Y	20	12	8	8	0	S	Adjacent to historic woodland extraction track.	Hornbeam coppice, Birch regeneration with	10m to 15m	No other pathogens observed

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
													Rutting 10m to east of tree. Bramble to base of tree	Oak standards		
903	EM	660	22	10	Above Normal	Y	20	10	10	6	0	S	Middle of stand with no footpaths or historic tracks. Bramble and bracken to base of tree	Oak woodland stand of same age range with occasional stored Hornbeam coppice. Ash to periphery of stand	10 to 15 m	No other pathogens observed

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
904	M	820	22	14	Above Normal	Y	25	15	10	8	0	S	Adjacent to footpath on edge of stand. Compacted ground to north of tree.	Stored Hornbeam coppice with Oak standards and pioneer Birch.	15m	Pin hole borer
905	M	780	22	14	Normal	N	9	3	6	8	0	W	Adjacent to recent historic forestry extraction route, heavily rutted – to west of tree.	Small stand of Oak standards with nearby Ash standards adjacent to seasonal watercourse to north	10 to 12m	No other pathogens observed

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
906	EM	550	20	15	Above normal	N	15	4	11	6	0	N, W	Within open glade area, primarily of Oak standards with some semi mature Silver Birch. Heavy bracken and bramble understorey.	Edge of stand / glade area Oak standards with deteriorated birch coppice and occasional Birch pioneers.	15m-25m	No other pathogens observed
907	EM	690	22	10	Excessive	Y	35	15	20	7	1	N, E, S + W	Adjacent to footpath on edge of stand. Compacted	Oak and Sweet Chestnut standards with mix of	15-20m	Armillaria, Tapioca slime mold, Hypoxylon canker. Severe basal

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
													ground to east of tree.	overstored Hornbeam and Sweet Chestnut coppice with patches of planted Larch and Douglas fir		decay to south
908	EM	650	18	0	Standing dead	N	2	1	1	5	0	N	Adjacent to historical excavations of sand (1900s), with consequent 2m pit season	Area of Sweet Chestnut coppice, pioneer Aspen and Silver Birch with Occasi	25m - 30m	No other pathogens observed

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
													ally water filled. Tree recent standing dead	onal Oak and Sweet Chestnut standards		
909	EM	580	20	12	Excessive	Y	23	8	15	7	0	N, E, + W	Small stand of Oak standards approx. 50m from main public Bridleway. Bracken understorey	Oak standards with Hornbeam and Sweet Chestnut coppice with Birch pioneer	10m-15m	No other pathogens observed
910	M	800	22	16	Above normal	Y	13	10	3	7	0	N, E, S	On edge of small spinne	Scattered Oak standards	20m to	No other pathogens observed

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
												+ W	y surrounded by agricultural land. Land to west – approx. 10m, regularly ploughed	within Sweet Chestnut coppice on 10 - 20year rotation	30 m	
911	EM	560	16	8	Normal	N	0	0	0	0	0	n/a	Adjacent to public footpath. Growing on wood bank with associated ditch line immedi	Overstored Hornbeam coppice with Oak standards	12 m to 15 m	Tree suppressed by competing canopies

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
													ately to west.			
912	M	710	22	16	Normal	N	0	0	0	0	0	n/a	Adjacent to historic woodland extraction track. Rutting 10m to east of tree. Bramble to base of tree	Hornbeam coppice, Birch regeneration with Oak standards	10m to 15m	Tree directly adjacent to 902
913	EM	560	18	12	Normal	N	0	0	0	0	0	n/a	Edge of stand adjacent to road. tracks. Bramble to base of tree	Oak woodland stand of same age range with occasio	10 to 15m	Ivy clad, close to road.

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
														nal stored Hornbeam coppice. Ash to periphery of stand		
914	EM	650	20	12	Normal	N	0	0	0	0	0	n/a	Adjacent to woodland extraction track. Bramble understorey.	Stored Hornbeam coppice with Oak standards and pioneer Birch.	15m	Tree of good health and condition
915	EM	560	20	10	Normal	N	0	0	0	0	0	n/a	Heavy bramble understorey. Adjace	Small stand of Oak standards with nearby	10 to 12m	Tree on bounds of woodland adjacent to stream and

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
													nt to waterc ourse.	Ash standar ds adjace nt to season al waterc ourse to north		agricultural field
91 6	E M	5 4 0	18	1 0	Norm al	N	0	0	0	0	0	n/ a	Tree on edge of glade, dense brambl e growth to base.	Edge of stand / glade area Oak standar ds with deterio rated birch coppic e and occasio nal Birch	15 m- 25 m	No Pathogens noted

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
														pioneers.		
917	EM	590	18	8	Normal	N	0	0	0	0	0	n/a	Close to footpath and path formed by horse riders. Consequent compacted ground to north of tree.	Oak and Sweet Chestnut standards with mix of overstored Hornbeam and Sweet Chestnut coppice with patches of planted Larch and Douglas fir	15-20 m	Tree is situated adjacent to re-directed path area. No pathogens

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
918	M	790	22	14	Normal	N	0	0	0	0	0	n/a	Adjacent to ditch line and private concrete roadway/ bridleway.	Area of pioneer Silver Birch with Occasional Oak and Sweet Chestnut standards	25m	Dominant tree adjacent to concrete road. No pathogens
919	EM	570	18	10	Normal	N	0	0	0	0	0	n/a	Small stand of Oak standards approx. 50m from main public Bridleway. Bracken	Oak standards with Hornbeam and Sweet Chestnut coppice with Birch pioneer	10m-15m	Tree of good health and condition. No pathogens

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
													understorey			
920	EM	320	18	8	Normal	N	0	0	0	0	0	n/a	On edge of small spinney surrounded by agricultural land. Ditch line to east approx. 25m – seasonal water flow. Land to east– approx. 30m, to managed	Scattered Oak standards within Sweet Chestnut coppice on 10 - 20year rotation	20m to 30m	Younger tree. No pathogens.

Tag no.	Age	DBH 1.5m mm	Height metres	Crown Spread metres	Deadwood	Evidence of Agrilus	Number of cankers	No. of Recent cankers	No. of Old cankers	Ht. of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Microtopography	Woodland Management	Density of Oak trees	Other Pathogens
													grassland			

Appendix 5: 2014 Data relating to Oak trees throughout Writtle Forest

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent Cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
901	Above Normal	Y	19	5	14	7	2	S,W	No significant changes
902	Normal	Y	16	6	10	7	0	S, E	No significant changes
903	Above Normal	Y	14	8	6	6	1	S	More canker evident throughout the area of woodland
904	Excessive	Y	25	9	16	7	0	S, E	Tree in heavy decline, signs of possible Armillaria
905	Normal	N	7	2	5	7	0	W	No significant changes
906	Above normal	N	6	2	4	6	0	N, W	No significant changes
907	Excessive	Y	25	7	18	7	3	N, E, S + W	Tree in decline, bark is beginning to fall from main stem to
908	Standing dead	N	2	1	1	5	0	N	Possible Armillaria resultant from falling bark
909	Dead	Y	12	3	9	6	0	N,E, + W	Large areas of bark have come away from the main stem. Reveals Armillaria

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent Cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
910	Excessive	Y	15	8	7	8	0	N, E, S + W	Tree has rapidly deterioated in condition, heavy bleed.
911	Above Normal	N	1	1	0	3	0	W	Minor bleed noted
912	Normal	N	0	0	0	0	0	n/a	No significant changes
913	Normal	N	0	0	0	0	0	n/a	No significant changes
914	Normal	N	0	0	0	0	0	n/a	No significant changes
915	Normal	N	0	0	0	0	0	n/a	No significant changes
916	Normal	N	0	0	0	0	0	n/a	No significant changes
917	Normal	N	1	1	0	2	0	N	single bleed noted
918	Normal	N	0	0	0	0	0	n/a	No significant changes
919	Above Normal	N	2	2	0	3	0	N	All trees within area appear in decline
920	Normal	N	0	0	0	0	0	n/a	No significant changes

Appendix 6: 2016 Data relating to Oak trees throughout Writtle Forest

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
901	Above Normal	Y	8	1	7	4	2	S,W	No significant changes
902	Normal	Y	9	3	6	7	1	S, E	No significant changes
903	Above Normal	Y	12	3	9	6	3	S	No significant changes
904	Dead	Y	21	1	20	6	0	S	Standing dead with development of Hypoxylon canker, and Sulphur Tuft fungi
905	Normal	N	4	1	3	5	0	W	Reduced bleed, some lessions are forming callous tissue and 1 has healed entirely
906	Above normal	N	2	0	2	4	1	W	No significant changes
907	Excessive	Y	12	3	9	4	0	N, E, S + W	Large quantity of bark shed on main stem
908	Standing dead	N	2	1	1	5	0	N	No significant changes

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
909	Dead	Y	0	0	0	0	0	n/a	Tree has fallen, unable to ascertain presence of bleeds
910	Dead	Y	8	2	6	5	2	N,E	Tree is standing dead. It appears to have squirrel damage where the heavier bleed was situated to the east
911	Above Normal	N	2	0	2	3	0	W	No significant changes, though further crown deterioration
912	Normal	N	0	0	0	0	0	n/a	No significant changes
913	Normal	N	0	0	0	0	0	n/a	No significant changes
914	Normal	N	0	0	0	0	0	n/a	No significant changes
915	Normal	N	0	0	0	0	0	n/a	No significant changes
916	Normal	N	0	0	0	0	0	n/a	No significant changes
917	Above Normal	N	1	1	0	2	0	N	No significant changes
918	Normal	N	0	0	0	0	0	n/a	No significant changes
919	Above Normal	Y	4	2	2	3	0	N,W	Deteriorated condition
920	Normal	N	0	0	0	0	0	n/a	No significant changes

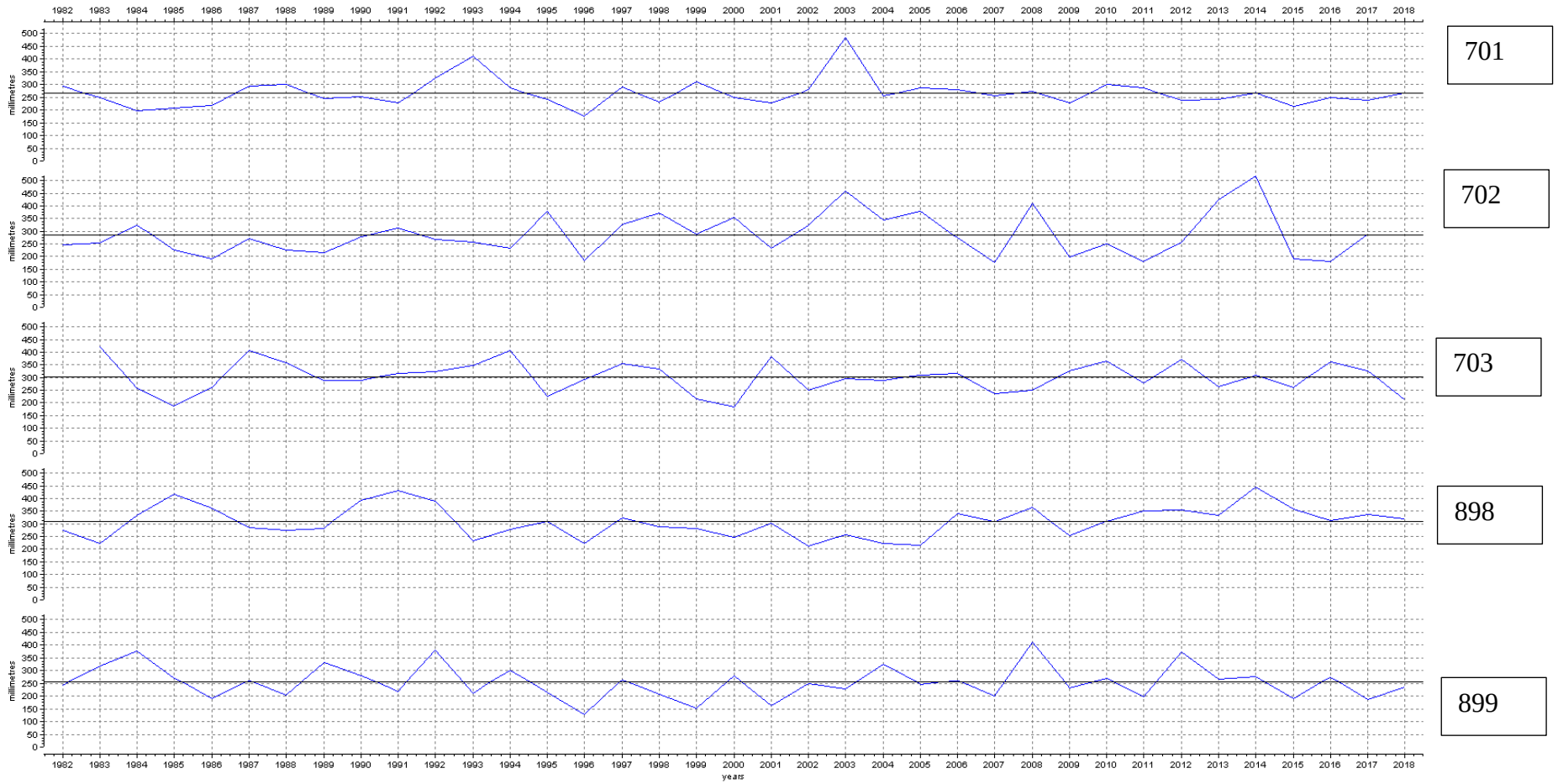
Appendix 7: 2018 Data relating to Oak trees throughout Writtle Forest

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
901	Above Normal	N	3	1	2	2	5	W	Fair to recovering condition
902	Normal	Y	7	4	3	7	3	S	No significant changes
903	Above Normal	Y	6	2	4	6	3	S	Far less recent bleed - as of other trees in area
904	Dead	Y	8	0	8	4	0	S	Large areas of bark have fallen away
905	Normal	N	4	0	4	5	2	W	No significant changes
906	Normal	N	2	0	2	4	1	W	All cankers are dried and tree appears to be recovering
907	Dead	Y	10	2	8	4	0	N, E + W	Other fungi present - Armillaria, Hypoxylon canker, Sulphur Tuft
908	Standing dead	N	0	0	0	0	0	N	Large areas of bark have fallen away
909	Dead	N	0	0	0	0	0	n/a	Tree has fallen, unable to ascertain presence of bleeds

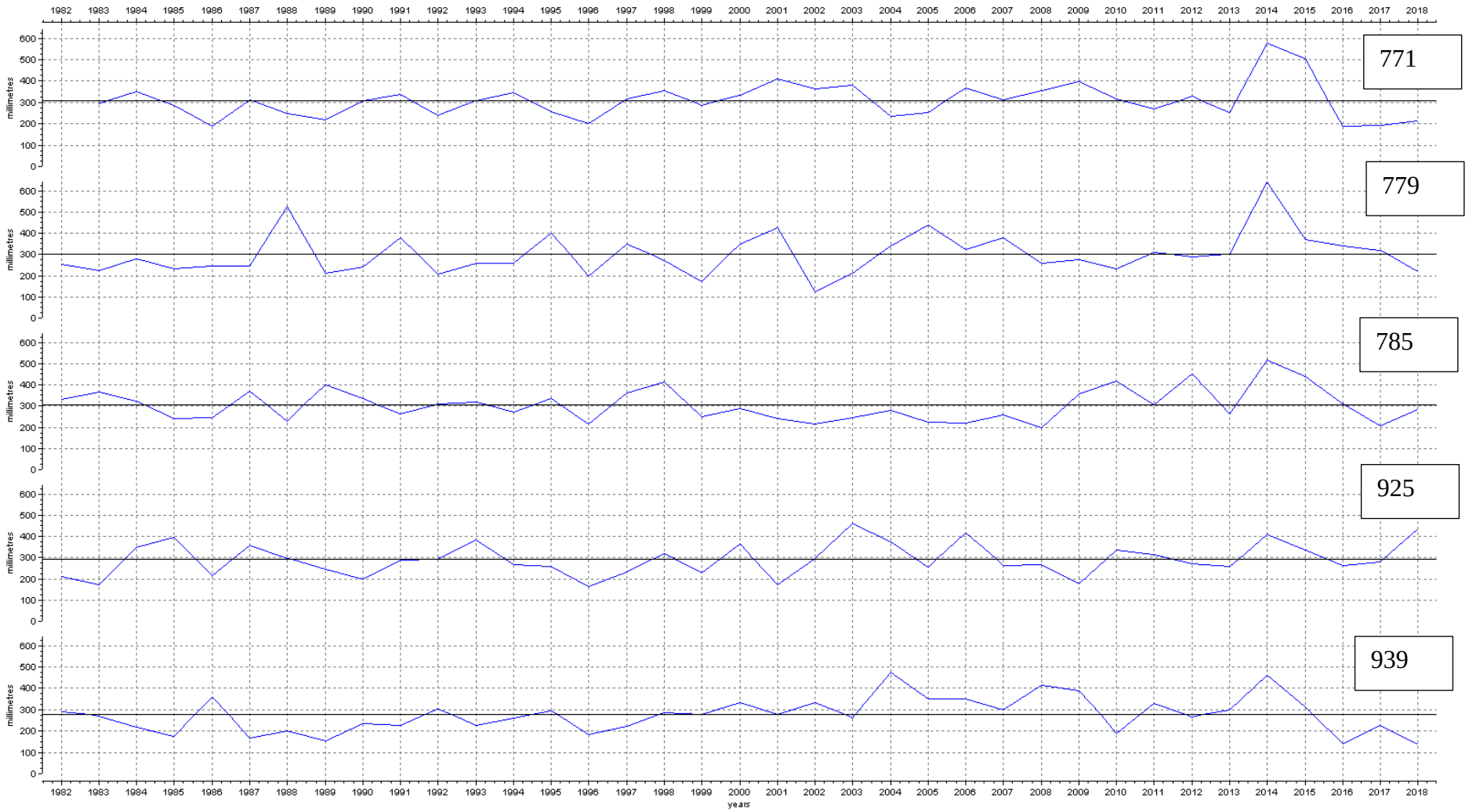
Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
910	Dead	Y	6	1	5	3	2	N, E	Large quantity of bark shed on main stem
911	Above Normal	N	0	0	1	3	2	n/a	No significant changes
912	Normal	N	0	0	0	n/a	0	n/a	No significant changes
913	Normal	N	0	0	0	n/a	0	n/a	No significant changes
914	Normal	N	0	0	0	n/a	0	n/a	No significant changes
915	Normal	N	0	0	0	n/a	0	n/a	No significant changes
916	Normal	N	0	0	0	n/a	0	n/a	No significant changes
917	Above Normal	N	0	0	1	n/a	1	n/a	No significant changes
918	Normal	N	0	0	0	n/a	0	n/a	No significant changes

Tag no.	Deadwood	Evidence of Agrilus	Number of cankers	Recent cankers	Old cankers	Height of highest canker on stem (m)	No. of callused cankers	Orientation of cankers	Comments
919	Above Normal	Y	7	3	4	4	0	N, W	Bleed symptoms persistent
920	Normal	N	0	0	0	n/a	0	n/a	No significant changes

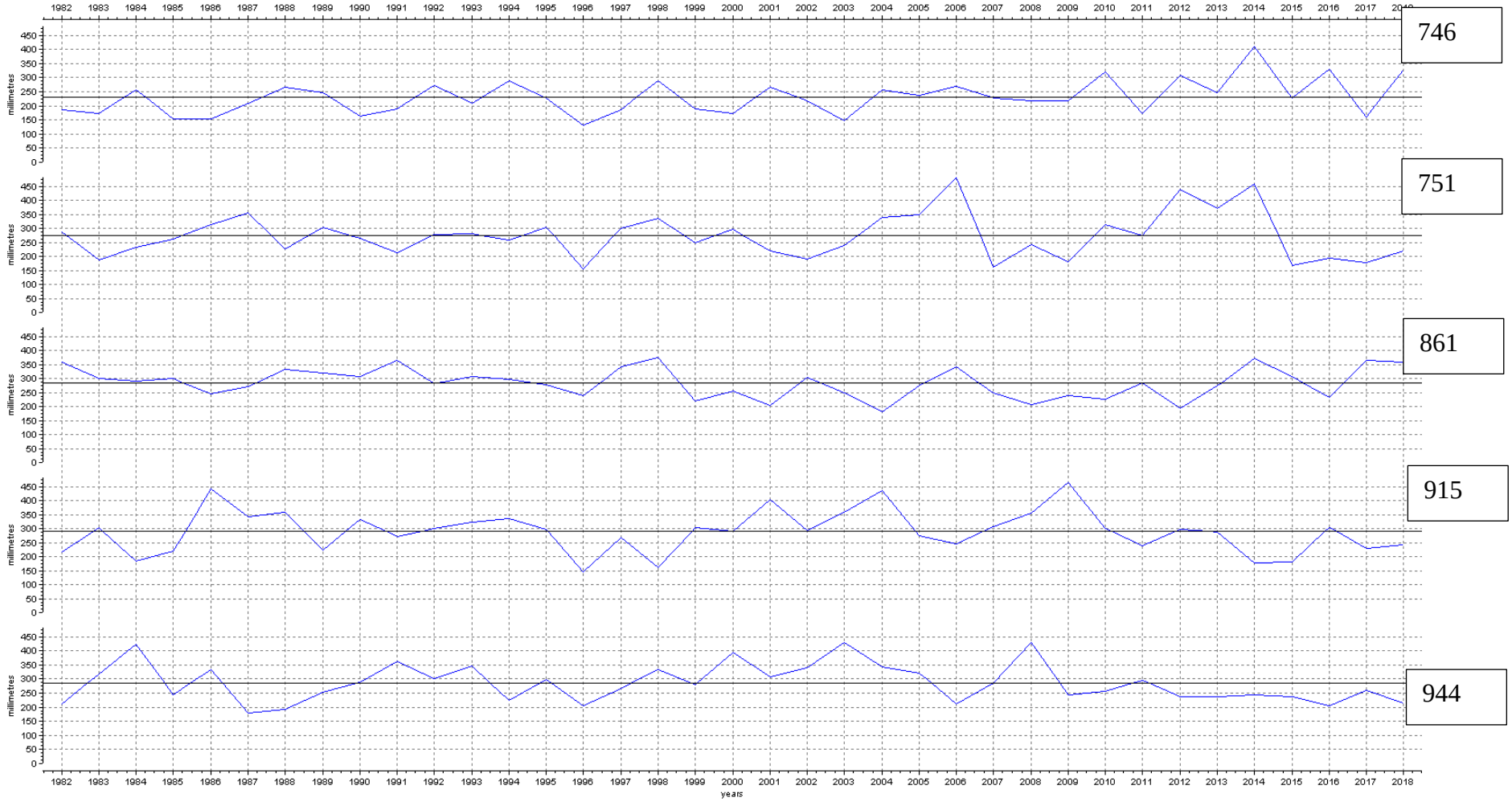
Appendix 8: Graphs and Statistic details relating to Chapter 5 Tree Rings Information



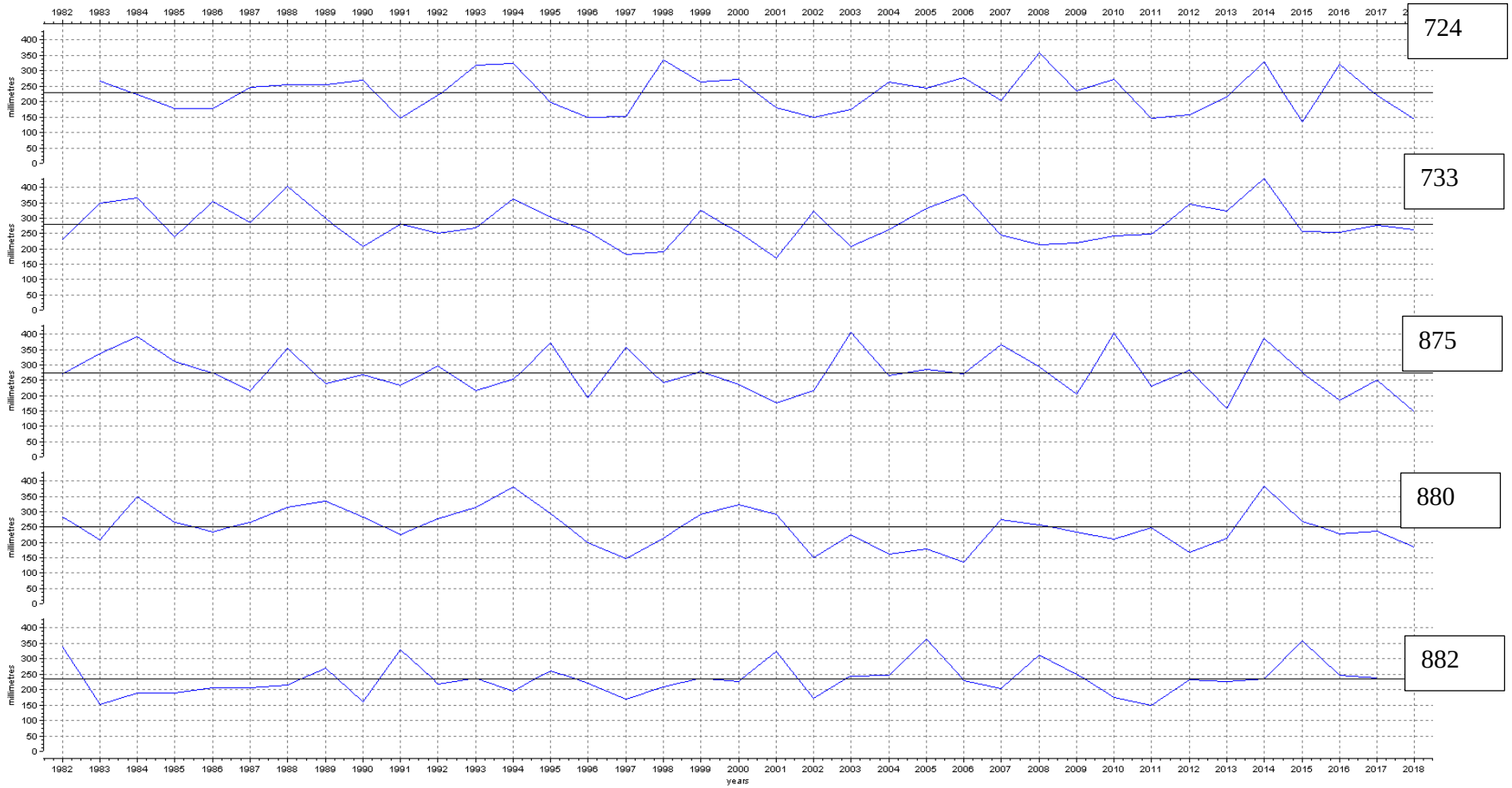
Pertaining to section 4.3.1 Annual Ring width of 5 trees with Zero OBC (Non-Symptomatic trees) from 2013 to 2019



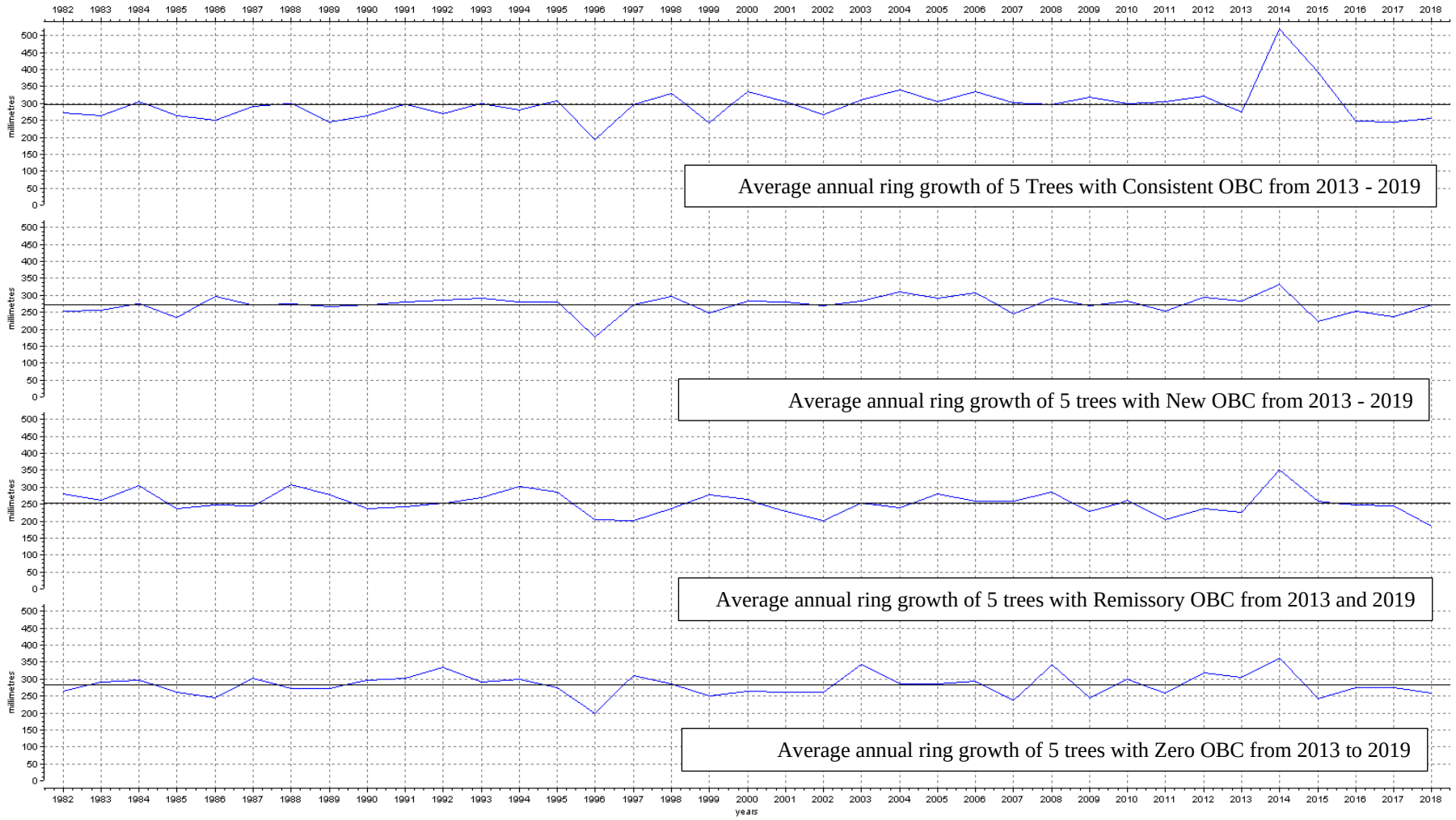
Annual Ring width of 5 trees with Consistent OBC (Symptomatic trees) from 2013 to 2019



Annual Ring width of 5 trees with New OBC from 2013 to 2019



Annual Ring width of 5 trees with Remissory OBC from 2013 to 2019



Average Annual Ring width growth for all 4 groups considered

Pertaining to section 4.3.5 & 4.3.6

Statistical Analysis of Ring width growth of all Four Groups

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Zero	37	10468	282.9189	1098.077		
Remissory	37	9387	253.7027	1163.159		
New	37	10076	272.3243	755.7252		
Consistent	37	10938	295.6216	2717.797		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	34908.72	3	11636.24	8.11629	**4.96E-05**	2.66744307
Within Groups	206451.3	144	1433.69			
Total	241360	147				

ANOVA (single factor) statistical analysis of the tree ring width of all four groups.

t-Test: Two-Sample Assuming Equal Variances		
	RW Zero	***RW Remissory***
Mean	282.9189189	253.7027027
Variance	1098.076577	1163.159159
Observations	37	37
Pooled Variance	1130.617868	
Hypothesized Mean Difference	0	
df	72	
t Stat	3.737247945	
P(T<=t) one-tail	0.000184952	
t Critical one-tail	1.666293696	
P(T<=t) two-tail	**0.000369904**	
t Critical two-tail	1.993463567	

t-Test: Two-Sample Assuming Equal Variances		
	RW Zero	***RW New***
Mean	282.9189189	272.3243243
Variance	1098.076577	755.7252252
Observations	37	37
Pooled Variance	926.9009009	
Hypothesized Mean Difference	0	
df	72	
t Stat	1.496764779	
P(T<=t) one-tail	0.069412756	
t Critical one-tail	1.666293696	
P(T<=t) two-tail	**0.138825512**	
t Critical two-tail	1.993463567	

t-Test: Two-Sample Assuming Equal Variances		
	RW Zero	***RW Consistent***
Mean	282.9189189	295.6216216
Variance	1098.076577	2717.797297
Observations	37	37
Pooled Variance	1907.936937	
Hypothesized Mean Difference	0	
df	72	
t Stat	-1.2508349	
P(T<=t) one-tail	0.107522101	
t Critical one-tail	1.666293696	
P(T<=t) two-tail	**0.215044202**	
t Critical two-tail	1.993463567	

t-Test: Two-Sample Assuming Equal Variances		
	RW Remissory	***RW New***
Mean	253.7027027	272.3243243
Variance	1163.159159	755.7252252
Observations	37	37
Pooled Variance	959.4421922	
Hypothesized Mean Difference	0	
df	72	
t Stat	-2.585794191	
P(T<=t) one-tail	0.00586932	
t Critical one-tail	1.666293696	

P(T<=t) two-tail	**0.01173864**	
t Critical two-tail	1.993463567	

t-Test: Two-Sample Assuming Equal Variances		
	RW Remissory	***RW Consistent***
Mean	253.7027027	295.6216216
Variance	1163.159159	2717.797297
Observations	37	37
Pooled Variance	1940.478228	
Hypothesized Mean Difference	0	
df	72	
t Stat	-4.092998173	
P(T<=t) one-tail	5.49347E-05	
t Critical one-tail	1.666293696	
P(T<=t) two-tail	**0.000109869**	
t Critical two-tail	1.993463567	

t-Test: Two-Sample Assuming Equal Variances		
	RW New	***RW Consistent***
Mean	272.3243243	295.6216216
Variance	755.7252252	2717.797297
Observations	37	37
Pooled Variance	1736.761261	
Hypothesized Mean Difference	0	
df	72	
t Stat	-2.40448097	
P(T<=t) one-tail	0.009384609	
t Critical one-tail	1.666293696	
P(T<=t) two-tail	**0.018769218**	
t Critical two-tail	1.993463567	

Testing variance of the tree ring width between each group in turn to ascertain difference between all groups using T-Test: Two-Sample Assuming Equal Variances.

Pertaining to section 4.4.3, 4.4.4, 4.4.5, 4.4.6 & 4.4.7

Statistical Analysis of Incremental growth of all Four Groups

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
RW Zero	37	3559553	96204.1351	3260671478		
RW Remissory	37	2848391	76983.5405	1951776895		
RW New	37	3321351	89766.2432	2671903810		
RW Consistent	37	3838381	103740.027	4972766759		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.4266E+10	3	4755243287	1.479411774	**0.22265822**	2.66744307
Within Groups	4.6286E+11	144	3214279735			
Total	4.7712E+11	147				

ANOVA (single factor) statistical analysis of the incremental growth of all four groups of trees

from 1982 - 2018

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
RW Zero	23	3015447	131106.391	1659341540		
RW Remissory	23	2377784	103381.913	1035013235		
RW New	23	2816370	122450.87	1159903069		
RW Consistent	23	3330938	144823.391	3196427564		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.0776E+10	3	6925437603	3.92894432	**0.01107675**	2.70818647
Within Groups	1.5512E+11	88	1762671352			
Total	1.7589E+11	91				

ANOVA (single factor) statistical analysis of the incremental growth of all four groups of trees from 1996 - 2018

t-Test: Two-Sample Assuming Equal Variances		
	IG Zero	***IG Remissory***
Mean	131106.3913	103381.913
Variance	1659341540	1035013235
Observations	23	23
Pooled Variance	1347177387	
Hypothesized Mean Difference	0	
df	44	
t Stat	2.561532733	
P(T<=t) one-tail	0.006962232	
t Critical one-tail	1.680229977	
P(T<=t) two-tail	**0.013924464**	
t Critical two-tail	2.015367574	

t-Test: Two-Sample Assuming Equal Variances		
	IG Zero	***IG New***
Mean	131106.3913	122450.8696
Variance	1659341540	1159903069
Observations	23	23
Pooled Variance	1409622304	
Hypothesized Mean Difference	0	
df	44	
t Stat	0.781791223	
P(T<=t) one-tail	0.219262055	
t Critical one-tail	1.680229977	
P(T<=t) two-tail	**0.43852411**	
t Critical two-tail	2.015367574	

t-Test: Two-Sample Assuming Equal Variances		
	IG Zero	***IG Consistent***
Mean	131106.3913	144823.3913
Variance	1659341540	3196427564
Observations	23	23
Pooled Variance	2427884552	
Hypothesized Mean Difference	0	
df	44	

t Stat	-0.944047927	
P(T<=t) one-tail	0.175150211	
t Critical one-tail	1.680229977	
P(T<=t) two-tail	**0.350300421**	
t Critical two-tail	2.015367574	

t-Test: Two-Sample Assuming Equal Variances		
	IG Remissory	***IG New***
Mean	103381.913	122450.87
Variance	1035013235	1.16E+09
Observations	23	23
Pooled Variance	1097458152	
Hypothesized Mean Difference	0	
df	44	
t Stat	-1.95200922	
P(T<=t) one-tail	0.028659622	
t Critical one-tail	1.680229977	
P(T<=t) two-tail	**0.057319244**	
t Critical two-tail	2.015367574	

t-Test: Two-Sample Assuming Equal Variances		
	IG Remissory	***IG Consistent***
Mean	103381.913	144823.3913
Variance	1035013235	3196427564
Observations	23	23
Pooled Variance	2115720399	
Hypothesized Mean Difference	0	
df	44	
t Stat	-3.055308176	
P(T<=t) one-tail	0.001905696	
t Critical one-tail	1.680229977	
P(T<=t) two-tail	**0.003811392**	
t Critical two-tail	2.015367574	

t-Test: Two-Sample Assuming Equal Variances		
	IG New	***IG Consistent***
Mean	122450.8696	144823.3913
Variance	1159903069	3196427564
Observations	23	23
Pooled Variance	2178165317	
Hypothesized Mean Difference	0	
df	44	

t Stat	-1.625617776	
P(T<=t) one-tail	0.05558666	
t Critical one-tail	1.680229977	
P(T<=t) two-tail	**0.11117332**	
t Critical two-tail	2.015367574	

Testing variance of the incremental growth between each group in turn to ascertain difference between all groups (1996-2018), using T-Test: Two-Sample Assuming Equal Variances.

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
RW Zero	17	2525394	148552.6	9.79E+08		
RW Remissory	17	1972187	116011	7.18E+08		
RW New	17	2344955	137938.5	5.17E+08		
RW Consistent	17	2810514	165324.4	2.52E+09		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.17E+10	3	7.25E+09	6.119753	**0.001001**	2.748191
Within Groups	7.58E+10	64	1.18E+09			
Total	9.75E+10	67				

ANOVA (single factor) statistical analysis of the incremental growth of all four groups of trees from 2002 - 2018

t-Test: Two-Sample Assuming Equal Variances		
	IG Zero	***IG Remissory***
Mean	116011	148552.6
Variance	7.18E+08	9.79E+08
Observations	17	17
Pooled Variance	8.48E+08	
Hypothesized Mean Difference	0	
df	32	
t Stat	-3.25749	
P(T<=t) one-tail	0.001331	
t Critical one-tail	1.693889	
P(T<=t) two-tail	**0.002662**	
t Critical two-tail	2.036933	

t-Test: Two-Sample Assuming Equal Variances		
	IG Zero	***IG New***
Mean	148552.6	137938.5
Variance	9.79E+08	5.17E+08
Observations	17	17
Pooled Variance	7.48E+08	
Hypothesized Mean Difference	0	
df	32	
t Stat	1.131658	
P(T<=t) one-tail	0.133094	
t Critical one-tail	1.693889	
P(T<=t) two-tail	**0.266188**	
t Critical two-tail	2.036933	

t-Test: Two-Sample Assuming Equal Variances		
	IG Zero	***IG Consistent***
Mean	148552.6	165324.4
Variance	9.79E+08	2.52E+09
Observations	17	17
Pooled Variance	1.75E+09	
Hypothesized Mean Difference	0	
df	32	
t Stat	-1.16846	
P(T<=t) one-tail	0.125626	
t Critical one-tail	1.693889	
P(T<=t) two-tail	**0.251252**	

t Critical two-tail	2.036933	

t-Test: Two-Sample Assuming Equal Variances		
	IG Remissory	***IG New***
Mean	116011	137938.5
Variance	7.18E+08	5.17E+08
Observations	17	17
Pooled Variance	6.17E+08	
Hypothesized Mean Difference	0	
df	32	
t Stat	-2.5734	
P(T<=t) one-tail	0.007454	
t Critical one-tail	1.693889	
P(T<=t) two-tail	**0.014909**	
t Critical two-tail	2.036933	

t-Test: Two-Sample Assuming Equal Variances		
	IG Remissory	***IG Consistent***
Mean	116011	165324.4
Variance	7.18E+08	2.52E+09
Observations	17	17
Pooled Variance	1.62E+09	
Hypothesized Mean Difference	0	
df	32	
t Stat	-3.57133	
P(T<=t) one-tail	0.000574	
t Critical one-tail	1.693889	
P(T<=t) two-tail	**0.001148**	
t Critical two-tail	2.036933	

t-Test: Two-Sample Assuming Equal Variances		
	IG New	***IG Consistent***
Mean	137938.5	165324.4
Variance	5.17E+08	2.52E+09
Observations	17	17
Pooled Variance	1.52E+09	
Hypothesized Mean Difference	0	
df	32	
t Stat	-2.04784	
P(T<=t) one-tail	0.024428	
t Critical one-tail	1.693889	
P(T<=t) two-tail	**0.048857**	
t Critical two-tail	2.036933	

Testing variance of the incremental growth between each group in turn to ascertain difference between all groups (2002-2018), using T-Test: Two-Sample Assuming Equal Variances.

Pertaining to section 4.4.9

Statistical Analysis of annual growth ring between all Four Groups 2012 - 2018

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
RW Zero	7	2038	291.1429	1649.143		
RW Remissory	7	1750	250	2597.333		
RW New	7	1896	270.8571	1355.143		
RW Consistent	7	2253	321.8571	10364.81		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	19682.39	3	6560.798	1.643648	**0.205699**	3.008787
Within Groups	95798.57	24	3991.607			
Total	115481	27				

ANOVA (single factor) statistical analysis of the tree ring growth of all four groups of trees from 2012 - 2018