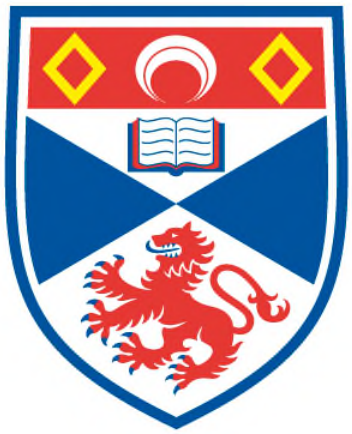
THE EPIDEMIOLOGY OF PHYTOPHTHORA RAMORUM AND P. KERNOVIAE AT TWO OUTBREAK SITES IN SCOTLAND

Matthew Elliot

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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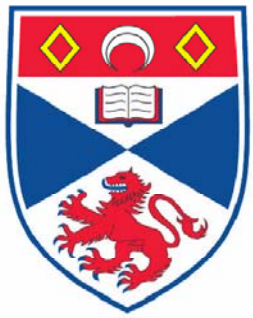

This thesis is submitted in partial fulfilment for the degree of PhD

at the

University of St Andrews

16th June 2013

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Abstract

This PhD examined the epidemiology of two potentially devastating plant pathogens, *Phytophthora ramorum* and *P. kernoviae*, at Benmore Botanic Garden in Argyll & Bute and Brodick Castle Garden in Arran. Spore traps, river baiting, bait plants and soil sampling were used to both confirm the presence of, and measure the amount of inoculum in the environment in order to quantify the relationship between inoculum levels and disease development. *P. ramorum* was detected in spore traps at high levels under a sporulating host throughout the year at Benmore. Also, findings at sites where infected hosts had been removed before the study led to the conclusion that the low level spore traps detect inoculum from soil splash. *Rhododendron* and *Vaccinium* bait plants were also infected with *P. ramorum* via soil splash at sites within Benmore where there was no sporulating host present. *P. kernoviae* was detected in spore traps at Brodick throughout the year but only where there was a sporulating host overhead. *P. kernoviae* infected bait plants only where an infected host is overhead. Water baiting confirmed the presence of *P. ramorum* in two streams at Benmore but *P. kernoviae* was not detected using this method despite the large-scale *P. kernoviae* infection at Brodick. Inoculum continued to be detected in soil in areas of both gardens where infected hosts had been removed two years previously, confirming that both of these pathogens can survive in soil for a considerable period. A number of statistical models were produced to describe conditions required for *P. ramorum* sporulation and infection. Sporulation occurred during warmer and wetter conditions and infection of bait plants occurred in wet conditions and where an infected host is in close proximity. A statistical model was also used to produce a *P. ramorum* risk map, uniquely at the garden scale, to enable garden managers at Benmore to visualise the areas most at risk within their garden. The findings of this study have clear management implications for the control of disease establishment and spread within the garden setting.

Contents

Chapter 1
Introduction

This study has investigated factors that enable the spread of two particularly devastating plant pathogens, *Phytophthora ramorum* and *P. kernoviae*, at two study sites in Scotland. This introductory chapter will describe the current situation regarding the discovery and spread of these pathogens and also explain the current understanding in a number of areas including the mechanisms of spread, biology and management.

1.1 Background

A number of particularly severe diseases have been introduced into forests and gardens over the past century including such well known examples as chestnut blight (*Cryphonectria parasitica*) and Dutch elm disease (*Ophiostoma* spp.) (Anagnostakis, 1987; Hubbes, 1999). North America has been particularly hard hit; chestnut blight on *Castanea dentata* reduced the population of this economically important tree to near extinction in the epidemic of 1904-1944 (Agrios, 2005). The widespread tree decline caused by these diseases has had cascading ecological effects (Liebhold, *et al*., 1995).

The nursery trade has had a part to play in the spread and introduction of diseases as it has significantly increased productivity and global trade networks (Pautasso, *et al*., 2010). In the example of chestnut blight in North America, the disease was found to be caused by the importation of *Cryphonectria parasitica* on infected Asian chestnut trees in the early 20th Century (Milgroom, *et al.*, 1996).

The introduction into Europe of fungal, viral and bacterial pathogens increased through the 20th Century from less than 5 per decade to over 20 (Wilkinson, *et al*., 2011). As potentially infectious plants and compost are now transported throughout the world, it is clear that the pathogens they may be carrying will be moved to parts of the world where they were not previously present (National Research Council 2002). Unfortunately, these conditions have contributed to the spread of new diseases and caused new outbreaks of existing diseases (Jones & Benson, 2001).

Current serious threats that cause concern in the UK include two recently discovered, potentially devastating fungal-like plant pathogens, *P. ramorum* (so called Sudden Oak Death) (Rizzo, *et al*., 2002; Grunwald, *et al*., 2008a) and *P. kernoviae* (Brasier, *et al.*, 2005). These unpredictable and destructive *Phytophthora* species affect forest tree species as well as ornamental trees and shrubs causing leaf necrosis, wilting, shoot tip die-back, bleeding cankers on infected tree trunks, and ultimately the death of a wide range of plants species.

The direct and indirect effects of such pathogens on the structure of plant communities are of increasing interest, particularly the reduction of mature tree populations by the direct influence of exotic pathogens (Dobson & Crawley, 1994). The resulting effects on relative abundance of the species involved within the wider plant community are discernible for many years after the initial disease outbreak has passed. This is particularly the case for *P. ramorum* and *P. kernoviae* as it is now known that *P. ramorum* can survive in soil in the absence of a host for at least two years (unpublished work, Alexandra Schlenzig, Science & Advice for Scottish Agriculture (SASA)) and *P. kernoviae* for at least a year (Widmer, 2011), thus recruitment is severely affected as seedlings become infected and die before maturity. Further to this, given the trade-off between disease resistance and other fitness components, the competitive abilities of genotypes are altered during epidemics which may affect the genetic composition of populations (Alexander & Holt, 1998).

The name *Phytophthora* (Greek for ‘plant destroyer’) was coined by Anton de Bary in 1876 with his description of *Phytophthora infestans*, the type species of this genus (Zentmyer, 1983). Gradually, more and more species of this fungal-like pathogen have been discovered and described thanks to improved diagnosis techniques and molecular genotyping. The number has accelerated recently; only c.43 *Phytophthora* species were listed in 1983 (Waterhouse, *et al*., 1983), c.50 in 1996 (Erwin & Ribeiro, 1996), over 70 in 2006 (Brasier, *et al*., 2006) to 101 in 2012 (Kroon, *et al*., 2012). Surveys of natural landscapes frequently find previously unknown *Phytophthora species*. For example, Jung, *et al*. (2011) found and described four species and one informally designated taxon during a survey of Australian vegetation and associated waterways. A comprehensive UK survey has yet to take place.

Concern about the impact of exotic plant pathogens in the wider UK environment has significantly increased recently after the discovery of infected plants in both heathland environments and commercial plantations. In 2008 *P. kernoviae* was discovered infecting wild heathland plants (*Vaccinium myrtillus*) in a Scottish woodland and Cornish heathland (Food and Environmental Research Agency (FERA), 2009b), whilst *P. ramorum* was found on the same species in Staffordshire (FERA, 2009a). These infections could have important implications for heathland biodiversity.

More recently, forest plantations have also found to be infected after the finding of *P. ramorum* on *Larix kaempferi* (Japanese larch) (Brasier & Webber, 2010). As of July 2010, *P. ramorum* has been found in a considerable number of larch plantations in England (Webber, *et al*., 2010) and has also been found infecting this host in Wales and Northern Ireland. The main concern with larch infection, apart from the commercial implications, is that many other plants have been infected as a result of growing in close proximity to the infected trees; the foliage of larch appears to be providing a platform for *P. ramorum* to sporulate heavily onto surrounding plants. The species affected include *Fagus sylvatica*, *Nothofagus obliqua*, *Castanea sativa*, *Betula pendula*, *Tsuga heterophylla* and *Pseudotsuga menziesii* (Webber, *et al*., 2010).

In addition to the concern caused by these infections, other new *Phytophthora* species are being introduced to UK landscapes at a worrying rate. For example, *Phytophthora lateralis* was found killing *Chamaecyparis lawsoniana* in 2010 at a number of locations in South West England, Yorkshire, Scotland and Northern Ireland (FC, 2011) and *Phytophthora austrocedrae* was found killing endemic and endangered *Juniperus communis* trees at the Upper Teesdale National Nature Reserve (NNR) in the North Pennines in England in 2011, and in single specimens of Lawson cypress and Nootka cypress trees at two sites in Scotland (FC, 2012).

1.1.1 The discovery of *P. ramorum* and *P. kernoviae*

P. ramorum was first described as a new species in 2001 (Werres, *et al.*, 2001) and was already known in Europe, initially being observed infecting *Rhododendron* in Germany and the Netherlands in 1993, but its significance as the cause of Sudden Oak Death (SOD) in California and Oregon was not understood until 2001 (Rizzo, *et al*., 2002).

The symptoms of SOD were originally noticed in 1994 (Venette & Cohen, 2006) and the disease soon reached epidemic proportions in forests along approximately 300km of the central coast of California (Rizzo, *et al*., 2002). The species initially infected by *P. ramorum* were tanoak (*Lithocarpus densiflorus*), coast live oak (*Quercus agrifolia*), California black oak (*Q*. *kelloggii*) and *Q. parvula* var. shrevei (McPherson, *et al*., 2000). It is particularly aggressive in *L. densiflorus* where it can cause cankers on very young stems as small as 5mm (Rizzo, *et al*., 2002).

The first cases in the UK of *P. ramorum* were found in nurseries in 2002 (Lane, *et al*., 2003) and from that point UK outbreaks in nurseries, gardens and the wider environment have steadily increased in number (Webber, 2008). During extensive surveys for *P. ramorum* in Cornwall in 2003, a new invasive *Phytophthora* species was discovered and named as *P. kernoviae* (Brasier, *et al*., 2005). At this point, this pathogen was found mainly within one 14 km^2 area, as well as a small number of other sites mostly with single infected plants involved (Webber, 2008).

As well as the afore mentioned heathland infections on *Vaccinium myrtillus*, a recent FERA study also concluded that the heathland species *Vaccinium vitis-idaea* and *Arctostaphylos uva-ursi* are highly susceptible to *P. kernoviae* (Beales, *et al*., 2009) although they have not yet been infected in the natural environment.

In Scotland, *P. ramorum* was first found in 2002 on plants within the horticultural trade. Seventeen more outbreaks were recorded that same year (Schlenzig, 2008). Phytosanitary emergency measures were initially successful at reducing the incidence of the pathogen to such an extent that there were no reported findings in 2006. There were, however, more findings in 2007 including the first finding in a garden open to the public, leading to an increased incidence; and new outbreaks are currently being recorded in public gardens (Pers. Comm., A. Schlenzig, SASA). The first instance of *P. kernoviae* in Scotland was recorded in January 2008 and there has been a slow but steady increase in outbreaks in gardens in the west of Scotland (Pers. Comm., A. Schlenzig, SASA).

1.1.2 The origin of *P. ramorum* and *P. kernoviae*

The recent development of reliable genotyping techniques have provided insights into the origins and movement of some *Phytophthora* species. *P. lateralis* for example has been affecting *Chamaecyparis lawsoniana* in the Pacific North West US since the 1920s where it has destroyed the commercial trade in *C. lawsoniana*. More recently, *P. lateralis* has been isolated from mature *Chamaecyparis* forests in Taiwan, which has led to the suggestion that this pathogen may originate from this region and was introduced from this area to the US in the early 20th Century and then from the US to Europe more recently (Brasier, *et al*., 2010).

Unfortunately the origins of *P. ramorum* and *P. kernoviae* are not yet as clearly understood. It is possible that *P. ramorum* may have originated somewhere in Asia (Goheen, *et al.*, 2006b) and been transported to where it is found now on collected ornamental plants. Asia is a centre of diversity for many of the known hosts of *P. ramorum* (Vannini, *et al*., 2009) and plant collectors have been bringing plants back from Asia for at least 150 years. More specifically, Yunnan Province in south-western China has been suggested as the possible origin for *P. ramorum* due to the abundance of hosts and favourable climate (Goheen, *et al.*, 2006b; Vannini, *et al*., 2009).

The origin of *P. kernoviae* is also unknown but it has been suggested that it could have been introduced to the UK from New Zealand where soil samples from several regions on North Island show that *P. kernoviae* is found in native and exotic forests (Ramsfeld, *et al*., 2007). Historical records show that *P. kernoviae* was found under *Pinus radiata* trees in New Zealand in the 1950s (as *Phytophthora* sp.); the only diseased plants found in New Zealand are *Annona cherimola*.

1.1.3 Detection and Identification

Pathogens have traditionally been identified morphologically (Gallegly & Hong, 2008) and the first comprehensive non-molecular keys for the identification of *Phytophthora* were produced in the 1960s (Waterhouse, 1963). Most *Phytophthora* species can be readily cultured on agar and identified under the microscope with the aid of a key based on the morphology of structures such as sporangia, mycelium, hyphal swellings, oospores and chlamydospores (Gallegly & Hong, 2008). A further complication in the

detection of *P. ramorum* and *P. kernoviae* is the recent discovery of asymptomatic aerial infection of leaves and fruit with some evidence of sporulation (Denman, *et al.*, 2007). This finding has serious management implications, particularly for the nursery trade.

Apart from the high level of expertise required, a number of problems exist when identifying *Phytophthora* species morphologically including an expanding host list, variable host symptoms, plasticity in colony morphology and variable culturing success (Hayden, *et al*., 2004). This has led to the development of molecular sequencing techniques to identify species by comparing regions of their DNA (Bailey, *et al*., 2002). This technology allows large numbers of samples to be processed relatively quickly whilst maintaining confidence in the identification of the pathogens.

Molecular techniques for taxon-specific detection include both qualitative and quantitative Polymerase Chain Reaction (PCR) techniques (Hayden, *et al*., 2004; Tooley, *et al*., 2006; Tomlinson, *et al*., 2005). Real-time PCR has been adopted as the preferred quantitative technique because of its advantages of speed, accuracy and sensitivity over conventional qualitative PCR based techniques (Schaad, *et al*., 2002).

1.1.4 Host species

According to FERA (2012), there are currently 157 known host species of *P. ramorum* and 37 of *P. kernoviae*, although it should be pointed out that this does not count the large number of cultivars involved (e.g. *Rhododendron* is counted as 1 when in fact there are many species and cultivars within this genus that are involved). The wide host range of both pathogens in the U.K. is particularly remarkable with infection on iconic species such as oak, beech, ash, birch and horse chestnut. Many traditionally popular garden species are also infected such as viburnum, camellia, pieris, magnolia, sweet chestnut and osmanthus.

A significant aspect of these host-pathogen relationships is that one of the most important host species for these pathogens in the UK is itself an invasive species, *Rhododendron ponticum* (Tracy, 2009; Sheppard, *et al*., 2006). *R. ponticum* plays an important role in the transmission of *Phytophthora* within woodlands (Tracy, 2009). In

California it was found that this role was played by bay laurel (*Umbellularia californica*) (Davidson, *et al*., 2005).

This *Rhododendron* is actually considered to be a hybrid of horticultural origin, a cross between *R. ponticum* subsp. *baeticum* and *R. catawbiense* (a US native) (Sheppard, *et al*., 2006). This weed species costs €3500 ha-1 in clearance costs because re-treatment of vigorous re-growth is required and each plant can produce 1 million very small seeds each year. Figure 1 shows a hillside in the west of Scotland colonised by *R. ponticum*.

Figure 1: *R. ponticum* colonisation in the west of Scotland.
Photo credit: A. Schlenzig, SASA

1.1.5 Biology of *Phytophthora*

1.1.5.1 Sporulation structures

The genus *Phytophthora* is classified as an oomycete.Oomycetes are members of the kingdom Chromista with fungal-like mycelium but they are not fungi (Hardham, 2007). Two orders, the Saprolegniales and Peronosporales contain the most important plant pathogenic oomycetes. Peronosporales includes several of the most important plant

pathogens including *Pythium*, *Phytophthora*, several fungi causing downy mildews, and *Albugo*, the cause of white rust on crucifers (Waterhouse, 1973). Oomycete plant pathogens show remarkable flexibility in their life cycles and ability to adapt to changing environmental circumstances (Jeger & Pautasso, 2008).

It may at first seem that *P. ramorum* has a remarkable ability to escape from nurseries and spread to infect plants, but its pathways of transmission are in fact consistent with other aerial *Phytophthora* species which disperse via rain splash from above ground infected parts (Hunter & Kunimoto, 1974; Grove, *et al*., 1985; Yang, *et al*., 1992; Maden, 1997). *P. ramorum* forms chlamydospores (fig.2a) whilst *P. kernoviae* forms oospores (fig. 2d), both species form sporangia (fig. 2b and 2c) and zoospores. Chlamydospores are produced asexually whilst oospores are produced through mating, both structures are thick walled resting spores associated with long term survival in the soil (Davidson, *et al.*, 2002; Hwang & Ko, 1978).

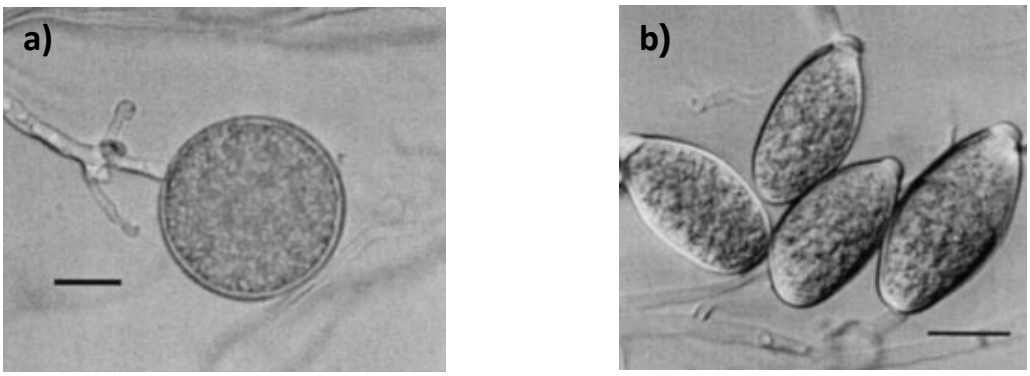


Figure 2): The a) chlamydospores and b) sporangia of *P. ramorum* (Werres*, et al*., 2001)
The c) sporangia and d) oogonia of *P. kernoviae* (Brasier, *et al*, 2005)

Phytophthora sporangia form on both solid and liquid substrates and are usually produced at or near to the air-substrate interface (Ribeiro, 1983). The complex process

of sporulation involves many factors including water potential, nutrients, sterols, light, temperature, etc. Water potential is perhaps the most significant factor influencing sporangia production with a relative humidity of 100% being highly conducive to sporangium formation (Ribeiro, 1983).

During studies of *P. ramorum*, Moralejo *et al*. (2006) described structures he termed sporangiomata. This is the first description of stromata produced by a *Phytophthora* species and it is thought to be a significant environmental adaptation in *P. ramorum*, particularly as the adaxial positioning suggests an adaptation for rain splash dispersal. The positioning could also protect from desiccation or solar radiation and clustering of sporangia may contribute to moisture retention (Moralejo, *et al*., 2006).

The most important developmental stage of oomycetes, which sets them apart from true fungi and allows oomycete diseases to become epidemics, is the formation of zoospores (Walker & Van West, 2007). Zoospores are asexual motile spores consisting of single nucleated wall-less cells which have two flagella that allow them to swim, one anterior and one posterior (fig. 3b). It is probable that the anterior flagellum is responsible for pulling the zoospore through the water and the posterior flagellum aids steering (Judelson & Blanco, 2005).

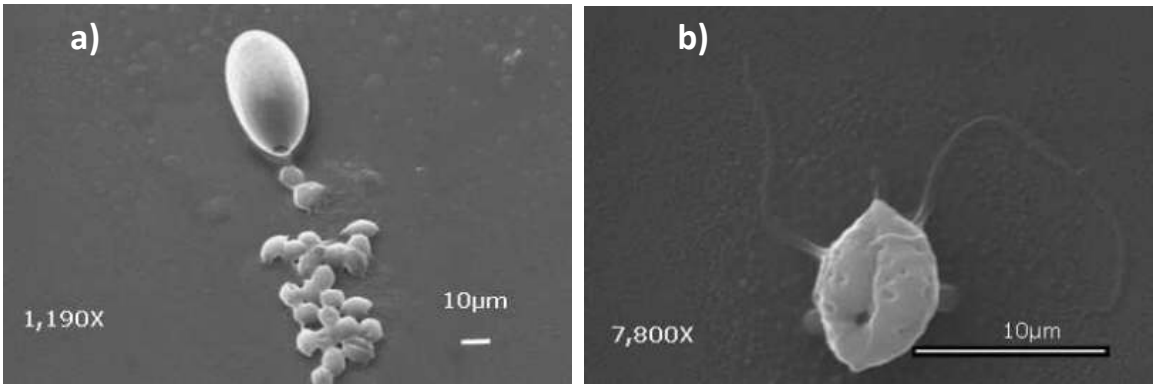


Figure 3: A *Phytophthora ramorum* sporangia releasing zoospores (a) and a zoospore (b).
Photo Credits: Edwin R. Florance, (suddenoakdeath.org)

Zoospores are released from sporangia (fig. 3a) and it is this element that drives *Phytophthora* epidemics. Multinucleated sporangia land on leaves and stems by air or through rain splashes, and germinate (Walker & Van West, 2007). Depending on the

species, a number of zoospores (3-8 or more) are formed within the sporangia and released during a period of low temperature (below 12 – 15^{o}C) through the sporangial apex. At temperatures above approximately 15^{o}C the sporangia germinate directly by producing a germ tube (Hardham, 2007).

Under the correct environmental conditions, zoosporogenesis happens extremely quickly and allows epidemics to spread rapidly (Walker & Van West, 2007). Once the motile zoospores are released they can swim for hours in the presence of an endogenous food reserve, thought to be lipids (Carlile, 1986). Although zoospores are small, up to 10µm in diameter, they can travel 25-35mm in waterlogged soils (Duniway, 1976).

Understanding zoospore behaviour is essential in understanding and predicting epidemic development and epidemiology, therefore models should take such behaviour into account (Jeger & Pautasso, 2008). Various predictive models involving outbreaks of *P. infestans* have shown how the consideration of zoospore activity can significantly improve forecasting (Johnson, *et al*., 1996; Aylor, *et al*., 2001; Bourgeois, *et al*., 2004).

1.1.5.2 The dispersal of *Phytophthora*

Phytophthora rarely kills its host plant immediately. Some hosts harbour the pathogen and support the formation of deciduous sporangia, allowing *Phytophthora* to infect surrounding susceptible plants. These host plants act as a platform for aerial dispersal within a suitable environment and are very important in the epidemiology of these pathogens (Denman, *et al*., 2008). Once produced, the most important means by which the sporangia are moved is via rain splash and wind-driven rain (Madden, 1997; Rizzo, *et al*., 2002).

Long distance dispersal of these pathogens is currently poorly understood, but it is clear that long distance dispersal is a fundamental aspect of plant disease epidemics that enables diseases to jump from one area to another, thwarting control and containment measures (Jeger & Pautasso, 2008). For example, *Peronospora tabacina* (tobacco blue mould) advances by 9-18km per day from the southern to the northern tobacco growing regions of the eastern US in an annual wave (Aylor, 1999).

The role of the horticultural trade in the introduction and spread of non-native plant pathogens is an important one. Jones & Baker (2007) found that 234 exotic pathogens were introduced into the UK between 1970 and 2004, 53% of these were found on ornamental crops and 15% on wild native species. Where the origin was known or strongly suspected, 47% came from the Netherlands. Brasier (2008b) states that the commercial movement of living plants, together with unlicensed specialist or amateur plant collecting, is now the pathway of highest risk.

Apart from the trade movement of plants, very little is known about how *P. ramorum* and *P. kernoviae* are transmitted from one outbreak site to another within the UK. Movement by wheeled vehicles and animals are potential pathways and many studies have found infested soil on people's shoes as they leave infected sites. Davidson *et al.* (2005) found that *P. ramorum* infested soil was carried on the shoes of one-third to one-half of hikers that had walked through infected areas during the rainy season in California.

There are already a number of historical examples of *Phytophthora* species being moved on machinery and shoes: *P. cinnamomi* in jarrah forests and native vegetation in Western Australia (Colquhoun & Hardy, 2000; Shearer & Tippett, 1989) and *P. lateralis* in Port-Orford-cedar areas in the Pacific Northwest (Hansen, *et al*., 2000; Goheen, *et al*., 2006a). No go areas and vehicle washing protocols have been enforced in these situations, actions that may need to be implemented in the future for *P. ramorum* and *P. kernoviae* and other invasive *Phytophthora* species (Brasier, 2008a).

1.1.5.3 Survival

An important factor in the pathogenicity of an organism is how long it can survive above or below ground in the absence of a host. There are many influential environmental conditions involved, solar radiation for example is the dominant factor in the survival of *Bremia lactucae* sporangia which will only survive on shaded leaves or cloudy days (Wu, *et al*., 2000). This is also the case with *Phytophthora infestans* spores where one hour of sun (SI > 600 W/m^2) reduces viability of sporangia by ~95% (Mizubuti, *et al*., 2000). The presence of water is also often an important factor; this is the case with

Plasmopora viticola which releases zoospores for up to seven days in the presence of water (Kast & Stark-Urnau, 1999).

The different biological structures of *Phytophthora* survive for different lengths of time. Chlamydospores and oospores are the most persistent, sporangia are intermediate and zoospores are the least persistent (Hwang & Ko, 1978). Shishkoff (2007) reported that *P. ramorum* chlamydospores survived in soil for at least 11-12 months, but in experiments at Science and Advice for Scottish Agriculture (SASA) they have survived for as long as 2.5 years (3 winters) (unpublished work, Alexandra Schlenzig, SASA). In hot, dry weather *P. ramorum* will survive within often symptomatic leaves on the host, but survival seems to depend on the specific environmental conditions of the infected site (Fichtner, *et al*., 2007).

1.1.5.4 Sexual reproduction

P. ramorum is heterothallic, which means that it is self-sterile and requires mycelial contact with another genetically distinct but compatible individual for sexual reproduction to occur, therefore two mating types are required to come together (Ko, 1978). Conversely, *P. kernoviae* is homothallic and therefore self-fertile and able to reproduce sexually without contact with a different mating type (Brasier, *et al*., 2005).

Comparisons of European and North American populations of *P. ramorum* have revealed that an A1 mating type occurs mainly in Europe and A2 primarily in North America (Grunwald, *et al*., 2012). In 2003, the A2 mating type was discovered in Europe (Werres & De Merlier, 2003), and some A1 isolates were reported in American nurseries (Hansen, *et al*., 2003), therefore suggesting that crossing between mating types would be possible. So far this has only been observed under laboratory conditions where it was achieved only with great difficulty suggesting that the mating system is not fully functional (Brasier & Kirk, 2004; Prospero, *et al*., 2009).

A number of differences have been observed between the two populations. American isolates, for example, are slower growing and more variable in morphology than the European isolates which grow faster and are more uniform (Werres & Kaminski, 2005). It has been suggested that the differences between mating types show that the populations are adaptively different which could be due to a number of processes or

events including association of intrinsic fitness factors with sexual compatibility type; introduction of genetically different founder populations; or differential adaptation and genetic behaviour of the two populations since introduction (Brasier, 2003).

Within these two *P. ramorum* populations it has been discovered, using microsatellite markers, that there are three clonal lineages, NA1 and NA2 in North America and EU1 in Europe, that have been evolutionarily separated for at least 100,000 years (Goss, *et al.*, 2009a). The NA1 lineage is found within the US nursery industry and forests and is the cause of the extensive mortality of oak and tanoak (Ivors, *et al*., 2006), whilst NA2 is more rare and has only been documented in North American nurseries (Ivors, *et al*., 2006; Elliott, *et al*., 2009). The EU1 lineage is found in nurseries, gardens and semi-natural landscapes and has also been found in a small number of North American nurseries (Grunwald, *et al*., 2008; Elliott, *et al*., 2009).

Recently introduced pathogens are expected to have low genetic diversity due to the genetic bottleneck experienced during introduction and establishment. However, using microsatellite markers, *P. ramorum* has been found to have enough genetic diversity that it's migration across the US can be traced to the state where the infection originated (Goss, *et al*., 2009b).

1.1.6 Management and Monitoring

1.1.6.1 The legislation and its application

The European Union Plant Health Directive (2000/29) aims to allow free movement of plants and plant products throughout the EU whilst trying to prevent the spread of harmful organisms (Hunter, 2005). This legislation allows member states to implement emergency measures should they be required; this is done in the UK under the Plant Health Act 1967 (OPSI, 2009).The import of plants and plant products into the UK from non EU countries are restricted to protect plant health, and phytosanitary certificates are required for such activities. This legislation is implemented in England by the Plant Health (England) Order 2005 (HMSO, 2005a), in Wales by the Plant Health (Wales) Order 2006 (HMSO, 2006) and in Scotland by the Plant Health (Scotland) Order 2005 (HMSO, 2005b).

In April 2002 the first confirmed case of *P. ramorum* in a managed garden in the UK set in motion emergency measures which were aimed at controlling imports of susceptible material from the US and the implementation of plant passports for susceptible material being moved within the EU (Hunter, 2005). The existing Plant Health Act (1967) was used to hold or destroy infected plant material.

In September 2002 the EU introduced emergency legislation in the form of Commission Decision 2002/757 (Byrne, 2002), later to be updated in 2004 (Byrne, 2004) and 2007 (Kyprianou, 2007). These current measures require that surveys are carried out, and where infected plants are found, they are to be eradicated. As well as these measures, import controls and internal movement controls on *Rhododendron* spp., *Viburnum* spp. and *Camellia* spp. are required (Slawson, *et al*., 2007).

Nurseries are subject to at least two inspections per year. If infected plants are found they are destroyed, as are all susceptible plants within a 2 metre radius and all associated growing media. In addition, plants growing within a 10m radius are subject to movement controls and are to be held for 3 months, not treated with anti-*Phytophthora* fungicides and inspected twice to confirm that they are free from the pathogen (Slawson, *et al*., 2007). All other susceptible plants at the nursery are also subject to official inspections.

These emergency measures were originally created as a result of the discovery of *P. ramorum,* but the same legislation and subsequent actions now apply to the more recently discovered *P. kernoviae*, although there is evidence to suggest that *P. kernoviae* is not spread as readily through the horticultural trade with only one nursery infection found to date (FERA, 2009b, Hunter, 2005). The *P. kernoviae* outbreak near Redruth in Cornwall was so extensive that new legislation was created and The Plant Health (*Phytophthora kernoviae* Management Zone) (England) Order 2004 came into force on 21 December 2004. This gives the Plant Health authorities power to prohibit the removal of host plants from the Management Zone, to gain access to check compliance and to close rights of way through infested areas. A Schedule to the Order sets out the geographic limits of the Management Zone (HMSO, 2004).

As there are no border controls within the EU, products can be moved freely within The Single Market; however plant passports have been introduced for consignments of

Rhododendron, *Viburnum* and *Camellia* to confirm that plants comply with the required official measures (DEFRA, 2005).

Legislation pertaining to the eradication of plants infected with *Phytophthora* extends to infections wherever they are found, including gardens. This has led to widespread clearances of *R. ponticum*, particularly in Cornwall, in some cases aided by grants from existing Forestry Commission schemes for woodland management (Hunter, 2005).

The regulations that were originally put in place when *P .ramorum* was first identified as the cause of sudden oak death have not always been effective and have had to be strengthened. In 2004 large numbers of infected camellias and rhododendrons were shipped across the United States from California and Oregon nurseries, increasing fears of endangering the oak resource in the eastern United States (Stokstad, 2004; Frankel & Oak, 2005; Goss, *et al*., 2009b). Instances like this have led to a broadening of national and international quarantines designed to prevent movement of the pathogen and to a renewed impetus to manage *Phytophthora* diseases in nursery settings (Rizzo, *et al*., 2005).

1.1.6.2 Management issues

Due to the recent discoveries of *P. ramorum* and *P. kernoviae*, understanding of these pathogens is limited, however, it is clear that management must focus on three non-exclusive levels: the individual plant, the landscape (or forest stand), and the regional to international scale (Rizzo, *et al*., 2005).

At individual plant level chemical controls are not allowed in the EU but have been used in the US to maintain the health of plants and lower inoculum pressure, although these controls have no use in forest outbreaks (Garbelotto, *et al*., 2002). The afore mentioned legislation is aimed at preventing further spread of the pathogen at the wider regional and international level. The most difficult level to manage *Phytophthora* however is at the landscape scale (Rizzo, *et al*., 2005).

The landscape scale level of management requires in-depth knowledge of *Phytophthora* and there have been many attempts to stop other exotic pathogens in landscape situations. For example, widespread clear-cutting was ultimately unsuccessful at stopping chestnut blight (*Cryphonectria parasitica*) across the north-

eastern United States in the early 1900s (Anagnostakis, 1987) as was large scale removal of host material attempted to control white pine blister rust (*Cronartium ribicola*) (Kinloch, 2003). It is thought, however, that breaking the connectivity of patches of woodland could be used to decrease the connectivity of susceptible hosts to *P. ramorum* and *P. kernoviae* and so control the spread (Jeger & Pautasso, 2008).

Sanitation programs have been tried against a number of pathogens including Dutch elm disease (*Ophiostoma ulmi, O. novo-ulmi*) in North America and Europe, *Phytophthora cinnamomi* in Australia, and *P. lateralis* in Oregon and California (Cannon, *et al*., 1977; Hansen, *et al*., 2000; Hardy, *et al*., 2001). Fungicides were also applied widely against *P. cinnamomi* in Australia to try to reduce the spread (Hardy, *et al*., 2001). The success of these programs was variable with some effective control on one hand and continued tree mortality on the other. The large scale application of chemicals can only be used in certain limited circumstances, it would be very difficult to treat large public areas with fungicides for example.

Programs are underway to attempt to breed trees with genetic resistance to *Phytophthora*. For example, the USDA Forest Service have been running a program since 1996 to develop resistance to *Phytophthora lateralis* in *Chamaecyparis lawsoniana*, and early results are encouraging, showing more than 40% higher survival in short-term glasshouse tests (Sniezko, *et al*., 2007). This is in addition to many other programs to develop resistance to other pathogens, particularly in western white pine (*Pinus monticola*), American chestnut (*Castanea dentata*), and American elm (*Ulmus americana*). However, these programs have to contend with the long-lived nature of trees and the potential of the pathogen to evolve (Sniezko, 2006).

Within the public garden setting, management has particular considerations and problems, such as the potential inadvertent spread of pathogens by visitors and staff. Management of infected plants in these gardens in the UK has so far focused on the eradication of infected plants upon their discovery. Removal of *R. ponticum* from gardens and parks has started, which is an important measure to reduce hosts and therefore sources of inoculum.

Managing forest diseases during climate change will bring further management challenges. Forest disease outbreaks are predicted to become more frequent and

intense as drought and other stressors are amplified under climate change (Sturrock, *et al*., 2011). This is by no means certain, however, as host-pathogen interactions are complex and difficult to predict. For example, resistance to two forest pathogens in the US actually increased under higher CO_2 concentrations (Runion, *et al*., 2010); therefore, a wide range of responses to increases in atmospheric CO_2 is expected worldwide.

1.1.6.3 Epidemiological Modelling

Epidemiology is concerned with studying the distribution and spread of pathogens and hosts. It is a multidisciplinary science because of the complexities of biotic and abiotic factors within the environment and the impact of human activity (Milgroom & Peever, 2003). A theoretical basis for the understanding of plant epidemics in time and space has been, and continues to be, one of the major goals in epidemiology (Jeger, 2000) although the theoretical basis for plant disease epidemiology has not yet been established to the same extent as for human or animal disease epidemiology (Michael, 1993).

When designing strategies for the management of disease, host-pathogen models are critical (McCallum, *et al*., 2001). A mathematical model for epidemics of plant disease was first developed in the 1960s by van der Plank (1963) in the form of a differential-delay equation. It was found that disease can be categorised into three elements: latent (not yet infectious), infectious, and removed (post infectious). Specifying three epidemiological parameters (an infection rate based on infectious diseased tissue, and latent and infectious period durations) within the equation enables a prediction to be made as to whether an epidemic will occur (the epidemic threshold). The initial growth rate of the epidemic and its final size can also be predicted with this model.

Since van der Plank in the 1960s a number of compartmental epidemiological models have been developed to model the progress of an epidemic. The most common are variations on SIR and SEIR models where the compartments are S (for susceptible), E (exposed), I (infectious) and R (removed or post infectious) (Jeger, *et al*., 1998). For example, Harwood *et al*. (2009) developed a spatial SEIS model to model pathogen dynamics and spatial dispersal of *P. ramorum* and *P*. *kernoviae* in the U.K. The removed (R) compartment in the standard model was replaced with (S) to show that if

plants are not removed they may recover but are still susceptible. S+E+I are defined as the total constant population size (N) where removed infectious plants are replaced by an equal input of new young plants therefore keeping the population size constant. This model suggested that inspections can control *Phytophthora* epidemics provided the pathogen is restricted to the nursery trade and that restricting the sale of particularly susceptible species would significantly reduce the spread.

Since the discovery of *P. ramorum*, disease risk models have been developed in order that risk maps can be produced to enable a more targeted response to disease prevention. In the US in 2002 for example, the US Department of Agriculture Forestry Service developed a national risk map based on (1) distribution of hosts known or likely to be susceptible to the pathogen, (2) climatic conditions adequate for survival and propagation of the pathogen, and (3) pathways for introduction of the disease outside the currently infested region (Smith, *et al*., 2002). The resulting map gives high, moderate and low areas of risk. The increase in the understanding of *P. ramorum* since 2002 has led to a number of updates in the risk map to include more factors as they become known (Koch & Smith, 2008).

The quality of the data which is gathered and used for creating risk maps is an important consideration as there may be constraints to the available knowledge in, for example, the biology of the species under investigation or the environmental conditions in a particular area (Venette, *et al*., 2010). Risk maps therefore reflect the current understanding of a pathogen invasion using the best available knowledge but they do not necessarily reflect all of the factors that affect pathogen risk.

1.1.7 Summary

Compared to large scale abiotic disturbances that affect forests, such as fires (Flannigan, *et al*., 2000; Bergeron, *et al*., 2001), pathogens have traditionally received less attention, which is surprising considering their important role in the dynamics of forest ecosystems (Dickman, 1992; Dobson & Crawley, 1994). The consideration of pathogens in modern landscape management, with its emphasis on the broader ecosystem, will need to be examined with much more care in the future (Castello, *et al*., 1995). The *Phytophthora ramorum* epidemics seem to have accelerated this process, particularly due to the widespread devastation caused in U.S. forests.

Our understanding of the spread of infection within public gardens and from one garden to another is still incomplete. These aspects are critical if these pathogens are going to be managed successfully as the movement of people through public gardens could be spreading infection both within the garden and from one garden to another. Also, before effective management techniques can be developed, more research is needed into the epidemiology of these two *Phytophthora* species to increase our understanding of how they are spread both within gardens and the wider environment.

1.2 Approach

In order to better understand the conditions that facilitate the local spread of these pathogens in Scotland, a range of diagnostic methods have been applied to study local epidemiology. The intention has been to adapt a set of standardised methods for field investigations that are based on those applied successfully in England and Wales. These methods measure the amount of inoculum in the environment in order to quantify the relationship between inoculum levels and disease development, environmental conditions such as weather, sporulation, dispersal and further outbreak development. This standardisation will allow comparisons with the studies in England and Wales so that there can be a specific focus on the identification of factors that may be driving or limiting disease development in Scotland.

1.3 Objectives

a) Identify sources and pathways/mechanisms of potential pathogen spread and quantify impact of biological and environmental factors on infection and spread.

b) Quantify effects of host, environment, season and climate on life stages present in infected plants and contaminated substrates and determine impacts on pathogen survival and dispersal.

c) Determine relative risks from *P. ramorum* and *P.kernoviae* to Scottish gardens and ecosystems through comparison with data from outbreaks in England and Wales.

d) Investigate measures required for successful eradication and containment of *P. ramorum* and *P. kernoviae* and identify how these vary according to pathogen/host combinations, seasonal factors and climatic conditions.

Chapter 2
Materials & Methods

2.1 Introduction

In order to avoid duplication throughout this thesis, universal materials and methods deployed during this study have been brought together and are outlined in the present chapter. Materials and methods specific to particular aspects of the study have been integrated into the relevant subsequent chapters.

2.2 Site selection

Permission for this study was sought and given by the owners of two gardens in the west of Scotland.

Brodick Castle Garden on the Island of Arran (figure 4, page 22) had an extensive *P. kernoviae* infection. Much of the original infection area had been cleared of the problematic host *Rhododendron ponticum* although it was starting to re-grow in places. The worst affected areas of the garden have been closed to the public. A *Vaccinium myrtillus* infection at Merkland Wood, less than a mile from Brodick, was also studied. This is a managed mature *Fagus sylvatica* woodland with a small area of infected *V. myrtillus* which has been sprayed with herbicide to eradicate this host species, although some re-growth was evident. This area was also closed to the public to reduce the risk of inadvertent spread.

Benmore Botanic Garden in Argyll & Bute (figure 5, page 24) was selected because both *P. ramorum* and *P. kernoviae* were present, although *P. kernoviae* was at a much lower level. The infection area was relatively small and the garden contained many of the known host species so further spread of the infection around the garden could be easily followed and documented.

2.3 Study locations at each garden

The specific sites where the spore traps were located at each garden are shown in detailed photographs in appendix H. They were selected as follows:

BrodickCastle Garden

Brodick Castle Garden is on the east coast of Arran at the foot of Goatfell Mountain. The garden is on a steep hill which goes down from the castle to the road. The garden contains many host species; it holds three national collections of *Rhododendron* for example. The worst affected area along the bottom of the garden was once a *R. ponticum* windbreak which became infected and was removed.

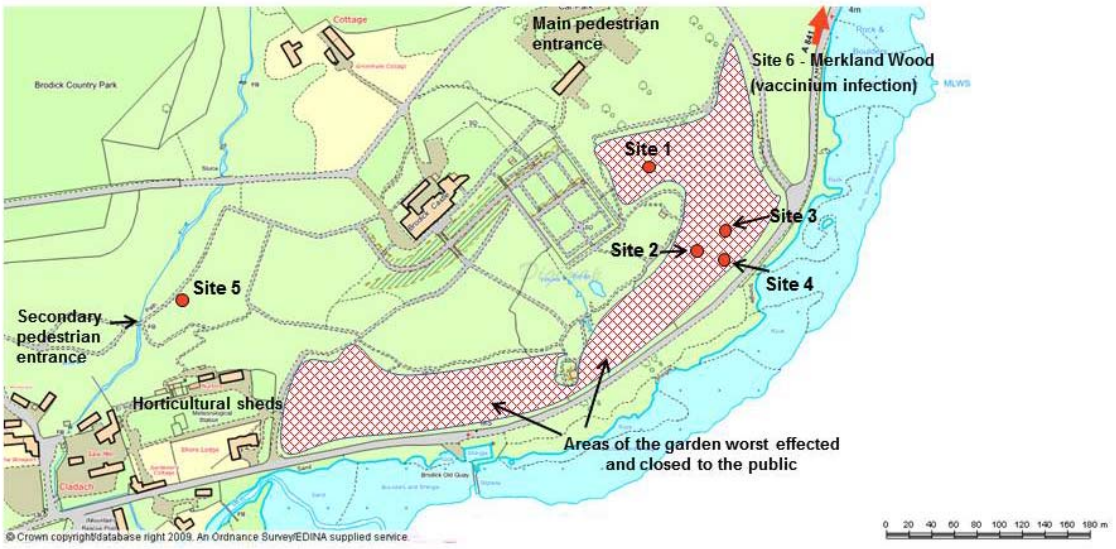


Figure 4: A map of Brodick Castle Garden showing the study sites.
Base map provided by Edina digimap (2012)

Site 1 was located within a stand of *R. ponticum* just below a cleared infection area approximately 50 metres from a main path in the open part of the garden. This area was the closest to the garden entrance and so onward spread from here to the wider garden was thought possible.

Site 2 was also within a stand of *R. ponticum* but lower down the hillside and closer to the main infected area of the garden

Site 3 was a location that has seen a number of *Rhododendron* infections and also the infection of a mature *Drimys winteri* which was removed in early 2008.

Site 4 was located in the heart of the infection within the area which was previously covered by *R. ponticum*, although it has since been cleared, there was some infected re-growth.

Sites 3 and 4 were chosen due to their proximity to the area that was most widely infected with *P. kernoviae*.

Site 5a (initially site 5 until a larger infection was discovered on the other side of the path). This site contained an infected *Pieris japonica* that was removed in 2009.

Site 5b was within a grove of larger *Pieris japonica*. It was selected because of its close proximity to the pedestrian entrance of the garden and because of a number of infected *P. japonica* in the immediate area.

Sites 5a and 5b were in the part of the garden open to the public.

Site 6 was against the stump of the removed large *P. kernoviae* infected *Drimys winteri*, the first confirmed infected plant at Brodick. There were also signs of infected regrowth.

The Merkland Wood site contained a group of infected *Vaccinium myrtillus*. This was the only confirmed infection on a heathland species and therefore an important site for assessing the potential impact of *P. kernoviae* on Scottish heathlands.

Benmore Botanic garden

Benmore Botanic Garden is situated in a valley at the foot of a hillside to the east. All of the streams in the garden flow from this hillside and down into the garden apart from a large river at the pedestrian entrance. The planting is typical of this period of garden and many of the species used are hosts including *Rhododendron, Pieris, Magnolia* and *Osmanthus*.

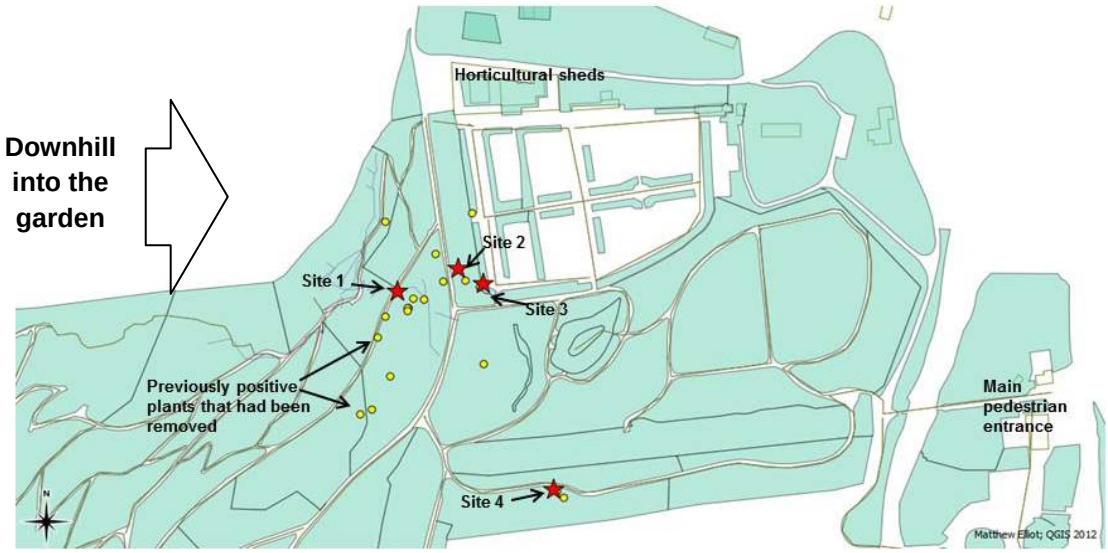


Figure 5: A map of Benmore Botanic Garden showing the study sites
(Map created using QGIS (2012)

Site 1 was at the centre of the original infection. The main species affected (by both *Phytophthora* species) in this area was *Rhododendron* 'Elizabeth Hobbie', which has since been cleared. It was on a hillside with a small stream running through it. A number of infected plants had also been removed from below this site. As this site was uphill of host plants and had a stream running through it, it was selected to assess whether infection could be found to move downhill.

Site 2 was within a lawn at the side of a main path under a single infected *Magnolia kobus* which has been left *in situ* by mutual agreement. There was another *Magnolia* and an *Acer palmatum* in close proximity. This site was selected because inoculum in the environment around the infected tree could be monitored for seasonal variation in intensity.

Site 3 was at a cleared *P. ramorum* infection on two removed *Kalmia latifolia* less than 10 metres from site 2.

Site 4 was separate from the main infection at this garden and was at the location of two removed *Osmanthus* species infected by *P. ramorum.* As this site is some distance

from the other infections it could show how long *P. ramorum* stays in soil in the absence of a host.

Six Gemini Tinytag Plus 2 dual channel temperature/humidity data loggers (recording temperature and relative humidity at 5 minute intervals) were placed in the two gardens as follows:

- Brodick – at sites 1, 3 and 5 (attached to shrubs as close to the spore traps as possible)
- Benmore – on a *Rhododendron* just below site 1, on the *Magnolia* at site 2 and on an *Enkianthus* as close to the cleared infected *Osmanthus* as possible at site 4

2.4 Monitoring inoculum and extracting DNA

2.4.1 Spore traps

Two types of spore traps were designed in order that rain and water splashes, potentially containing sporangia and zoospores, could be caught. Low level traps were located at ground level and constructed by digging a 2 litre water bottle into the ground and placing a funnel on top (fig. 6). Wire mesh was placed around the top of the funnel in order to prevent the funnel becoming blocked by fallen leaves and other debris.

High level water traps were approximately 1 metre above ground level. They consisted of a 2 litre bottle on the ground, or slightly dug in to give it stability, a funnel and a length of hose connecting the two (fig. 7). Wire mesh and muslin was used around the top of the funnel to avoid blockage from fallen leaves.

Figure 6: Low level spore trap

Figure 7: High level spore trap

The low level traps are designed to record mainly rain splash dispersal from the surrounding soil whilst the high level traps record dispersal from rain and leaf splash from the nearby plants.

The 2 litre bottles were recovered to the quarantine unit at SASA every 2 weeks and processed as described in appendix C. Real-Time PCR was used to quantify the amount of *Phytophthora* DNA in the resulting sample (see appendix E).

2.4.2 Bait plants

To test the viability of the inoculum in the environment and to test for a link between inoculum levels and infection, one potted *Rhododendron* sp. plant was placed monthly at each site in both gardens for a 4 week period in order to see whether they become infected.

During the study evidence became available (Pers. Comm. Phil Jennings, FERA) that *Vaccinium myrtillus* bait plants were considerably more susceptible to *P. kernoviae* than *Rhododendron*, *V*. *myrtillus* plants were sourced for use as baits in the hope that this species would be more sensitive to the *P*. *kernoviae* infections and therefore give us more accurate results. In April 2011 *Pieris* plants were also tried as bait plants because a number of infected *Pieris* have been found at both Benmore and Brodick throughout 2010 and 2011.

The bait plants were recovered to the quarantine glasshouse at SASA after the 4 week period in the gardens (fig. 8). The glasshouses were kept between 6^{o}C and 18^{o}C and were fitted with misting equipment in order to match optimal environmental conditions for the pathogens.

Figure 8: *Rhododendron* bait plant in the quarantine facility. It is infected with *P. ramorum* after being placed at site 2 in Benmore for 4 weeks. Photo credit: SASA, Crown copyright

The plants were kept for 3 months post-exposure and were checked daily for symptoms. Infected leaves were plated onto agar medium (see appendix B), followed by PCR testing if required (see appendix E).

2.4.3 Water baits

Local spread of *Phytophthora* species is often facilitated by watercourses (Beales, 2007; Werres, *et al*., 2007); therefore, leaves from healthy *Rhododendron* plants were used as bait traps for detection of pathogens in watercourses. Three leaves were placed in a square of muslin which was then tied with twine. The twine was about one metre long which allowed it to be anchored in the ground next to the stream (Figure 9).

Figure 9: Water bait (highlighted) in a stream

Leaf baits were then placed in watercourses around each garden for a minimum of 4 hours (ideally up to 24 hours).

As with the bait plants, the use of *V. myrtillus* and *Pieris* as water baits was tried to establish levels of *P. kernoviae* and *P. ramorum* inoculum in watercourses at both gardens.

Once the leaf baits had been recovered, the leaves were taken from the muslin bags and dipped in alcohol to reduce contaminants, such as *Pythium* spp., and cut into small pieces (about 2mm^2) with sterile scissors. Approximately 10 of these pieces were transferred aseptically onto V8 agar + antibiotics (recipe in appendix B) which is semi-selective for *Phytophthora* species.

The plates were checked under a compound microscope after 5 days for the presence of mycelium and sporulation structures. If the presence of either of these *Phytophthora* species was suspected, the Nucleospin® Plant Kit protocol for DNA isolation from plants was used to extract any DNA and Taqman PCR was used for confirmation (appendix E).

2.4.4 Soil samples

Initially, samples of soil were taken from the known infected areas at both gardens in order to develop a reliable detection method. Bags (23 cm x 14cm) were filled with soil that was taken from the surface, no deeper than 15cm. A number of DNA extraction and sampling methods were tried during this period and a final protocol was developed (see appendix D) that was an adaptation from methods provided by E. Gilroy (The James Hutton Institute (JHI)) and P. Jennings (FERA). Once our method was developed, areas for study could be systematically sampled every 3 months at increasing distances from the foci.

Brodick

Soil samples were taken at the worst affected area along the bottom of the garden around sites 2, 3 and 4. This area had been largely cleared of hosts so it was the long term survival of *P. kernoviae* in soil that was being investigated.

Site 5 and Merkland Wood are the other two areas where soil samples were taken. Initially a sample was taken at the spot where the infected plant once stood (where the spore traps were) then a sample was taken every 2 metres to the north, then every 2 metres to the south, east and west to a distance of 10 metres. This sampling method led to the discovery of high inoculum levels under a grove of mature *Pieris* about 5 metres from site 5 so a decision was made to establish an additional site 5b in this area of potentially high infection.

Benmore

Soil samples were collected every 2 metres along a transect between the worst infected site 1 and the *Magnolia kobus* at site 2. Random samples were also taken around the transect.

Samples were taken at site 4 where two infected *Osmanthus* spp. had been cleared. 3 samples were taken in a north, south, east and west direction at 2 metre intervals (one sample could not be taken as it fell on a concrete path hence 11 samples not 12).

When the soil samples at each site were collected, additional soil samples were also collected from previously infected parts of both gardens in order to establish how long these pathogens can survive in soil in the absence of a host.

Soil samples were recovered to the quarantine unit at SASA and homogenised using a planetary ball mill (such as the Retsch type PM500) using a JHI protocol described in appendix D. As with the other samples, Real-Time PCR was then used to quantify *Phytophthora* DNA in the sample (see appendix E).

2.5 PCR

Once the samples were processed using the extraction processes described above, Taqman Real-Time PCR, using a thermo-cycler (e.g. Stratagene MX3005 Pro), was used to quantify the levels of a target pathogen DNA sequence, therefore quantifying the presence of pathogens. This PCR method uses fluorophore-labelled DNA probes to measure the amount of amplified product in a sample in real time, giving results in cycle thresholds (Ct) values. The Ct value is the number of cycles needed to get a fluorescent signal that is significantly higher than background levels. The lower the number of cycles, the more copies of the target DNA sequence present in the sample.

In order to enable conversion of the Ct to a more useful measurement (e.g. the amount of DNA per sample in picograms), four standards of known concentration were included in each PCR run along with the samples. The Real-Time thermo-cycler software[1] can then give the amount of DNA per sample by extrapolating from the known amounts in the standards. Details of the reaction mixtures, primers and thermo-cycler settings can be found in appendix E.

[1] In this instance a Stratagene Mx3005p thermal cycler was used with MxPro v. 3.20, build 340, schema 74 (2006) Software

Chapter 3
Inoculum phenology and dispersal

3.1 Introduction

Whilst it is impossible to stop the aerial dispersal of pathogens, it is now possible to anticipate their movement using predictive modelling and pest risk mapping (Venette, *et al*., 2010). Such maps are useful visual tools which can inform management decisions by providing information on the potential arrival, establishment and spread of an alien pathogen. Such models have been used to predict the introduction and spread of many diseases including apple scab (*Venturia inaequalis*), Karnal bunt (*Tilletia indica*) and Asian soybean rust (*Phakopsora pachyrhizi*) (Aylor, 1998; Stansbury, *et al*., 2002; Pan, *et al*., 2006).

P. kernoviae and *P. ramorum* disperse aerially for short distances within rain splash and wind driven rain due to the formation of deciduous sporangia that are easily dislodged from infected tissue and spread onto surrounding vegetation (Denman, *et al*., 2008). It is the movement of these sporangia, and the zoospores that they contain and release, that rapidly drive epidemics within a suitable environment (Walker & Van West, 2007).

Risk maps predicting the potential spread of *P. ramorum* and *P. kernoviae* have been developed for a number of environments. This has particularly been the case with *P. ramorum* in the United States. Meentemeyer, *et al*. (2004), for example, developed a geographic information system (GIS) infection risk model that was rule based (i.e. a rule-based model uses research data and expert input, rather than statistical inference, to determine the importance of predictor variables) to predict *P. ramorum* spread based on host susceptibility and weather variables (precipitation, relative humidity, maximum temperature, and minimum temperature).

The quality of the data that is gathered and used for creating risk maps is an important consideration as there may be constraints to the available knowledge in, for example, the biology of the species under investigation or the environmental conditions in a

particular area (Venette, *et al*., 2010). Risk maps therefore reflect the current understanding of a pathogen invasion using the best available knowledge, but they do not necessarily reflect all of the factors that affect pathogen risk.

Before modelling techniques can be applied to better understand the potential for spread from an initial infection, the presence of the pathogen needs to be confirmed, usually prior to symptom development. In the case of *Phytophthora*, this can be achieved through use of spore traps. There are many designs of spore trap devices available with differing specifications depending on their application. Traps are required to be reliable, specific and accurate; the operators must be able to quickly and accurately identify the pathogens present (Jackson & Bayliss, 2011).

The spore trap design that has been developed for detecting *P. ramorum* and *P. kernoviae* in a number of field studies is the passive rain trap (Davidson, *et al*., 2005; Turner, 2007; Turner & Jennings, 2008; Valachovic, *et al*., 2008). This is often a rudimentary device which collects rain and water splash in a bottle via a funnel. The collected water can then be analysed for the presence and amount of inoculum collected during a given period. Susceptible *Rhododendron* plants are also used as water baits and sentry plants (e.g. Turner, *et al*., 2008a; Fichtner, *et al*., 2007a) in order to determine whether *Phytophthora* spores in the environment are viable.

Understanding the environment, and in particular the temperature thresholds at which these pathogens can survive, and the ideal moisture requirements for sporulation are very important aspects of their life history that need to be established before accurate modelling can start. The optimal temperature for *P. ramorum* is about 20^{o}C, although extent of growth observed at 15^{o}C and 25^{o}C is only slightly less than at 20^{o}C; with minimal growth at 30^{o}C and no growth at 35^{o}C (Rizzo, *et al*. 2002). The minimum temperature for growth is 3^{o}C, although it is thought that *P. ramorum* can withstand short periods of much lower temperatures (Tooley & Kyde, 2005).

The optimal temperature for growth of *P. kernoviae* is approximately 18^{o}C with an upper limit for growth of 26^{o}C (Brasier, *et al*., 2005). Water potential is perhaps the most significant factor influencing sporangia production in *Phytophthora* species with a relative humidity of 100% being highly conducive to sporangium formation (Ribeiro, 1983).

The current chapter outlines how inoculum levels at the study sites were measured in order that links with environmental factors in the west of Scotland could be explored. The viability of inoculum was established using bait plants, and watercourses were also tested for the presence of pathogens by using water baits. Results from this study, combined with the known biological and environmental conditions, were then used to conduct analysis to attempt to determine whether Scottish conditions in particular are conducive to higher inoculum levels. This would determine whether conditions in the west of Scotland favour these pathogens and also if there were any seasonal patterns in their establishment and spread.

3.2 Materials & Methods

P. ramorum and *P. kernoviae* inoculum was assayed in rain water collected in both low and high level rain traps as described in section 2.4.1 and the viability of the inoculum was established using bait plants (section 2.4.2).

The four sites at Benmore are shown in figure 10; detailed photographs of the sites can also be found in appendix H. At the beginning of this study, infected plants at sites 1, 3 & 4 had been removed. The infected *Magnolia kobus* at site 2 remained in situ so that inoculum levels under an infected tree could be measured and compared to levels in the other areas that did not contain hosts. Both low and high levels traps were deployed at site 2 to trap sporangia that were splashed off the leaves of the infected *M. kobus*. At sites 1, 3 & 4 only low level traps were used because there was very little overhead canopy at these sites.

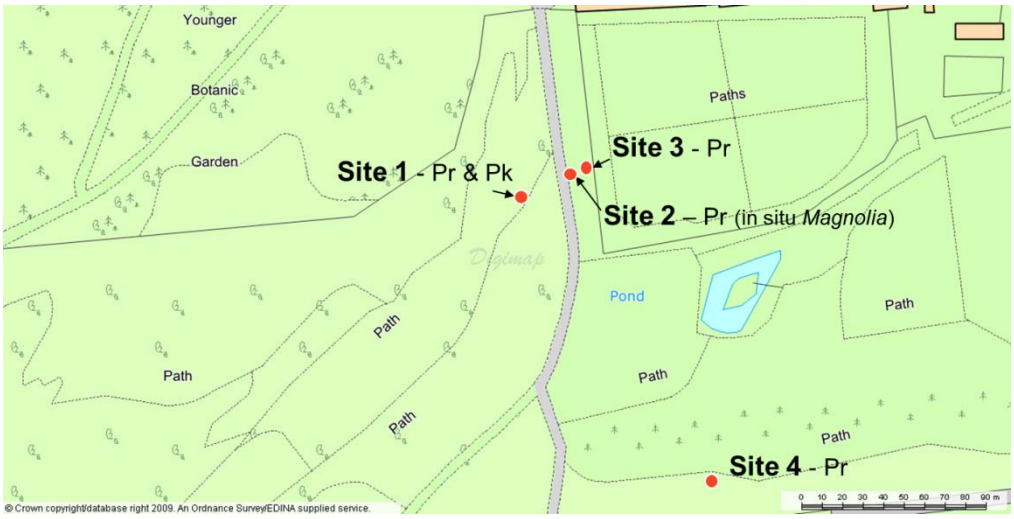


Figure 10: Map showing the study sites at Benmore. Base map - Edina digimap 2012

Due to the unexpectedly high amount of rainfall at both gardens the 2 litre bottles were not big enough to catch all of the rain in some of the months. The bottles were therefore changed half way through the month by the gardeners so that up to 4 litres of rain could be collected each month.

At Brodick (fig. 11; detailed photographs of the sites can also be found in appendix H) all of the known infected plants at the sites had been removed before the study started. Most of the sites contained both high and low level traps except for site 6 which was put in later (August 2010) in order to measure the inoculum released from the regrowth on a large previously infected and felled *Drimys winteri*. This regrowth was low down on the stump so just the low level trap was deployed. A low level trap was also placed at Merkland Wood to establish if inoculum was being released from infected *Vaccinium myrtillus* which grows close to the ground.

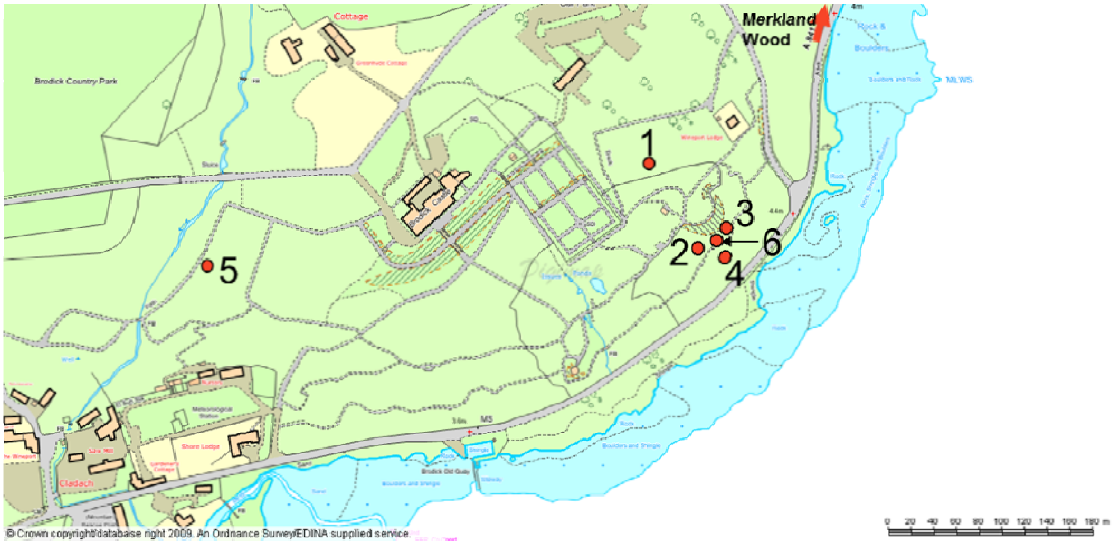


Figure 11: Map showing the study sites at Brodick. Base map - Edina digimap 2012

Initially site 5 was set up on an area where a single infected *Pieris japonica* had been removed but after exploration of the immediate area a more severely infected area was identified under a grove of mature *Pieris* about 6 metres from the original site 5 (Fig. 11). Leaves infected with *P. kernoviae* were found in the leaf litter directly under these *Pieris* so it was assumed that one or more of these trees were infected. The spore

traps were therefore moved in July 2010 to the new site 5b so that inoculum levels at this more infected site could be monitored.

Rhododendron ponticum and *Vaccinium myrtillus* bait plants were placed at each site every month and left in place for the duration of the month to test the viability of the inoculum in the environment as described in chapter 2.4.2. This allowed the link between inoculum dispersal and plant infection to be studied.

The presence of *P. ramorum* in watercourses at Benmore was established using a water baiting technique described in section 2.4.3. The baits were *Rhododendron* leaves in muslin bags and they were placed around the garden as shown in figure 12. Baiting for *P. kernoviae* at Brodick was attempted but was unsuccessful.

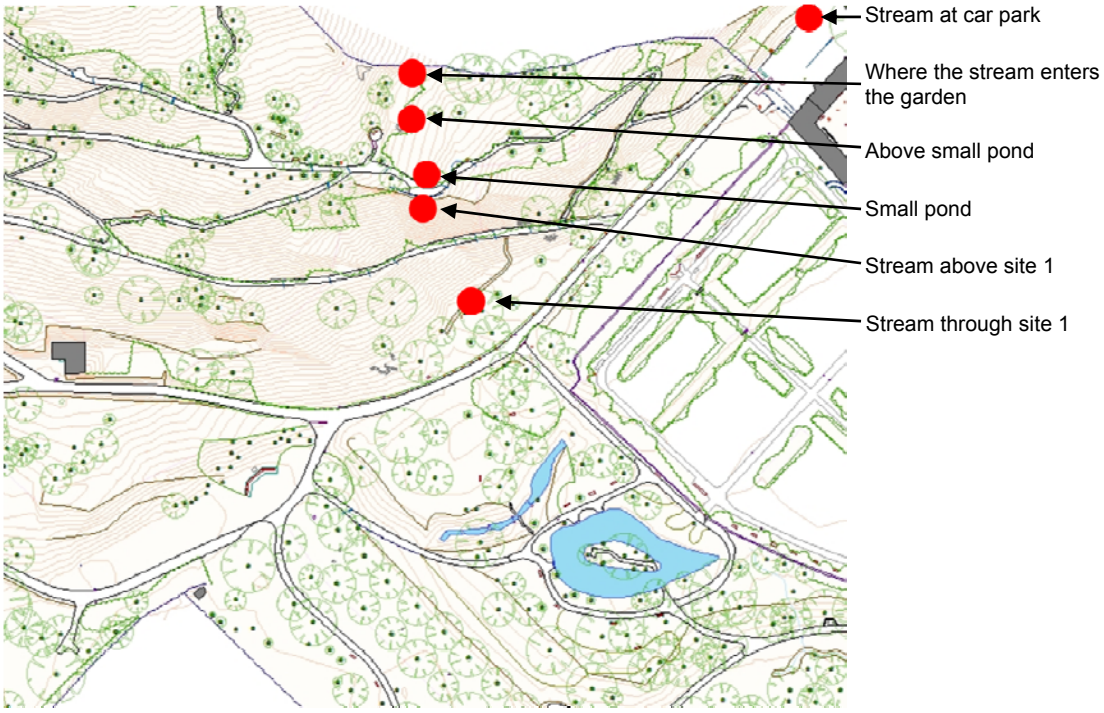


Figure 12: A map showing the watercourse baiting locations at Benmore. Created using ArcGIS 10

After initial baiting trials around Benmore were carried out, a small stream that enters above, then flows through, the main infection site was selected for regular monthly

baiting. This is shown in figure 12 from the point where the stream enters the garden down to the stream through site 1. A watercourse from a different source was also regularly tested and shown on the map as ‘stream at car park’.

3.2.1 Methods of analysis

3.2.1.1 Spore trap model

The links between the incidence of inoculum in the spore traps and the weather conditions were explored with a binomial generalised linear model (glm). Analyses were conducted using the R Project statistical software package (version 2.14.2; R Development Core Team 2011, Vienna, Austria) and the full code is in Appendix F(2). The weather data were supplied by the Met Office as they have weather stations at both gardens which collect rain and temperature data.

Inoculum was not found in sufficient amounts on enough occasions to allow the inoculum levels per month at each trap to be statistically modelled therefore it was decided that the response variable was the proportion of traps that contained *P. ramorum* on a given sampling event.

The explanatory variables included in model selection were monthly average and standard deviation of rainfall, relative humidity and temperature. The reliance on water and conducive temperature for *Phytophthora* sporulation is well understood (Ribeiro, 1983; Rizzo, *et al*. 2002; Tooley & Kyde, 2005; Brasier, *et al*., 2005). This led to the inclusion of a squared function for rain in the model and an interaction term between rain and temperature in order to explore these relationships in a way that is not simply additive. The *vif* function (Variance Inflation Factor) in R was used to check for collinearity between variables to insure that collinear variables were not included in the model. The full model is shown in appendix F(2).

The model was fitted using backwards stepwise selection, with the variable/factor with the highest p-value (determined by an analysis of variance) being dropped at each step. The best fitting model was then determined using AIC. A two sided runs test was used to check for autocorrelation and none was found ($p = 0.06671$).

3.2.1.2 Bait plant model

A generalised estimating equation (GEE) was used to model the relationship between bait plant infection and environmental conditions using the *geeglm* function in R. The response variable was binary; whether a bait plant became infected (1) or not (0) in a particular month. Full code in appendix F(3).

The explanatory variables included in model selection were the monthly average of relative humidity, the monthly average of relative humidity the previous month, standard deviation of rain, standard deviation of rain the previous month and the presence or absence of a host. The full model is shown in appendix F(3).

The model was fitted using backwards stepwise selection, with the variable/factor with the highest p-value (determined by an analysis of variance) being dropped at each step. The GEE approach was adopted because after fitting a poisson Generalise Linear Model (GLM) significant temporal autocorrelation was found in the model residuals (determined by “runs” test in R, p=5.082e-05). A correlogram was used to examine the extent of autocorrelation and it was found that the temporal autocorrelation declined to close to zero after 1 month, and therefore we included a 1 month blocking unit in the GEE.

3.3 Results

3.3.1 Benmore spore traps

Over the two years, 125 spore traps were emptied and tested from the 4 sites. *P. ramorum* was detected 33 times as shown in figure 13. Of these, 11 findings were at site 2 in both the high and low level traps under the *M. kobus*. Four of these findings were in the high level and seven in the low level. When inoculum was recorded in the high level traps it was also recorded in the low level traps in three of the four instances (April 2010, December 2010 & November 2011). The higher number of positive cases from the low level trap confirms that these traps catch inoculum from both soil splash and from the infected leaves of the tree. Also, proportionally more inoculum was collected in the low level trap (average 1.75pg/µl) compared to the high level trap (average 0.5pg/µl), in fact the high level trap on average contained the least amount of inoculum found at any site throughout the study.

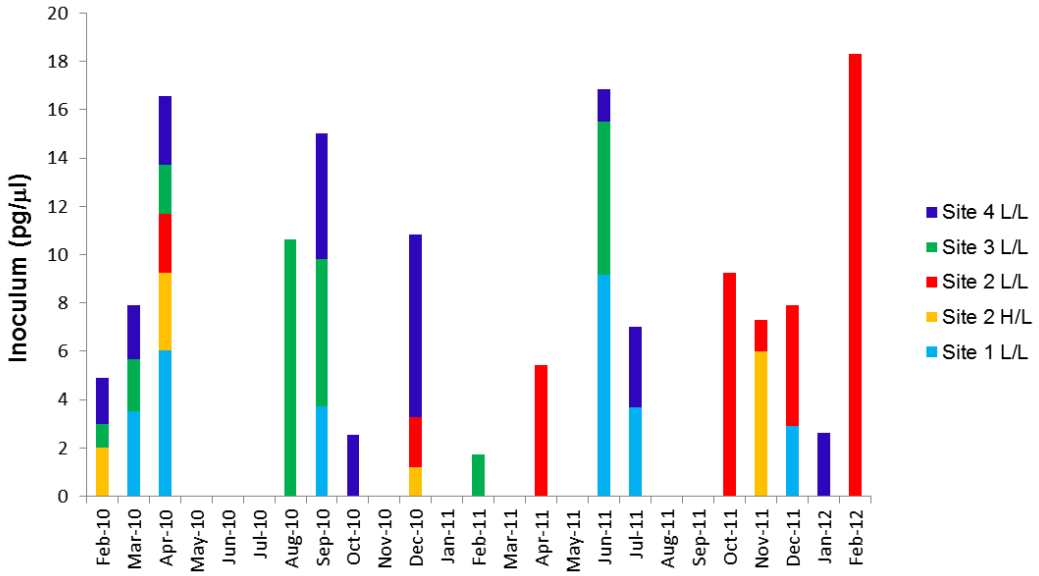


Figure 13: The incidence of *P. ramorum* in spore traps at Benmore

Inoculum was recorded in the other low level traps on nine occasions at site 4, seven at site 3, and six at site 1. These data show that *P. ramorum* is readily splashed from soil into the low level traps.

P. kernoviae was only recorded on four occasions in spore traps at Benmore (fig. 14).

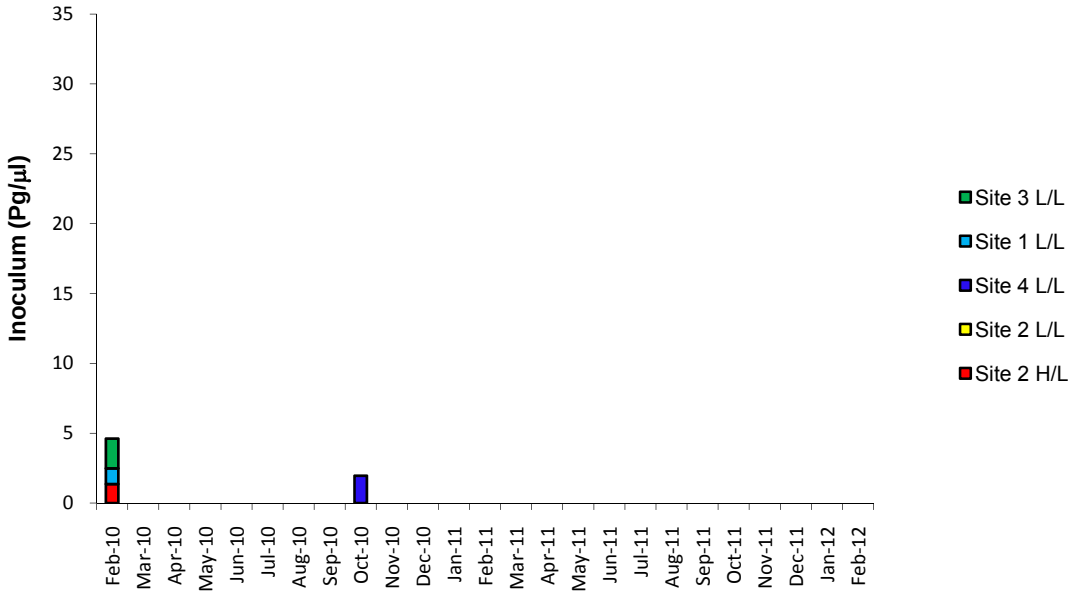


Figure 14: The incidence of *P. kernoviae* in spore traps at Benmore

These four incidences were at very low levels (between 1.12pg/µl and 2.13pg/µl) and occurred at four different spore trap locations.

3.3.2 Brodick spore traps

At Brodick *P. kernoviae* was recorded on 12 occasions at site 5; 9 of these were in the low level trap and 3 in the high (fig. 15). The only other findings were in March 2010 at site 3 and site 4.

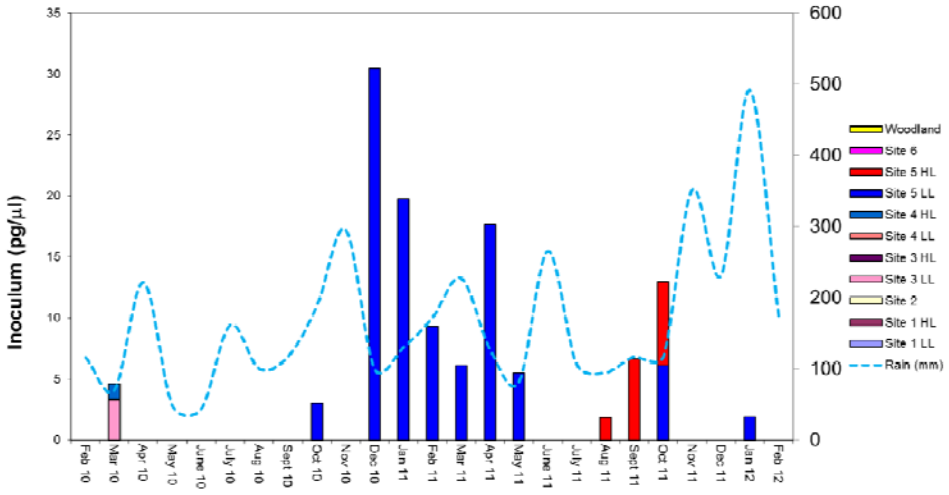


Figure 15: The incidence of *P. kernoviae* in spore traps at Brodick and total monthly rainfall (mm)

The high levels of inoculum recorded in the site 5 low level traps in December 2010 (30.4pg/µl) and January 2011 (19.7pg/µl) were preceded by a month of high rainfall in November 2010 (total of 296mm). The *Pieris* trees had formed a thick canopy at this site so the high level of rainfall could wash inoculum through the canopy into the trap although the high level traps did not record inoculum during this period so these findings were more likely due to water splashing off the soil and leaf litter around the low level trap.

The low level trap findings at site 5 in December 2010 and January 2011 also coincided with a cold period at the garden (e.g. average of 2.2^{o}C in January2011) whereas the three times that the high level traps contained inoculum at site 5 were during a warm period between August and October 2011 (e.g. average 13.9^{o}C in September 2011) (fig. 16).

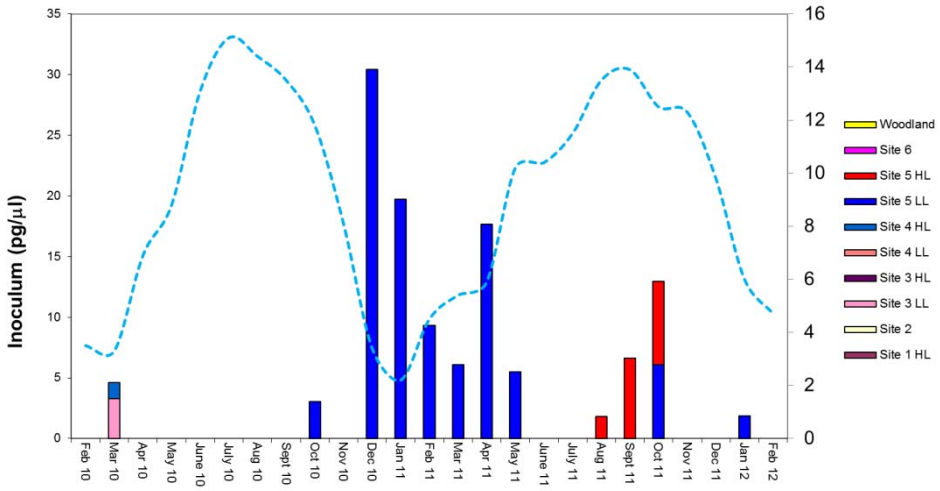


Figure 16: The incidence of *P. kernoviae* in spore traps at Brodick plotted with average monthly temperature (^{o}C)

3.3.3 Water baiting

Baits containing *Rhododendron* leaves were placed in streams and ponds at both gardens. A map of the bait sites can be seen in section 3.2. *P. kernoviae* was not recovered from either garden. *P. ramorum* was recovered from watercourses at Benmore as shown in figure 17.

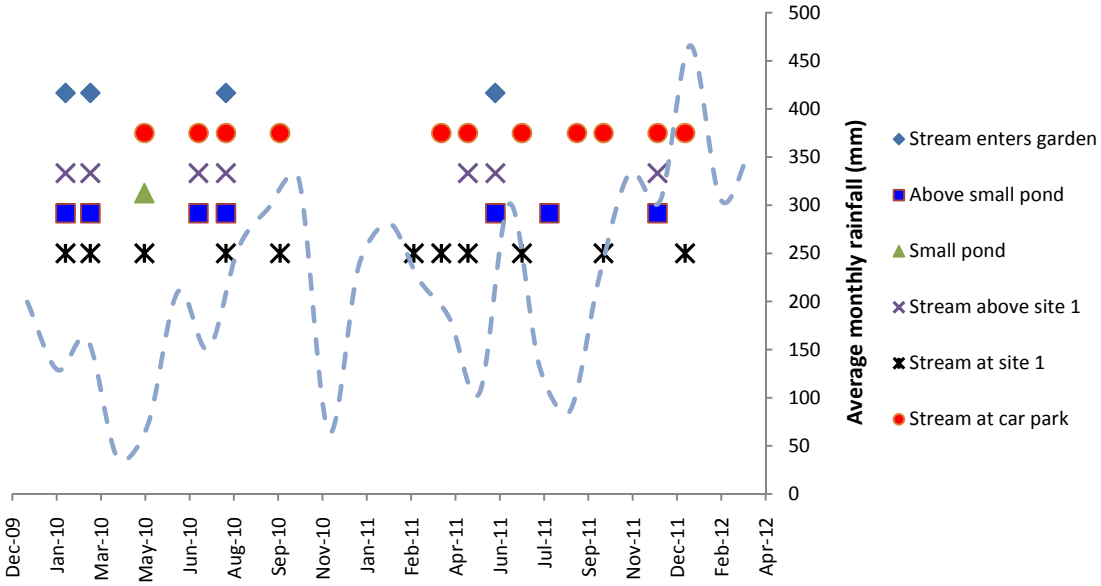


Figure 17: The incidence of *P. ramorum* in watercourses at Benmore established using *Rhododendron* baits and average monthly rainfall. The months where no results are shown are due to lack of water or frozen water in the watercourses.

The main focus of the baiting was along a stream which ran from where it entered the garden to a small pond and then down to site 1. The five baiting locations along this stream are shown in figure 17 as running from the light green block, through yellow, green and black, and finally to red at site 1. *P. ramorum* was isolated 4 times where the stream enters the garden, 7 times at the next point, once in the small pond, 7 times above site 1 and then 11 times at site 1 (figure 18).

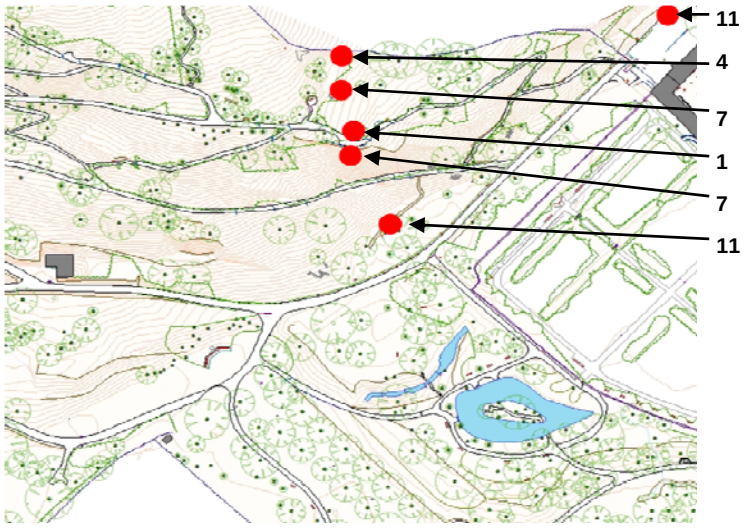


Figure 18: The number of times *P. ramorum* was isolated from watercourses at Benmore. Each dot is a location in the garden (see figure 12) and the numbers are the number of times each site yielded an infected bait

The other regularly tested watercourse was from a different source and is shown as the blue block in figure 17. This stream was near to the car park and horticultural sheds and *P. ramorum* was isolated 11 times from it (fig. 18). It originates from outside the garden at a former commercial forestry plantation above the site.

There is variation in findings from watercourses, but it does not appear to be seasonal. In August 2010 for example, *P. ramorum* was isolated from all of the baits but in August 2011 only one. The most important factor is whether there was enough rain to cause the streams to fill enough for baiting to take place. The period between November 2010 and February 2011 shows this, as a lack of results here was not due to baits not becoming infected but due to the very cold weather where the streams were frozen so baiting could not be done. If there was enough water in the streams for baiting, *P. ramorum* was usually found.

3.3.4 Linking inoculum levels with infection

One of the reasons for placing bait plants at the sites was to explore whether the presence of inoculum in the environment, established using the spore traps, is linked to infection in the surrounding plants. Do bait plants become infected more often under a known infected host plant and can bait plants become infected in the absence of infected hosts? These factors are important in understanding disease spread.

3.3.4.1 Benmore bait plants

Rhododendron sp., *Pieris japonica* and *Vaccinium myrtillus* bait plants were trialled during the study. For continuity, the *Rhododendron* plants were used throughout whereas the other species were tried as they became available. This means that, at times, three bait plants were placed at each site (one of each species) but at other times, particularly at the start of the study, there would have just been the *Rhododendron* plant. At Benmore the *Pieris* bait plants did not become infected with either pathogen during the study. The *Rhododendron* and *Vaccinium* became infected together when they were placed at the site and there was no advantage to using either, although the *Vaccinium* were smaller so easier to transport and store. The infection of bait plants at Benmore is shown in figure 19.

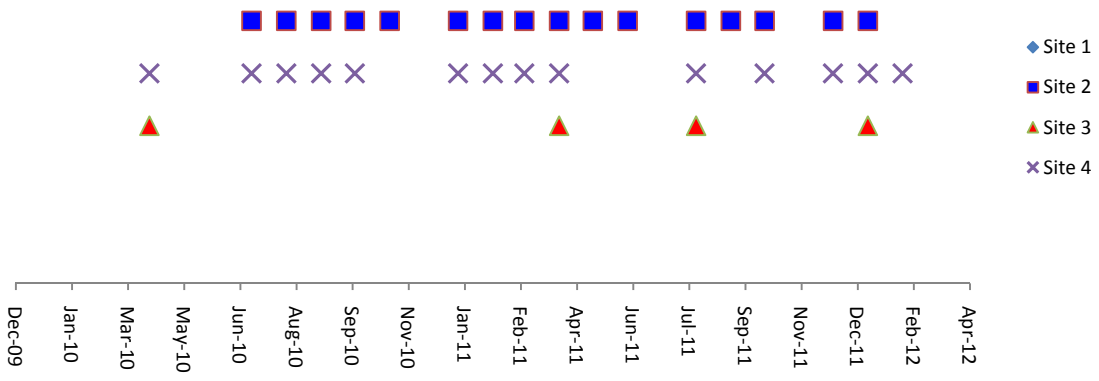


Figure 19: *P. ramorum* infected bait plants recovered from Benmore

Bait plants were found to be infected with *P. ramorum* on 16 occasions at site 2 under the *M. kobus* over the 2 years. This is not surprising because of the presence of an overhead sporulating host at this site. Site 4 bait plants, however, became infected almost as often (14 times) despite the removal of the host before the start of the study. This is also the case with the 4 infections at site 3. The bait plant infections at sites 3 and 4 have most likely resulted from inoculum splash from the surrounding infested soil although the presence of undetected infected plants in these areas cannot be ruled out.

The infection of bait plants informs us that the inoculum recorded in the spore traps at site 2 in April 2011, October 2011, December 2011 and February 2012 was viable because these were the months that the bait plants became infected (fig. 20). The month where a bait plant became infected when inoculum level was at its lowest was in December 2011 with 5pg/µl, this was in the low level trap. In the months where both inoculum was found and bait plants were infected, the incidence of infection was more prevalent when the low level traps contained inoculum (4 occasions) rather than the high level traps (0 occasions).

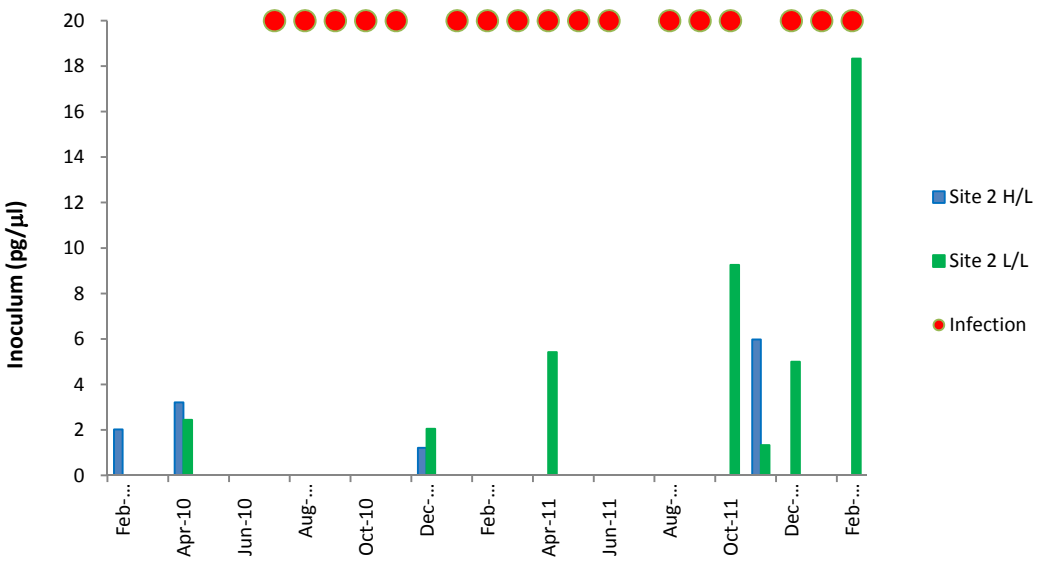


Figure 20: Months where the bait plants were infected with *P. ramorum* (red dots) at site 2 and the inoculum recorded in the spore traps

At site 4, there were four occasions when bait plants became infected and inoculum was recorded in the low level trap (April 2010, September 2010, October 2010 & January 2012) (fig. 21). There were far more bait plant infections than inoculum

detections in the trap with a total of ten instances of infection with no spore trap inoculum. The low level trap at site 3 only recorded inoculum with a bait plant infection once in April 2010. There were also only three more bait plant infections at site 3 which all occurred when inoculum was not recorded in the spore trap.

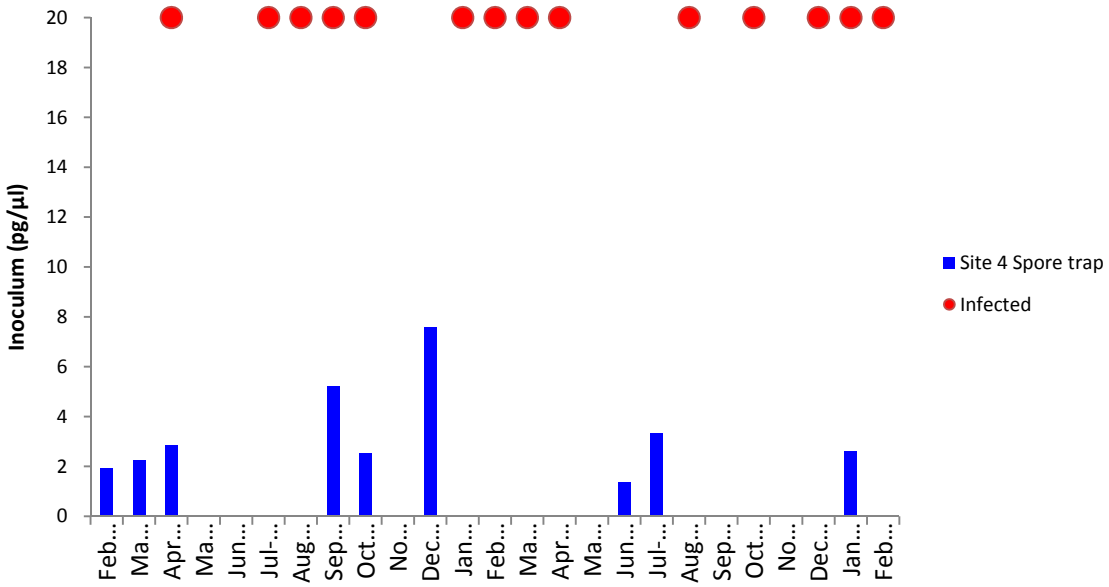


Figure 21: Months where the bait plants were infected with *P. ramorum* (red dots) at site 4 and the inoculum recorded in the spore traps

There were other environmental factors that may have led to bait plant infection which are explored in section 3.3.4.2. None of the bait plants at Benmore became infected with *P. kernoviae*.

3.3.4.2 Brodick bait plants

All three bait plant species were also tried at Brodick to detect *P. kernoviae*. Once again the *Pieris* did not become infected. The *Rhododendron* bait plants were also ineffective at Brodick, which was surprising because of the high prevalence of *P. kernoviae* infection of planted *Rhododendron* and *R. ponticum* at this garden. This could be because the bait plants were only exposed for one month whereas the *R. ponticum* in the garden may have been exposed for a number of years before succumbing to infection. It was not until a particularly susceptible batch of *Vaccinium myrtillus* plants were placed in the garden in January 2011 that infection started to be recorded. Infection is shown in figure 22.

Once the *Vaccinium* bait plants were placed at site 5 in January 2011 infection was recorded in 10 of the subsequent 12 months. The bait plants were often almost dead upon their recovery from site 5 because of the extent of their infection. Infection was also recorded on five occasions at site 4 which is at the bottom of the garden in the heart of the original infection although the symptoms on the bait plants here were more subtle.

Figure 22: *P. kernoviae* infected bait plants recovered from Brodick

Figure 23 shows that of the eleven instances of bait plant infection, inoculum was recorded in a spore trap (high and/or low) on six occasions. The lowest amount of *P. kernoviae* inoculum recorded whilst a bait plant was infected was 1.836 pg/µl in August 2011 in the low level trap.

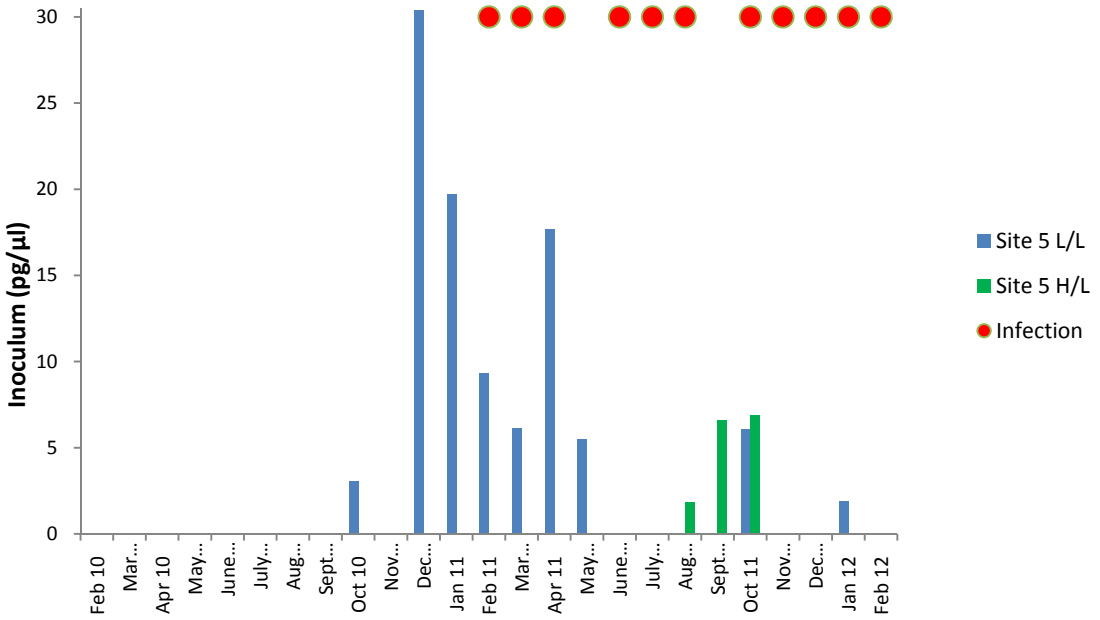


Figure 23: Months where the bait plants were infected with *P. kernoviae* (red dots) and the inoculum recorded in the spore traps at site 5 Brodick

3.3.5 Modelling

3.3.5.1 Spore traps

A generalised linear model was fitted using the *glm* function in R. The method for choosing the best fitting model for *P. ramorum* incidence in spore traps at Benmore is outlined in section 3.2.1.1 and the detailed code is shown in appendix F(2). Results from the best fitting model are shown in table 1, the overall deviance explained was 33% and the AIC was 78.129.

The average rain and temperature interaction term included in the model was significant (p=0.0016) (table 1). This term was included because it allows for the effects of temperature and rain to act simultaneously in a way that is not just simply additive. The coefficient estimate in this model was positive, therefore temperature and rain were interacting to augment each other. This means that for a given amount of rain, higher temperatures increase the effect of rain over and above the effect of rain on its own; and for a given temperature, higher amounts of rain increase the effect of temperature over and above the effect of temperature on its own. This means that a

spore trap is more likely to contain inoculum on a warm and wet day than a cool dry day.

Table 1: Results from the spore trap GLM

	Estimate	Std. Error	P value
(Intercept)	5.17412	1.87784	0.005863
Average temp	-0.34572	0.10266	0.000759
SD of relative humidity	-0.06211	0.02925	0.033710
Previous month avg RH	-0.02819	0.01412	0.045945
Average rain	-0.40775	0.12506	0.001112
Temp/rain interaction	0.04444	0.01407	0.001592

Standard deviation of relative humidity was also significant (p=0.03); the estimate was negative (table 1) which means that the lower the standard deviation, the more often inoculum was found in the spore traps, this suggests that a constant relative humidity significantly increases the probability of inoculum in the environment. The average relative humidity the previous sampling month was significant (p=0.04), although less so than the other variables, and it also had a negative estimate. This was unexpected, and at this time cannot be explained, as the effect of relative humidity would not be expected to be this long term.

3.3.5.2 Bait plants

A generalised estimating equation was fitted using the *geeglm* function in R. The method for choosing the best fitting model for *P. ramorum* infection of bait plants at Benmore is outlined in section 3.2.1.2 and the detailed code is shown in appendix F(3).

According to the best fitting model (table 2), the most significant variable determining whether a bait plant becomes infected in a given month is whether an infected host is present (p=0.000036). Increased variability of rain also increases the probability of infection as shown by the positive estimate of the standard deviation of rain variable (p=0.018). High relative humidity the previous month was not statistically significant but removing this variable during backwards stepwise selection worsened model fit so it remains in the model.

Table 2: Results from the bait plant GEEGLM

	Estimate	Std.err	P value
(Intercept)	-5.3048	1.5592	0.00067
Average RH previous month	0.0244	0.0160	0.12773
Host	2.3267	0.5634	0.000036
Standard deviation of rain	0.1957	0.0627	0.00180

There was not enough infection to produce sufficient data to model *P. kernoviae* infection of bait plants at Brodick or Benmore.

3.4 Discussion

The overall findings of inoculum in spore traps were relatively low with only 13% of traps recording the presence of inoculum. Spore traps have been used widely for detecting the presence of plant pathogens in other studies (Lievens & Thomma, 2007), including studies of *P. ramorum* in forests (Davidson, *et al*., 2005; Hansen, 2008, Turner, *et al*., 2008a); but it could be the case that the removal of garden hosts as soon as they become infected reduces the inoculum in the environment and therefore the spore traps.This appears to be particularly true for *P. kernoviae* at Brodick, because inoculum was only found under the sporulating host at site 5b; no inoculum was found in other spore traps despite them being placed in areas previously affected by this pathogen.

The low level traps at Benmore where there was no sporulating host present, but where *P. ramorum* inoculum was still recovered, show that inoculum was most likely splashed from soil during rain. This is further confirmed by the infection of bait plants under sporulating hosts, as seen at site 2 Benmore and site 5b Brodick, through soil splash as shown by more frequent infection of bait plants than inoculum findings in the spore traps. This argument is strengthened by the frequent infections of bait plants at sites where the sporulating host had been removed, however, it must be noted that *P. ramorum* could be sporulating from an undetected infected host nearby causing infection of bait plants. Infection of conifer seedlings and *Rhododendron* plants via soil splash have also been found to occur under infected Californian bay laurel in the US (Chastagner, *et al*., 2008).

The infection of bait plants by both pathogens during the winter was intriguing. This happened under the deciduous infected *M. kobus* at Benmore and the evergreen *Pieris* at Brodick. It also occurred where no host was present at Benmore site 4. These infections could therefore be occurring from sporulation from infected foliage (under the *Pieris*) and infected bark (under both the *M. kobus* and *Pieris*) and from infested soil splash (at all sites). If this experiment were to be repeated, plastic collars could be placed around the rim of the pots in order to prevent soil splash infection whilst still allowing aerial infection. The exploration of these infection processes in more detail could then be carried out to establish whether hosts can be infected from the bark of nearby infected deciduous trees in winter. The process of soil infection and inoculum spread is explored in more detail in the next chapter.

The findings of more inoculum in the low level trap than the high level trap under the infected *Magnolia* at Benmore was surprising given that inoculum is being released from the leaves of the tree around the high level trap. This could be because the lower trap contains more chlamydospores as, of the three *Phytophthora* spore types, chlamydospores are the most persistent in soil, sporangia are intermediate and zoospores the least persistent (Hwang & Ko, 1978). The high level trap is perhaps more likely to contain just sporangia and zoospores and this in some way may account for the differences found here.

The water baiting for *P. ramorum* was successful at Benmore but *P. kernoviae* was not picked up at Brodick through baiting despite the heavy infection in this garden. This is consistent with studies that have found that water baiting is less effective at detecting *P. kernoviae* than *P. ramorum* in watercourses (CSL, 2008; DEFRA, 2008a). It could be the case that leaves from other species of host plant would make better *P. kernoviae* water baits (DEFRA, 2009) but time did not allow for much experimentation in this area during the study.

The seasonal variations found with the *P. ramorum* water baiting at Benmore partially concurs with other work carried out in both the UK and California where the most bait infection occurred during the winter months with reduced detection during the summer (Turner, *et al*., 2007; Tjosvold, *et al*., 2002). This study found that there was inoculum in the streams at Benmore throughout the year; if there was running water, inoculum was recovered from watercourses.

The isolation of *P. ramorum* from baits at a place where the stream enters Benmore suggests that this could point to the infection being carried into the garden from outlying forests. The forested area above the garden was briefly explored but no infected plants were found and no baits were positive further up the stream. There was a large area of *R. ponticum* in the understory but far more experimental work would have to be carried out in order to determine whether infection was present.

The model describing bait plant infection by *P. ramorum* during periods of variable rainfall andhigh humidity and a host present supports current understanding of the biological conditions required for infection (Turner, *et al*., 2008; Tooley, *et al*., 2005). The high rainfall managers however these data reinforce the need for host removal in gardens around known infected plants which is achievable in gardens in order to reduce spread.

The average rain and temperature interaction term used in the spore trap model ($p=0.001$) leads to the conclusion that a spore trap is more likely to contain inoculum on a warm and wet day than a cool dry day. This finding concurs with current biological knowledge on *P. ramorum* sporulation conditions (Englander, *et al*., 2006; Davidson, *et al*., 2005).

The lack of data from the spore traps and other potential problematic effects, such as a dilution effect in the traps due to the high rainfall that occurs in the west of Scotland, mean that modelling these variables is complex. For example, if it were possible to empty the traps weekly the resulting data would allow for a more robust model. Also, if infected plants were left in situ and not immediately removed, the measurement of sporulation on them would improve our understanding of the sporulation process in Scotland. This said, the results shown here have given a number of insights into sporulation, particularly of *P. ramorum* in gardens, which should be followed up with more experimental work in the future to better understand this process.

Chapter 4
Survival of inoculum in the soil

4.1 Introduction

The ability of pathogens to survive when a host is not present or during periods that are unfavourable to host infection has important implications for disease management. The capability of *P. ramorum* and *P. kernoviae* to survive in soil prior to infecting a host is influenced by many factors but in particular soil moisture. These pathogens survive and remain viable for much longer periods in moist conditions (Davidson, *et al*., 2002). It is the chlamydospores of *P. ramorum* and the oospores of *P. kernoviae* that are able to survive for longer periods than the short-lived sporangia and zoospores. This allows them to serve as inoculum reservoirs by enabling long term survival in soil and potting media waiting for the ideal conditions for growth (Fichtner, *et al*., 2007a).

It has been reported that *P. ramorum* survives in infected leaf debris for at least two years (Turner, *et al*., 2005). Experiments at Science and Advice for Scottish Agriculture (SASA) have shown that it can survive in soil for at least 2.5 years under Scottish conditions (unpublished work, Alexandra Schlenzig, SASA). *P. kernoviae* has also been shown to survive for at least a year in soil (Widmer, 2011).

P. ramorum has been found to survive in wood and wood chips for some considerable time in the Netherlands. The pathogen was detected in wood chips for up to 2 years after a number of rhododendrons were cut down and chipped (Steeghs, 2008). In addition, *P. ramorum* was found on logs that had been air dried for six months in California and in the exposed wood of trees for two years in the UK (Shelly, *et al*., 2006; Brown & Brasier, 2007).

Widmer (2011) found that when UK isolates of *P. kernoviae* were held in sand for one year at 4, 10, 20 or 30^{o}C, survival was 86, 75, 82, and 78% respectively showing that *P. kernoviae* can survive for a year at a wide range of temperatures. Widmer (2011) also concluded that the ability of *P. kernoviae* to survive for long periods in soil and leaf litter is most likely linked to the ability to produce new sporangia and oospores.

It is important to understand the conditions required for survival and the periods of time that these pathogens can survive in soil because this aspect of the disease cycle has implications for human mediated spread over both short distances on footwear, machinery and tools, and long distance on car wheels and in the compost of transported plants.

This part of the project therefore aimed to assess the survival, viability and possible seasonal variation of inoculum in the soil at the two study sites as well as identifying management practices that may facilitate or reduce inoculum survival and spread.

4.2 Materials & methods

Soil samples were taken at three month intervals from selected areas of each garden as described in detail in chapter 2.3.2.4. DNA was extracted from a 2ml sub-sample of each soil sample (appendix D) and quantified using real-time PCR (appendix E) to give an amount of DNA in picograms per ml of sample (expressed as *x* pg/ml). DNA amount was taken as indicative of the number of pathogen organisms in the soil.

At the beginning of this study infected plants at sites 1, 3 & 4 at Benmore had been removed and therefore the level of inoculum in the soil in the absence of a host was measured. The infected *M. kobus* at site 2 remained in situ so that seasonal variation in inoculum levels could be assessed (fig. 24).

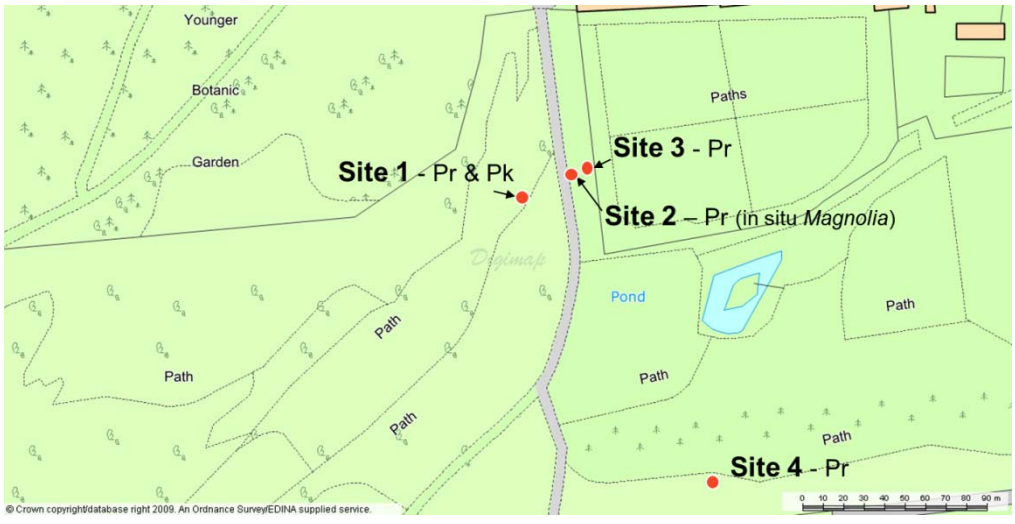


Figure 24: Map showing the study sites at Benmore

In addition to collecting soil samples from each site at Benmore, a sample was taken every 2 metres along a transect between sites 1 and 2. Also, samples at site 4 were taken every 1 metre in a north, south, east and west direction for 3 metres (centred on the spore trap) giving 12 samples per sampling event.

At Brodick, infected plants had been removed from all of the sites before the study started. Soil samples were taken from the study sites and a transect was set up between sites 3 and 4 in the worst affected area of the garden (see fig. 25). Initially site 5 was set up on an area where a single infected *Pieris* had been removed but after exploration a more severely infected area was identified under a grove of mature *Pieris* about 6 metres from the original site 5. Leaves infected with *P. kernoviae* were found in the leaf litter directly under these *Pieris* so it was assumed that one or more of these trees were infected. Both of these areas continued to be monitored and therefore the original site 5 was renamed 5a and the new site 5b.

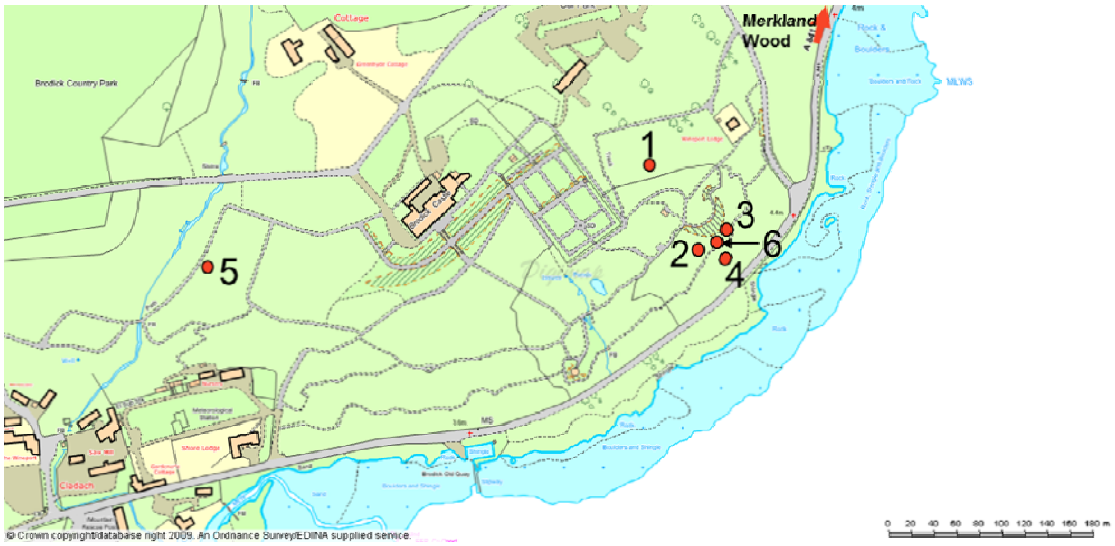


Figure 25: Map showing the study sites at Brodick

Bait plants were placed at each site every month (described in chapter 2.4.2) and left in place for the duration of the month to test the viability of the inoculum in the soil and also whether plant infection from soil splash was occurring.

4.3 Results

4.3.1 Benmore

P. ramorum remained in the soil after the removal of the host plants at sites 1, 3 & 4 at Benmore for 2 years (fig: 26). There was some evidence of seasonal variation in inoculum levels under the infected *M. kobus* at site 2 with peaks of 11205pg/ml in August 2010 and 15805pg/ml in August 2011 (fig: 26), which coincides with low levels of rain. This variation was not reflected in site 3, which is only a short distance away (~10m), where the average levels remained relatively low (average 112pg/ml).

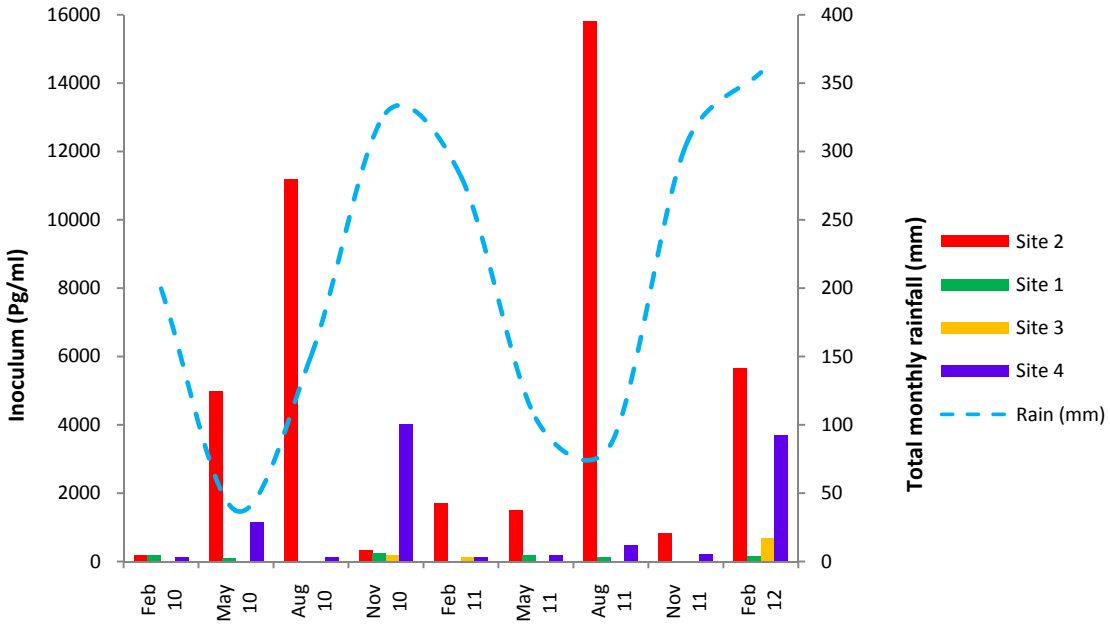


Figure 26: *P. ramorum* inoculum levels in soil at Benmore (pg/ml) and total rain per month (mm) over the 2 year study period.

As multiple samples were taken at site 4 during each sampling event the average inoculum level for this site is shown in Figure 26. The peak of 4010.5pg/ml at site 4 in November 2010 and 3706pg/ml in February 2012 coincide with particularly wet months at the garden. This site is in a depression in the ground where the soil was often found to be saturated.

It may be expected that of all of the sites where the host had been removed, site 3 would contain the highest levels of inoculum (average 112pg/ml) because it is only c10

metres from the heavily infested site 2 but much higher levels were actually recorded at site 4 (average 1129pg/ml), the furthest site from the infected *M. kobus*. Site 4 soil sampling is discussed in more detail in section 4.3.1.2.

The overall level of *P. kernoviae* inoculum in the soil at Benmore was low. It was only consistently found throughout the study period in the soil at site 1 with an average of 136.7pg/ml. The inoculum level also depleted from 559pg/ml in May 2010 to 74pg/ml in Feb 2012 (fig. 27). This site is where *P. kernoviae* infected *Rhododendron* plants had been removed in 2009.

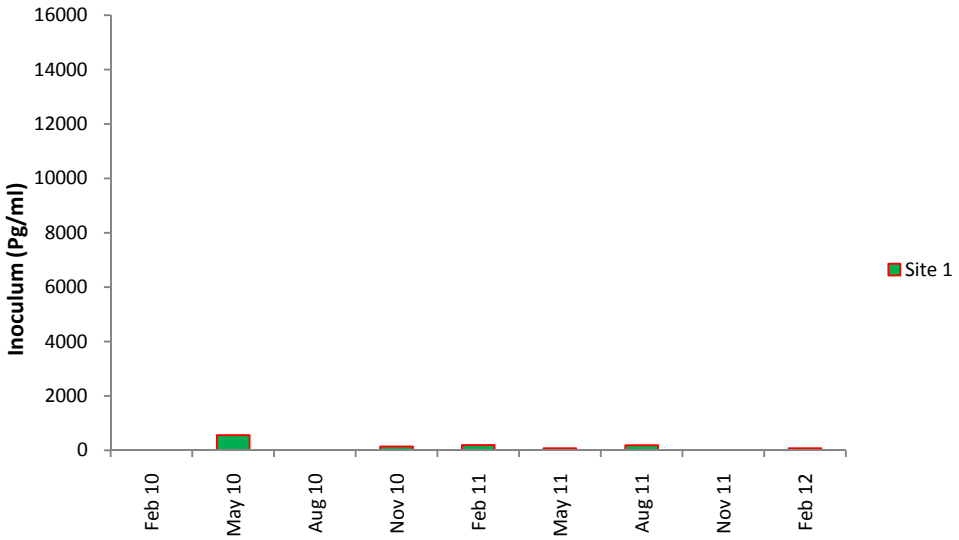


Figure 27: *P. kernoviae* inoculum level in soil (pg/ml) at site 1 Benmore.

4.3.1.1 Benmore transect

A transect was sampled between two infected sites at Benmore down a steep slope from site 1 to site 2. It was expected that high inoculum levels would be found at each end of the transect, and that some inoculum would have washed down the slope from site 1. The soil under the infected *M. kobus* was indeed heavily infested with an average inoculum level of 4692pg/ml (table 3, over page). The inoculum in the soil at site 2 also increased markedly over the 2 years from 200pg/ml to 5645pg/ml. Infested soil was detected at various points along the transect throughout the study period (table 3) and a number of *Rhododendron* shrubs became infected and were removed. Of the 153 samples taken along the transect during the 2 years 55 contained *P. ramorum* and 11 contained *P. kernoviae* inoculum.

Eight of the 11 positive *P. kernoviae* results were at the site 1 end of the transect, which is where infected plants were removed in 2009. There were also three occasions where *P. kernoviae* inoculum was recorded downhill from site 1; this inoculum could have been transported down the hill from site 1 in rain water.

Table 3: The transect at Benmore. Site 1 was at the top of the hill then each point was 2m down the hill to site 2. Each black value is *P. ramorum* and blue is *P. kernoviae* (given in pg/ml), empty boxes are negative samples.

	Site 1	1->2-1	1->2-2	1->2-3	1->2-4	1->2-5	1->2-6	1->2-7	1->2-8	1->2-9	1->2-10	1->2-11	1->2-12	1->2-13	1->2-14	1->2-15	Site 2
Feb-10	99	73	-	60	-	-	-	59.5	-	-	-	76	-	-	62	-	218
May-10	99 990	51.5 51.5	5230	-	-	-	149 140	126 80	-	56	144.5	-	-	-	117.5	98.5	6035
Aug-10	-	306.5	-	-	-	-	-	-	37.5	-	274.5	-	109	-	-	-	11205
Nov-10	143.5	-	-	-	203.5	252 12.5	-	-	-	170	-	-	-	695	137	250.5	3220
Feb-11	191	-	-	-	-	-	-	-	-	-	-	-	-	81.5	228	170	1718
May-11	179 73	-	-	-	-	-	-	-	-	-	-	-	65	-	52.5	172.5	5680
Aug-11	120 190	106.5 206.5	-	-	-	-	-	-	248.5	114	-	-	-	-	-	-	6740
Nov-11	-	-	-	-	-	-	143.5	-	117	-	-	-	1140.5	928.5	-	500.5	836
Feb-12	155.5 24.35	-	-	-	539	-	-	-	-	-	2356	-	215.5	-	-	401	5645

4.3.1.2 Site 4 soil sampling

Two *P. ramorum* infected *Osmanthus* plants were removed from this site in June 2009 and a further ailing *Osmanthus* was removed in March 2011 although it had not been confirmed as infected. *P. ramorum* infested soil was found throughout the site over the 2 years (fig. 28).

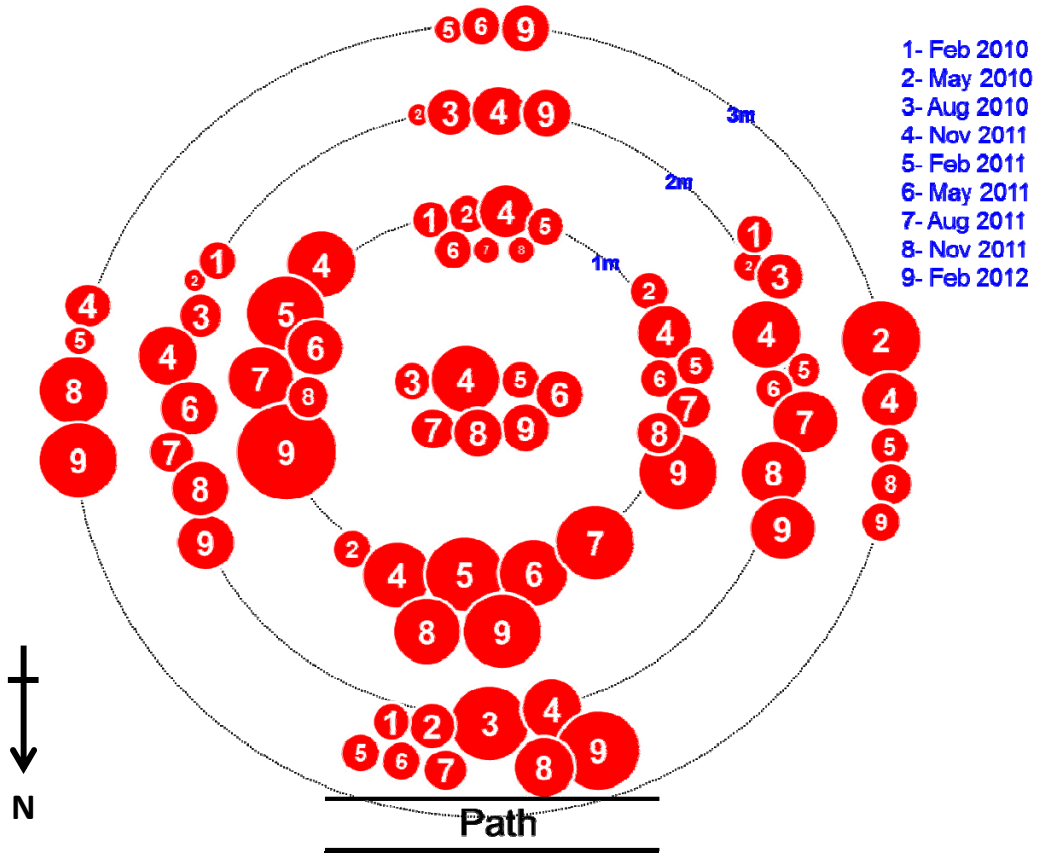


Figure 28: Findings of *P. ramorum* in soil at site 4, Benmore. A sample was taken from the centre then every 1m in a north, south, east and west direction for 3m. Each red circle represents a positive sample and the number within circle represents the month the sample was obtained. The size of the circle is relative to the amount of inoculum found in that sample.

Site 4 is in a depression which remained wet for long periods of time. The most inoculum recorded was in the samples in a northerly direction towards the path (average of all samples of 1182.5pg/ml); the soil in this direction was particularly saturated most of the year. The next most infected area was to the east (average for all samples 655pg/ml) and then to the west (average for all samples 587pg/ml), which is where the ailing *Osmanthus* was removed in March 2011. The least amount of inoculum was recorded to the south (average for all samples 114pg/ml), this area rises

slightly from the depression and is under a large *Abies* which means the soil is considerably drier.

4.3.1.3 Findings from random soil sampling

In October 2010 very high levels of *P. ramorum* inoculum (8540pg/ml) were found near to a pond despite there being no confirmed cases of plant infections in this area (see fig. 29). This sample was followed up with 6 more samples in November 2010 to check if there was an error with the initial sampling or processing. All 6 of these samples were positive, with the highest level among these samples at 4670pg/ml. Once it had been established that the area was infested the garden managers were able to confirm that the area had been redesigned the previous year and that they had added their own compost to the soil in order to improve it. The compost heaps in the garden were subsequently tested in the following three months and *P. ramorum* was found to be present at an average level of 238pg/ml. These compost findings were followed up by testing the wood chip piles at Benmore which would be used for mulching the beds. In February 2011 787pg/ml of *P. ramorum* inoculum was detected in the wood chips and 406.5pg/ml in April 2011.

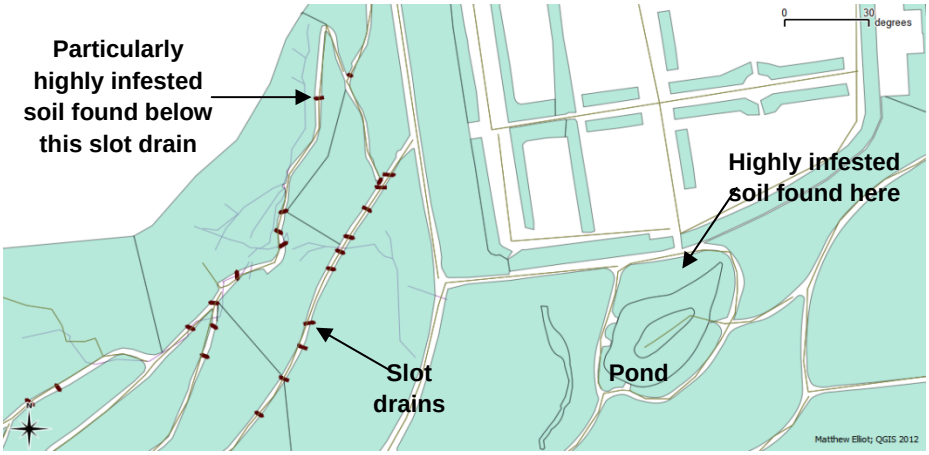


Figure 29: Areas at Benmore where *P. ramorum* inoculum was found to be high after random sampling events.

On a number of occasions the downhill sides of wooden slot drains were tested and found to contain high levels of *P. ramorum* inoculum, particularly the slot drain below the first confirmed case in the garden (highlighted in fig. 29). This particular drain was

first tested in November 2010 and a high inoculum level of 3975pg/ml was found, testing then continued over the next year revealing that the area was heavily infested with average inoculum levels ranging from 3488pg/ml to 6305.5pg/ml. Furthermore, the highest inoculum level found during this whole study (19760pg/ml) was found at this site in February 2012. This is higher than any of the samples taken from under the infected *M. kobus* (max. 11205pg/ml). These drainage channels across the paths increase the amount of water on the downhill side of paths which in turn appears to concentrate the inoculum.

4.3.2 Brodick

P. kernoviae remained in the soil at infected sites at Brodick throughout the two-year study period (fig 30). Inoculum levels at site 5b were particularly high with an initial average of 2347.5pg/ml in May 2010, a maximum of 4380pg/ml in November 2011 and an average over the 2 years of 2579.5pg/ml. These findings combined with symptomatic leaves on the ground led to the conclusion that one or more of the *Pieris* at this site were infected.

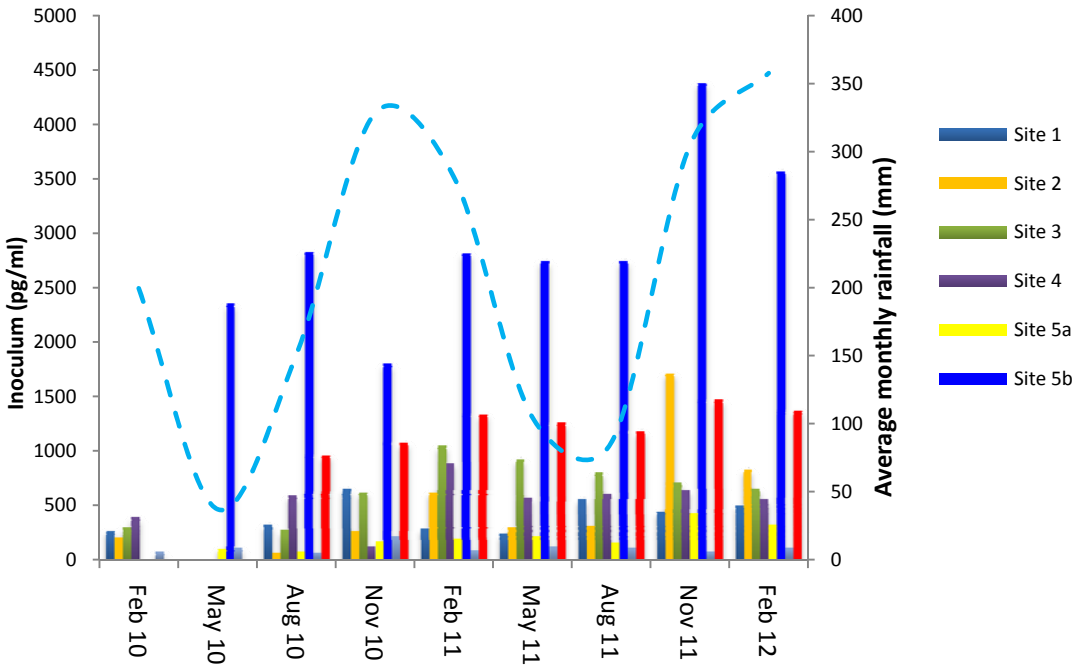


Figure 30: *P. kernoviae* inoculum levels (pg/ml) at Brodick throughout the 2 year study period

Site 6 was added in August 2010 to measure inoculum around the stump of a large infected *Drimys* that had been removed before the study started. This is in the heart of the worst affected area and the soil inoculum levels here remained high with an average of 958pg/ml at site 6, 480.7pg/ml at site 4 and 586.5pg/ml at site 3.

The soil at the *V. myrtillus* infected site in Merkland Wood showed low levels of infection over the two years with an average of 102.4pg/ml. In addition, only small patches of soil were found to contain inoculum as opposed to parts of Brodick garden where whole areas were infested, around site 6 for example.

4.3.2.1 Transect at Brodick

The transect ran between sites 3 and 4 in the heart of the worst affected part of the garden which formerly contained a large area of *R. ponticum,* the large infected *Drimys* and a number of other *Rhododendron* cultivars. Table 4 shows that *P. kernoviae* was found throughout the area between these study sites.

Table 4: The transect at Brodick. Each value is the amount of inoculum in the sample (pg/ml) from along the transect taken at 2m intervals. Empty cells were negative samples.

	Site 4	4 -> 3-1	4 -> 3-2	4 -> 3-3	4 -> 3-4	4 -> 3-5	4 -> 3-6	4 -> 3-7	4 -> 3-8	4 -> 3-9	4 -> 3-10	Site 3
Feb-10	382.5	-	-	381	251	2315.5	422	1861.5	-	-	349	293
May-10	-	-	-	-	-	-	-	-	-	-	-	-
Aug-10	582	107.5	1185.5	110.3	260	266.5	184	116.5	-	740	625	266.5
Nov-10	114	-	233	485	-	945	435	995	1275	-	-	615
Feb-11	885	374	475	62	808	896	438.5	1292.5	401	1525.5	2122.5	1042.5
May-11	567.5	185	1071.5	101.5	711	602	1646.5	1689	1218.5	1676.5	1313	916
Aug-11	603	-	217	237	973	839.5	-	265.5	1264	1729.5	3133	796
Nov-11	637.5	220.5	-	-	994.5	593.5	1109	1564.5	1033.5	2455	1241.5	705.5
Feb-12	555	437	-	816.5	242	478	267	456	330	2061.5	1484	644.5

The highest levels of inoculum were found at the site 3 end of the transect with an average level of 1140pg/ml at point 4 -> 3-10. The half of the transect between point 4->3-6 and site 3 was more infected (average 814.8pg/ml) than the other half towards site 4 (average 411pg/ml). This could be due to the presence of the *Drimys* stump in the more infected half of the transect because this tree would have released high amounts of inoculum into the soil immediately surrounding it whilst it was infected and before it was removed. There was some infected regrowth from the *Drimys* stump during the study. The less infected half of the transect was sparsely planted with just a partially cleared clump of *R. ponticum* at site 4.

Average inoculum over the whole transect in February 2010 was 521pg/ml and in February 2012 it was 647pg/ml with some variation within the study period. No samples were collected in this area in May 2010 due to adverse weather conditions.

4.3.2.2 Findings from random soil sampling

P. kernoviae was detected throughout the lower part of the garden at Brodick (shaded area in fig. 31) with 169 of the 211 samples collected containing inoculum. Most of the infections within this area were on planted rhododendron species and cultivars or *R. ponticum* except for site 6 which is where the infected *Drimys* once stood.

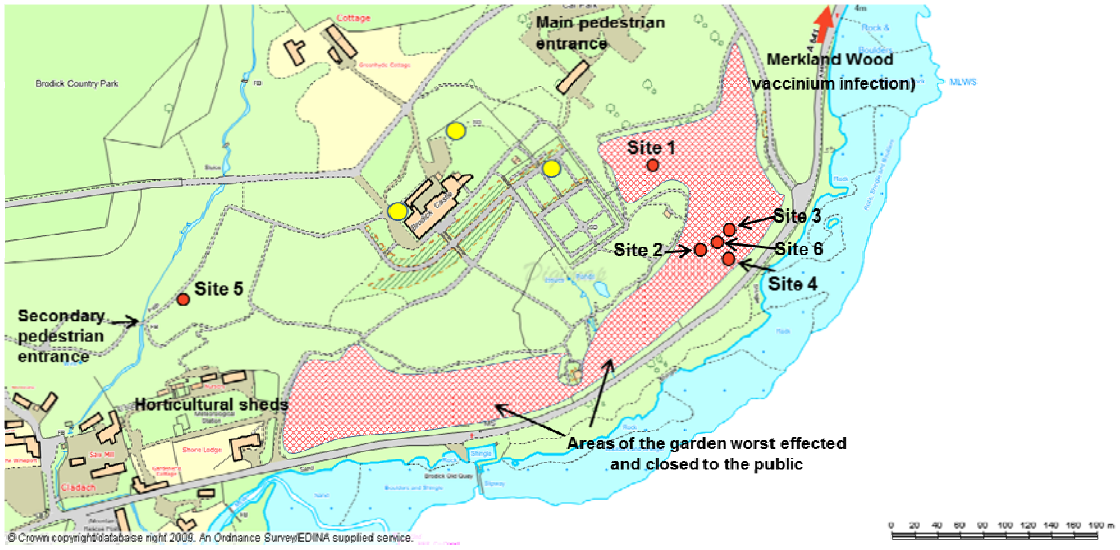


Figure 31: Map of Brodick garden showing the areas of infection. Yellow dots are areas where infested soil was found as a result of random sampling

Infested soil was also found in moderately high concentrations (510pg/ml) in higher areas of the garden that contained no previously infected plants (yellow dots in fig. 31). It is assumed that *P. kernoviae* was introduced into these areas by horticultural activity.

At Merkland Wood random sampling uncovered no new infested areas of soil and the original patches of known infestation remained limited.

In February 2011 *P. ramorum* was detected for the first time in soil samples collected from site 5 at Brodick. Initial samples contained 254.5pg/ml but follow-up sampling over the next 12 months found that *P. ramorum* inoculum levels were actually much higher, an average of 1248pg/ml was recorded in November 2011 for example. In December of the same year the first confirmed *P. ramorum* infected plants were found by plant health inspectors at Brodick near to both the main entrance and secondary pedestrian entrance (fig. 31).

4.3.3 Soil inoculum levels and bait plant infection

Rhododendron sp., *Pieris japonica* and *Vaccinium myrtillus* bait plants were trialled during the study. For continuity, the *Rhododendron* plants were used throughout whereas the other species were tried as they became available. This means that at times 3 bait plants were placed at each site (one of each species) but at other times, particularly at the start of the study, there would have just been the *Rhododendron* plant.

As described in chapter 3, section 3.3.4, there were links between inoculum findings in spore traps under sporulating hosts and bait plant infections. The incidence of bait plant infection where there were no sporulating hosts present provides evidence for bait plant infection via infested soil splashing onto the lower leaves of the bait plants allowing the initiation of infection.

The amount of inoculum required to be present in the soil for bait plant infection via soil splash was explored qualitatively. *P. ramorum* bait plant infection at site 2 Benmore, where the host is present, and the inoculum levels are shown in figure 32 and table 5.

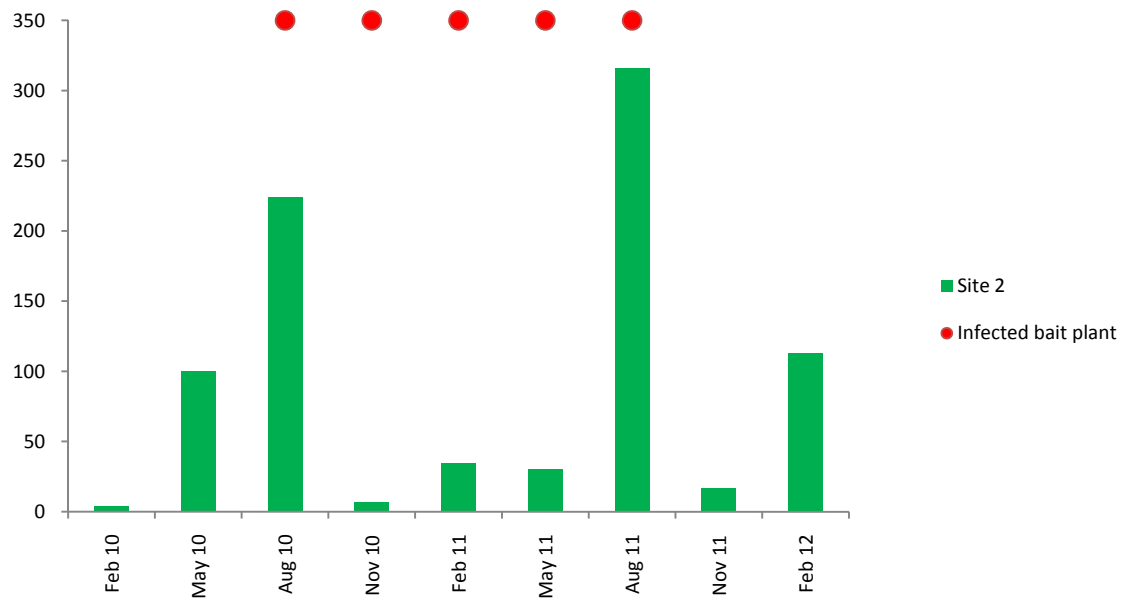


Figure 32*: P. ramorum* soil inoculum levels (soil samples taken as bait plants are recovered) and the incidence of bait plant infection at site 2 Benmore.

Table 5: *P. ramorum* soil and spore trap inoculum levels (H/L is high level trap and L/L is low level) and the incidence of bait plant infection at site 2 Benmore.

Date	Bait plant infected	Soil inoculum level(pg/ml) upon bait plant recovery	Spore trap inoculum findings (pg/µl)	Total monthly rainfall (mm)
Feb-10	N	4.01	2.02 (H/L trap)	199.5
May-10	N	100	0	37.1
Aug-10	Y	224.1	0	150.4
Nov-10	Y	6.5	0	328.2
Feb-11	Y	34.36	0	281.6
May-11	Y	30.02	0	104.6
Aug-11	Y	316.1	0	85.9
Nov-11	N	16.72	5.98 (L/L), 1.33 (L/L)	304.5
Feb-12	N	112.9	18.33 (L/L)	357.7

High soil inoculum levels in both August 2010 and August 2011(224.1 & 316.1 pg/ml respectively) resulted in bait plant infection but bait plants have also become frequently infected when soil inoculum levels were lower, November 2010 for example (6.5 pg/ml). Bait plants also became infected throughout the winter when the leaves of this infected deciduous tree were not present, however inoculum was recorded in the high and low level spore traps in winter (February 2010, November 2011 and February 2012). Therefore inoculum is either still being released from the bark into the high level trap or being splashed into the low level trap from the soil.

Resistance to bait plant infection during periods of relatively high soil inoculum was shown in May 2010 and February 2012 and whilst this could be explained by dry conditions in May 2010, this was not the case in February 2012.

Site 4 Benmore definitively shows that bait plants become infected from soil splash where the host was absent as shown in figure 33 and table 6. The host at site 4, *Osmanthus*, was removed in 2009 yet bait plants continued to be infected into 2012.

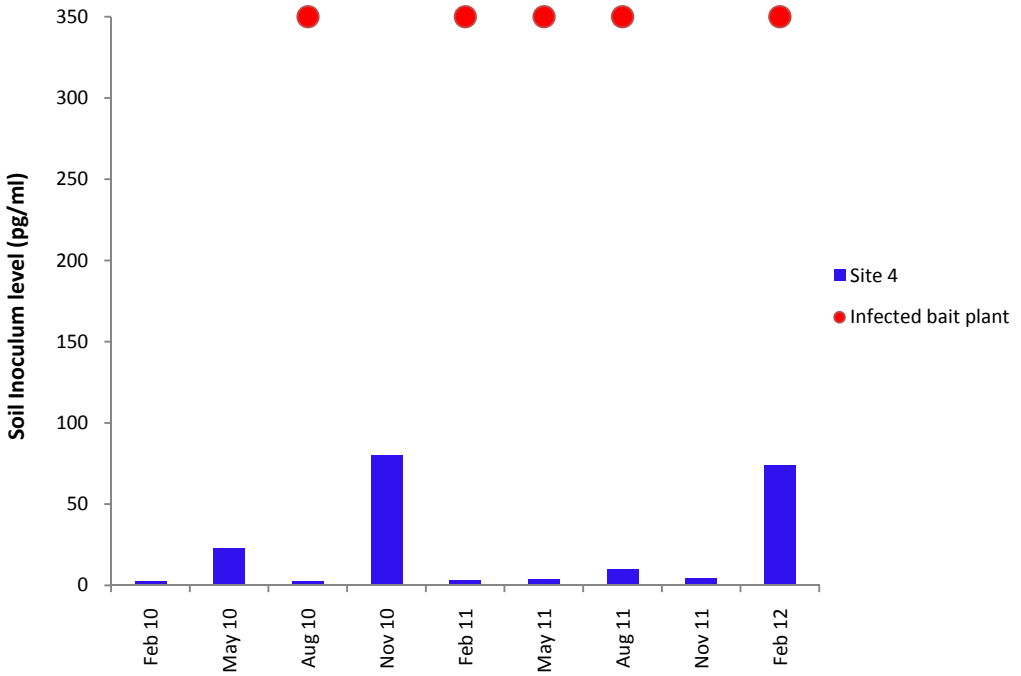


Figure 33: *P. ramorum* soil inoculum levels and the incidence of bait plant infection at site 4 Benmore.

Table 6: *P. ramorum* soil and spore trap inoculum levels and the incidence of bait plant infection at site 4 Benmore.

Date	Bait plant infected	Soil inoculum level (pg/ml) upon bait plant recovery	Spore trap inoculum findings (pg/µl)	Total monthly rainfall (mm)
Feb-10	N	2.3	1.92	199.5
May-10	N	23.2	0	37.1
Aug-10	Y	2.6	0	150.4
Nov-10	N	80.21	0	328.2
Feb-11	Y	2.9	0	281.6
May-11	Y	3.76	0	104.6
Aug-11	Y	9.84	0	85.9
Nov-11	N	4.36	0	304.5
Feb-12	Y	74.12	0	357.7

It is clear from these data that bait plants can become infected with *P. ramorum* from very low soil inoculum levels as shown in August 2010 (2.6 pg/ml) and also when spore trap inoculum was low and where an infected host is not present. Bait plants have also not become infected during periods where inoculum was high such as November 2010 (80.21 pg/ml) but this could be due to low levels of rainfall.

The incidence of *P. kernoviae* bait plant infection at Brodick does appear to increase as inoculum levels increase at site 5b (figure 34) however this effect could be due to the use of a new batch of *Vaccinium* bait plants from February 2011 that proved to be particularly susceptible to infection.

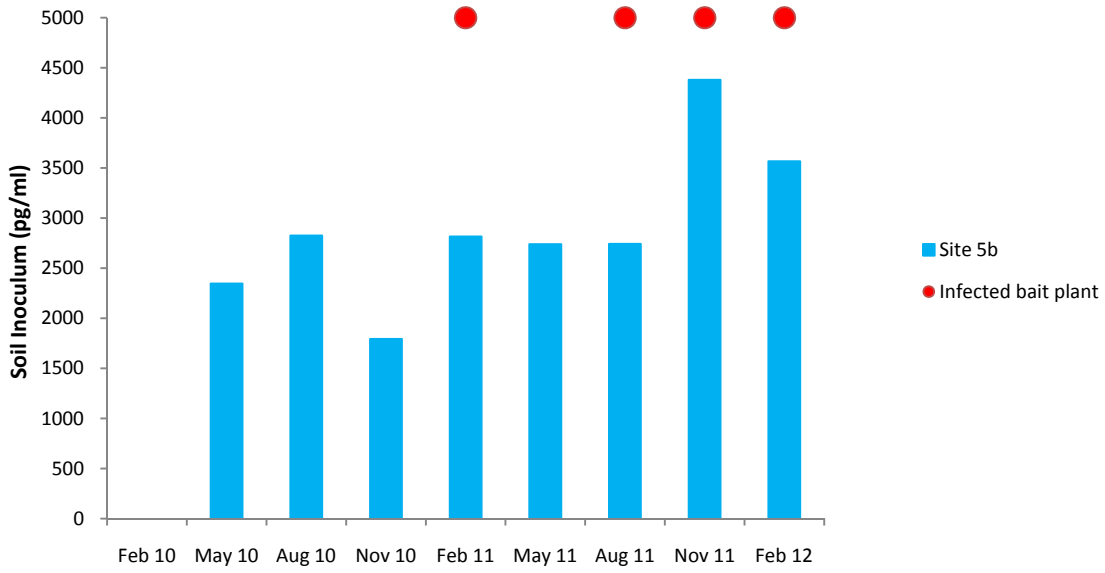


Figure 34: *P. kernoviae* soil inoculum levels and the incidence of bait plant infection at site 5b Brodick.

Table 7 shows that inoculum was also found in the high level spore trap in August 2011 at site 5b so bait plant infection may have occurred throughout the year from the infected leaves of the evergreen *Pieris* at this site. Bait plants were infected during a period of relatively low rainfall in August 2011 (85.9mm) and periods of high rainfall, for example in February 2012 (357.7mm).

Table 7: *P. kernoviae* soil and spore trap inoculum levels (H/L is high level trap and L/L is low level) and the incidence of bait plant infection at site 5b Brodick.

Date	Bait plant infected	Soil inoculum level (pg/ml)	Spore trap inoculum findings (pg/µl)	Total monthly rainfall (mm)
Feb-10	N	0	0	199.5
May-10	N	2347.5	0	37.1
Aug-10	N	2826.5	0	150.4
Nov-10	N	1795	0	328.2
Feb-11	Y	2816.5	466.8 (L/L)	281.6
May-11	N	2740.5	275 (L/L)	104.6
Aug-11	Y	2743	91.8 (H/L)	85.9
Nov-11	Y	4380	0	304.5
Feb-12	Y	3566.5	0	357.7

It appears that lower levels of *P. ramorum* inoculum in soil were required to infect bait plants at site 2 Benmore (e.g. 6.5 pg/ml in November 2010) compared to the levels of *P. kernoviae*required to infect bait plants at site 5b Brodick (e.g. 2743 pg/ml in August 2011), although this was not tested statistically. Furthermore, *P. kernoviae* bait plant infection only occurred under infected evergreen hosts whereas *P. ramorum* infection occurred where there were no known infected hosts in the area (site 4 Benmore for example).

4.4 Discussion

The results of this investigation show that both *P. ramorum* and *P. kernoviae* persist in soil in the west of Scotland for at least two years after their host is removed. This concurs with work carried out in other parts of the UK and further afield that have also found that these pathogens persist in soil for at least two years (Turner, *et al*., 2005; Shiskoff, 2007; Widmer, 2011; unpublished work, Alexandra Schlenzig, SASA). Evidence of seasonal variation of inoculum levels in soil was not found at these sites over the two years; it may be possible that variation would be found to occur if inoculum levels were observed over a longer period of time.

The level of *P. kernoviae* at the lesser infected site 1 at Benmore did deplete over time which could mean that the soil inoculum level after a less severe infection would be lower and survive in the soil for less time. This however was not the case with the less severe infection at Merkland Wood where soil infestation remained constant throughout the study. It may therefore be the case that the extent of the initial infection is only one factor for inoculum survival in soil that works in combination with environmental factors. Site 1 Benmore is in the open and prone to drying whereas Merkland Wood is a damp shaded Beech forest. These factors will influence inoculum survival.

There appeared to be an effect of soil water content at site 4 Benmore. The area that consistently contained the most inoculum was toward the front of the site where the soil remained saturated for long periods (personal observation, no soil water content measurements taken) whereas the least inoculum was detected at the higher and drier area to the back of the site under an *Abies* tree. Inoculum in an area that does not dry out would be expected survive for longer (Maloney, *et al*., 2002), which suggests that

inoculum levels would remain higher post host removal than they would in areas that frequently dry out.

Another important consideration of inoculum survival in soil at Brodick is that although the above ground parts of the main host, *R. ponticum*, had been largely removed, there was some evidence of regrowth and reinfection at some sites around the garden. This, combined with the possible asymptomatic infection of some of the other *Rhododendron* species present in the most infested parts of the garden, could effectively 'top up' inoculum in the soil. This is an important aspect for the management of this disease because it backs up the current UK legislation which requires infected plants to be removed in whole from a garden upon their discovery therefore reducing the chance of inoculum build up in the soil. However, although complete removal of infected plants is the ideal, quite often the whole plant is not removed, just the above ground parts. If the host is *R. ponticum* the removal process is made more challenging because it is extremely difficult to completely remove from an area and it often takes a number of herbicide applications to kill it. There is also an *R. ponticum* seed bank in the soil which means that once a mature plant is removed seedlings germinate in their place and become new hosts for the pathogens.

The practice of removing hosts to prevent inoculum build up in soil is also confounded by a number of other issues. The findings of very high inoculum levels at site 5b in Brodick where the *Pieris* at the site are presumed to be infected but no *Phytophthora* has been isolated from the trees is one such issue. If this study had not randomly taken samples from that area to determine if the soil was infested, it would have been impossible for the garden managers to know that site 5b was infectious at all. This shows that inoculum can build up in soil in close proximity to an asymptomatic host. The infected *M. kobus* at Benmore also shows very little evidence of infection, even after almost 3 years have passed since diagnosis. If the plant health inspectors had not already been in this garden due to other infections it is doubtful that the *M. kobus* would have been identified as infected with a pathogen.

Infection of bait plants at site 4 (Benmore) and site 5 (Brodick) show that inoculum in infested soil can be splashed onto the lower leaves of hosts and infect them. A DEFRA (2005a) study found that infection occurred on adaxial leaf surfaces of *Rhododendron* (where the stomata occur) when they were inoculated, but did not occur on unwounded

upper leaf surfaces with no stomata. Furthermore, infection of bait plants under the *M. kobus* at site 2 during the winter could also point to infection by means of soil splash as there would be no inoculum from the leaves of this deciduous tree during the winter and sporangial production on infected bark of trees probably does not occur, or occurs rarely and is thought to be insignificant (Davidson, *et al*., 2005 & 2008; Garbelotto, *et al*., 2003; Tjosvold, *et al*., 2002a). In this study the instance of infection from soil splash onto bait plant leaves at site 4 (14 occasions) was almost as common as infection from both an overhead sporulating host and infested soil as found at site 2 (16 occasions).

Significantly more bait plants became infected with *P. ramorum* at Benmore (17% infection from 3 of the 4 sites) than became infected with *P. kernoviae* at Brodick (5% infection from 2 of the 6 sites - only 16 infections from more than 300 plants placed around the garden). In addition only bait plants in close proximity to a *P. kernoviae* infected host at Brodick became infected whereas bait plants at Benmore became infected with *P. ramorum* in areas where the hosts had been removed. This suggests that either *P. kernoviae* is not able to infect a host from soil splash as readily as *P. ramorum* or that *P. kernoviae* inoculum does not live for as long in the soil after host removal than *P. ramorum* inoculum.

Inoculum levels in the environment (inoculum pressure) have an important bearing on infection (Hansen, *et al*., 2005). The infested soil to host plant infection process however appears to be more complex than inoculum pressure alone because bait plants have become infected during periods where measurements of inoculum in the soil have been low and also not become infected when high inoculum levels have been recorded. Infection has also occurred during periods of both high and low rainfall. This was not expected because current understanding is that higher inoculum pressure and wet conditions are required for bait plant infection (Hansen, *et al*., 2005; DEFRA, 2005a). More work is required to understand the processes that allow host plant infection from infested soil.

There were also some differences in bait plant susceptibility depending on the species used as *P. kernoviae* infections at Brodick were far more common on *Vaccinium myrtillus* bait plants than Rhododendron bait plants. Furthermore, none of the *Pieris* bait plants used at Brodick became infected and only two became infected (with *P. ramorum*) at site 2 Benmore during the study.

These issues have important management implications for the replanting of affected areas as planting host species would leave them at high risk of becoming infected from the soil, particularly from *P. ramorum*. In addition, the lower branches of host species already present in an infected area should be removed to prevent infection via soil splash onto lower leaves. These findings concur with current DEFRA advice on the removal of leaf litter and the lower branches of susceptible plants (DEFRA, 2008).

The *Vaccinium myrtillus* infection at Merkland Wood seems to have been controlled by the application of herbicide to remove the host as the infestation of *P. kernoviae* in soil remains low and was confined to small patches of ground. It remains to be seen if the infection reoccurs as the host re-establishes in the area.

Perhaps the most worrying finding from the soil studies at the gardens was evidence of the movement and introduction of *P. ramorum* by horticultural activity at Benmore into areas of the garden not previously infected. *P. kernoviae* was also found during random sampling at Brodick in areas that have no history of infection and contain no hosts. This leads to the conclusion that infected compost may have been introduced to these areas at some point. Correct composting techniques do kill both of these pathogens. In large scale compost heaps Noble *et al*. (2011) found that no *P. ramorum* had survived after 5 days at a mean temperature of 41.9^{o}C (32.8^{o}C for *P. kernoviae*) or for 10 days at 31.8^{o}C. If these composting procedures cannot be followed correctly, horticultural practices should be modified in gardens that have become infected by these pathogens so that infected material is burned and no compost or leaves are gathered to be used again.

Chapter 5

Assessing *Phytophthora ramorum* infection risk at an important heritage garden; a case study at Benmore Botanic Garden

5.1 Introduction

In Europe, *Phytophthora ramorum* was initially discovered within the nursery industry and was found to infect only container grown *Rhododendron* and *Viburnum* plants (Werres, *et al*., 2001). The extent of the host range and the damage that this pathogen could cause in gardens was not immediately apparent, and it was not until outbreaks in established parks and gardens were discovered in southern England that the true potential for damage became evident (Brasier, 2008b). During surveys undertaken to find *P. ramorum* in 2003, *P. kernoviae* was found infecting trees and shrubs in woods in Cornwall (Brasier, *et al*., 2005). It soon became clear that *P. kernoviae* could cause as much damage as *P. ramorum* in gardens, particularly if the garden contained a large number of *Rhododendron* species (Webber, 2008).

The trees and shrubs that have traditionally been grown in UK historic gardens are particularly susceptible to both pathogens. Initially, ornamental *Rhododendron* species and the invasive *R. ponticum* were found to be most susceptible; but as garden infections developed many more commonly used horticultural species became infected such as *Magnolia*, *Pieris*, *Osmanthus*, *Fagus* and *Camellia*.

According to the Food and Environment Research Agency (FERA) (2012), there are now 157 known host species of *P. ramorum* and 37 of *P. kernoviae*, although it should be pointed out that this does not count the large number of cultivars involved (e.g. *Rhododendron* is counted once when in fact there are many cultivars). Newly discovered susceptible species are frequently being added to the host lists.

The availability of large numbers of hosts and apparently suitable weather conditions in parts of the UK make conditions ideal for establishment of these pathogens in gardens and for potential spread from one garden to another (Brasier, 2008a; Turner, *et al.*, 2008a;).

Benmore Botanic Garden is situated in Argyll & Bute in the west of Scotland and is owned by the Royal Botanic Garden Edinburgh (RBGE). In common with most gardens designed during the Victorian era, it contains many of the host species frequently infected by these two pathogens. The first plant confirmed to be infected with *P. ramorum* at the garden was a mature *Pieris japonica* in August 2008, and the first *P. kernoviae* infection was on a group of *Rhododendron* 'Elizabeth Hobbie' in October 2008. As of July 2012, 36 *P. ramorum* and 3 *P. kernoviae* infections have further occurred on *Magnolia*, *Osmanthus*, *Rhododendron*, *Pieris* and *Kalmia*. The current situation is shown in figure 35. This figure shows that most of the infections are confined to a relatively small area of the garden which is at the bottom of the hillside on which Benmore is situated.

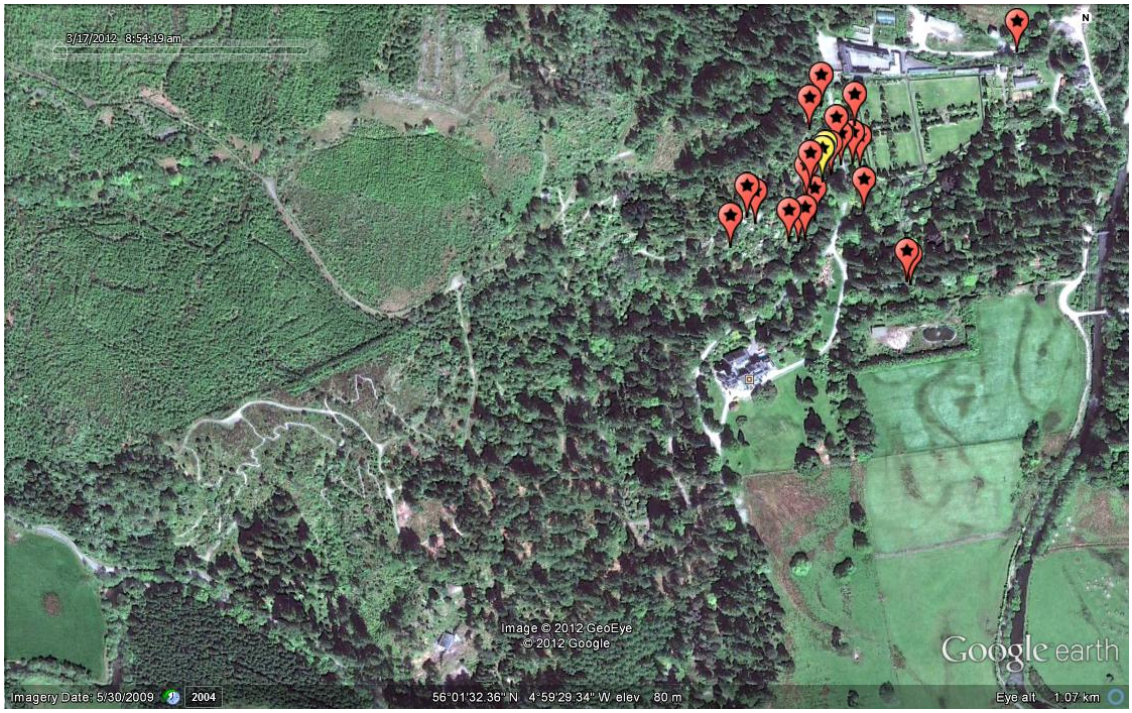


Figure 35: A map showing the infections from both pathogens at Benmore (red marker shows *P. ramorum*, yellow *P. kernoviae*).

To add to the potential threat to Benmore, the garden is in risk zone 2 of the Forestry Commission *P. ramorum* larch infection risk map and very close to risk zone 1as shown in figure 36. These risk zones were created by the Forestry Commission in order to visually show the areas most at risk from larch infection. The risk of infection was calculated due to the proximity to larch infections at the time (Oct 2011), conducive temperature and high rainfall. Larch within zone 1 are most at risk from *P. ramorum* infection and zone 3 the least.

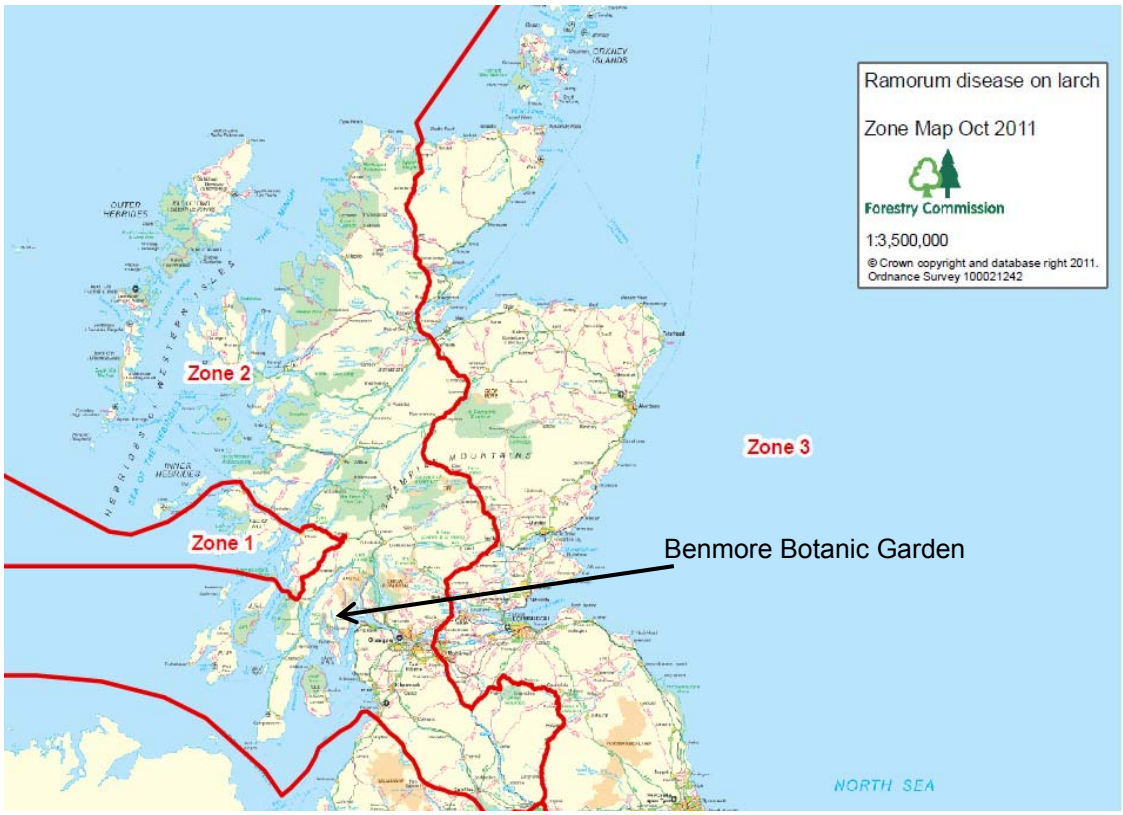


Figure 36: Forestry Commission zone map of *P. ramorum* on larch disease threat (Oct 2011). Zone 1 is most at risk and zone 3 the least. Reproduced by kind permission of the Forestry Commission

The larch disease situation is fast moving and the map shown in figure 36 is already out of date as diseased larch have recently been discovered near Strachur about 16km north of Benmore and 12km south east in Greenock (fig. 37). This would potentially move risk zone 1 to include Benmore in future risk assessments.

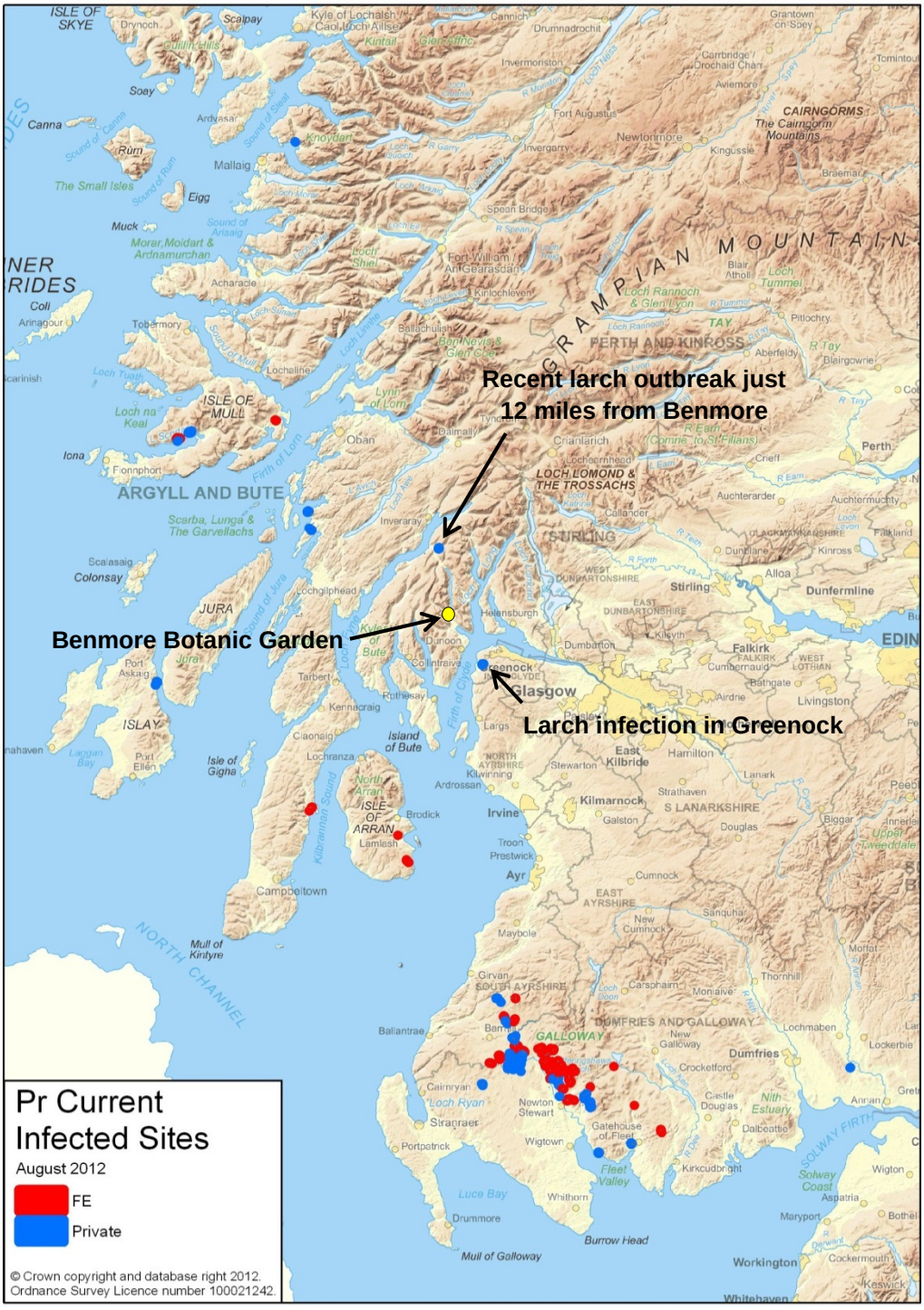


Figure 37: Larch outbreak sites as of July 2012. Red dots are Forestry Commission owned sites and blue are privately owned sites. Reproduced with the kind permission of the Forestry Commission

5.1.1 Mapping disease risk

Epidemic mapping of plant diseases using Geographical Information Systems (GIS) is now a common epidemiological tool used to assess risk and inform management decisions (Venier, *et al*., 1998; Jaime-Garcia, *et al*., 2001; White, *et al*., 2002). Globally, risk maps for *P. ramorum* infections have been created at the country, state and county scale (Magarey, *et al.*, 2007; Meentemeyer, *et al*., 2008; Harwood, *et al*., 2009). This chapter aims to present risk maps generated at the finer resolution of garden scale using Benmore as a case study, thereby aiding in the management and monitoring of *P. ramorum* at this site. This is the first time a risk map has been created at such a local scale and it could be taken as a case study on which to base the construction of localised risk maps at other sites.

5.2 Materials and Methods

Over the last 15 years RBGE has invested in a plant database system (BG Base) and GIS mapping technology. This primarily aids in plant information archiving at their gardens, but this existing technology is very useful for a number of applications including assessing potential for disease invasion and progression. RBGE provided the complete GIS map (created using ArcGIS 10 (ESRI, 2012) and shown in appendix G1) and the list of c.12000 plants present at Benmore (in Microsoft Excel 2010 format) from their database. The number of host plants in each bed was counted from the plant list and the proportion of host plants per bed for both pathogens was calculated (appendix G2).

Using ArcGIS, the layers that were considered relevant to risk were selected from the complete map provided by RBGE. These layers were paths, beds, streams, rivers, ponds and buildings. Layers specific to this project were added, which included data from spore trap sites and the positions of infected and removed plants. A GPS was used in the garden to map the smaller streams that were not present in the GIS map. These streams were then added as a layer to the map.

Due to the extensive plant database kept by Benmore Botanic Garden, spatial analysis could be carried out to identify factors that may lead to a bed within the garden becoming and remaining infected.

The proximity of each bed to features such as water, buildings and paths was established by using ArcGIS. Firstly, the centroid of each bed was calculated by the software using the *feature to point* function; and then the *near* function (input is centroids) could be used to give the proximity in metres to a given feature. These values were then exported for use in the analysis. The number of times a bed was sampled and the number of positive soil samples from each bed were recorded in MS Excel. These combined data give the proximity of a bed to given features, the host density in a bed and also a measure of the amount of *Phytophthora* in the soil in a particular bed. Due to the size of the garden, soil samples could not be taken from every bed and so only proximity to feature and host density values were available for some beds.

Analyses were conducted using the R Project statistical software package (version 2.14.2; R Development Core Team, Vienna, Austria). The response variable was the proportion of soil samples from each bed that was positive. Explanatory variables included in the model fitting were proximity to path, proximity to water, proximity to buildings and host density. Initially a binomial generalised linear model, using the *glm* function in R, was used and the model was fitted using backwards stepwise selection, with the variable/factor with the highest p-value (determined by an analysis of variance) being dropped at each step. The best fitting model was then determined using Akaike information criterion (AIC).

Given the spatial nature of the data, a correlogram was fitted to the residuals from the GLM model to check for spatial autocorrelation. Significant spatial autocorrelation was evident and therefore the INLA (Integrated Nested Laplace Approximations) approach was adopted (Rue, *et al.*, 2009) using the R-INLA package in R (full code in appendix F). An adjacency matrix was created whereby beds within 10m of each other were considered contiguous and this matrix was included in the INLA model.

Fitted values from the model were allocated to the beds for which soil data existed. For those beds with no soil sample data, predictions were generated based on the variables retained in the model. This allowed a risk map showing the probability of finding a positive soil sample in each bed to be produced.

5.3 Results

Benmore garden contains 113 beds ranging from approximately 36m^2 to 15,000m^2 in size and, according to the database, the beds contain 11,994 plants (as of July 2011). Of these, 3350 are *P. kernoviae* hosts and 3652 are *P. ramorum* hosts.

Figure 38 shows the beds where soil sampling took place and also how many of the samples were positive for *P. ramorum*.

Figure 38: *left*, the sampled beds at Benmore and the number of times they were sampled; *right*, the number of times that *P. ramorum* was found in each bed.

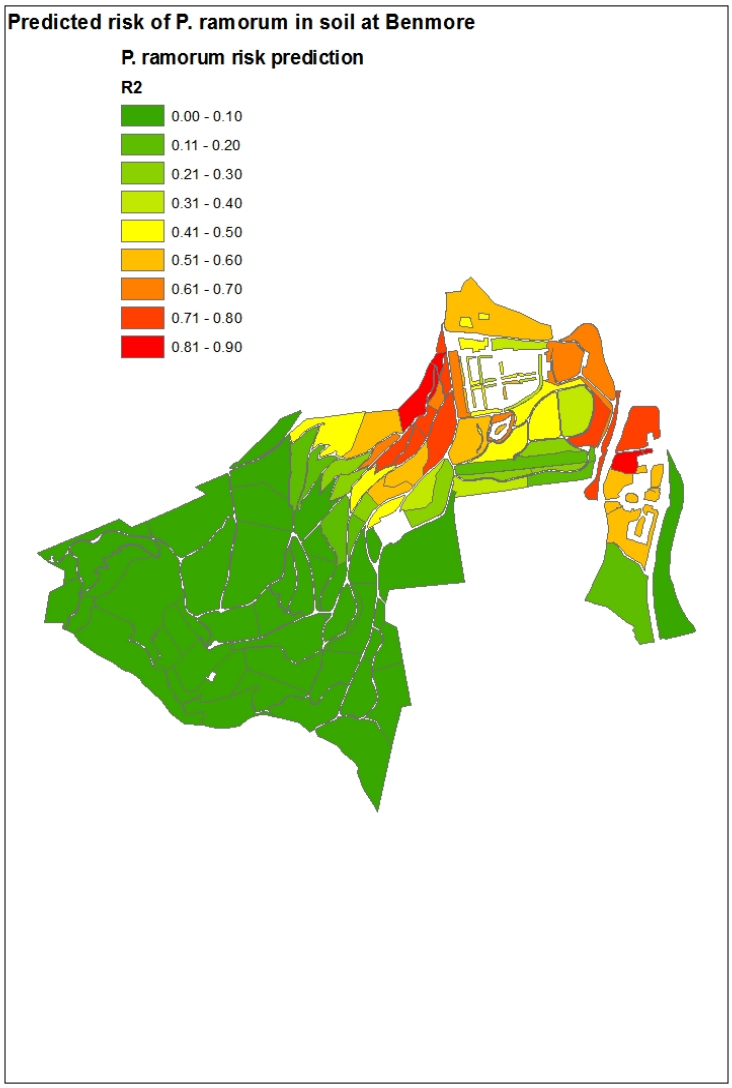
Results from the best fitting model using the INLA approach are shown in table 8, the DIC was 103.20.

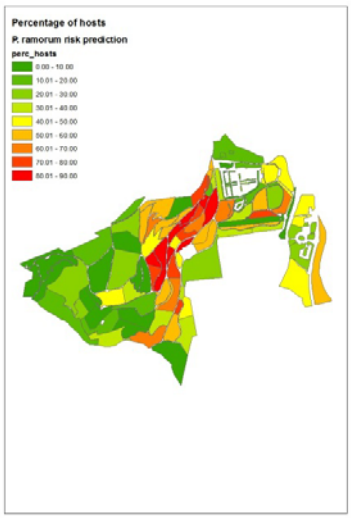
Table 8:Mean and quantile coefficient values from the INLA model fitted to proportion of soil samples that were positive

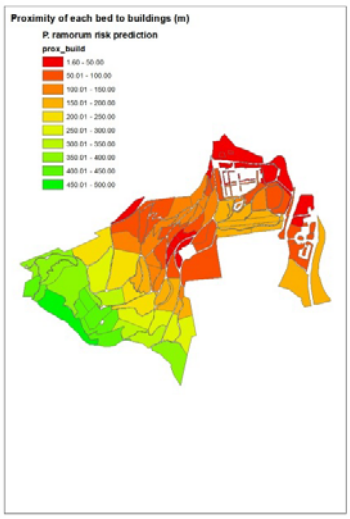
	Mean	0.025 quant	0.975 quant
Percentage hosts	0.01804778	0.002254191	0.034501035
Proximity to buildings	-0.01412324	-0.025687954	-0.003296421
Proximity to water	-0.02763758	-0.044513482	-0.012156102

The closer a bed was to water sources such as rivers and ponds (proximity to water) the greater the probability that *P. ramorum* would be found in the soil. The importance of water for *Phytophthora* establishment and spread is well understood (Davidson, *et al*, 2005; Reeser, *et al*., 2011) so this level of significance was expected. The higher the percentage of hosts present in a bed, the more likely that the soil will be found to contain *P. ramorum* (percentage of hosts), this corroborates past studies and confirms that hosts are essential for disease spread (for example Rizzo, *et al*., 2002a). The significance of buildings was an unexpected result (proximity to buildings); the closer a bed is to buildings the more likely the soil is to be infested.

A polygon shape file containing the boundaries and characteristics of each bed was imported into R and the fitted and predicted values (probability of a soil sample being positive) from the INLA model were allocated to the relevant bed,and this was used to create the Benmore risk map shown in figure 39 (over page).







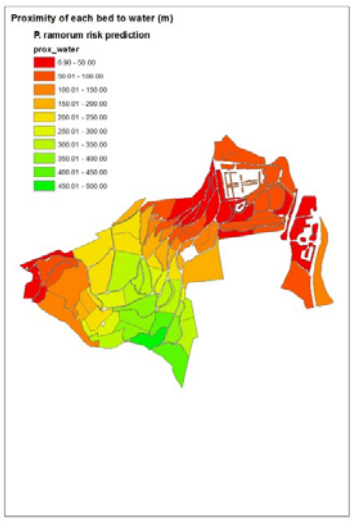


Figure 39: *Top*, predicted risk (the probability of a soil sample taken from a bed being positive for *P. ramorum*) for each bed at Benmore. *Below left*, percentage of hosts in each bed. *Below middle*, the proximity of each bed to buildings. *Below right*, proximity of each bed to water. Created with ArcGIS 10.

Figure 39 shows the complete risk map produced by the model (top) as well as a spatial representation of each explanatory variable (proximity to water and buildings and percentage of hosts), demonstrating visually how each is contributing to the overall map. The area of the garden that has had the most infection (highlighted in figure 38; YK6=8 infections & YL1=6 infections) is shown as a high risk area in the map. This is in part due to the stream that runs through the area but is also because of the high percentage of hosts here (>79.04%). Other areas that had a high proportion of positive soil samples are predicted to be lower risk due to the absence or low density of hosts and no water features; bed YN2 which was one of the study sites, is an example of this (see fig. 40 for position of YN2). Ninety four soil samples were taken in YN2 over the study period, 81 of these contained *P. ramorum* yet this bed remains relatively low risk due to the lack of hosts and no water features in close proximity.

Infection was present in the soil in some beds where host densities were relatively low (<53.13%), in beds YK5, YQ1, YN2 & YP6 for example (figure 40), this could be due to the introduction of infected material through horticultural practices or perhaps surveillance in these areas was more frequent. There are also a number of beds with high host density (>79.04%) in areas where infection on plants is yet to be encountered (YL6 and YG2), surveillance should be increased in these areas.

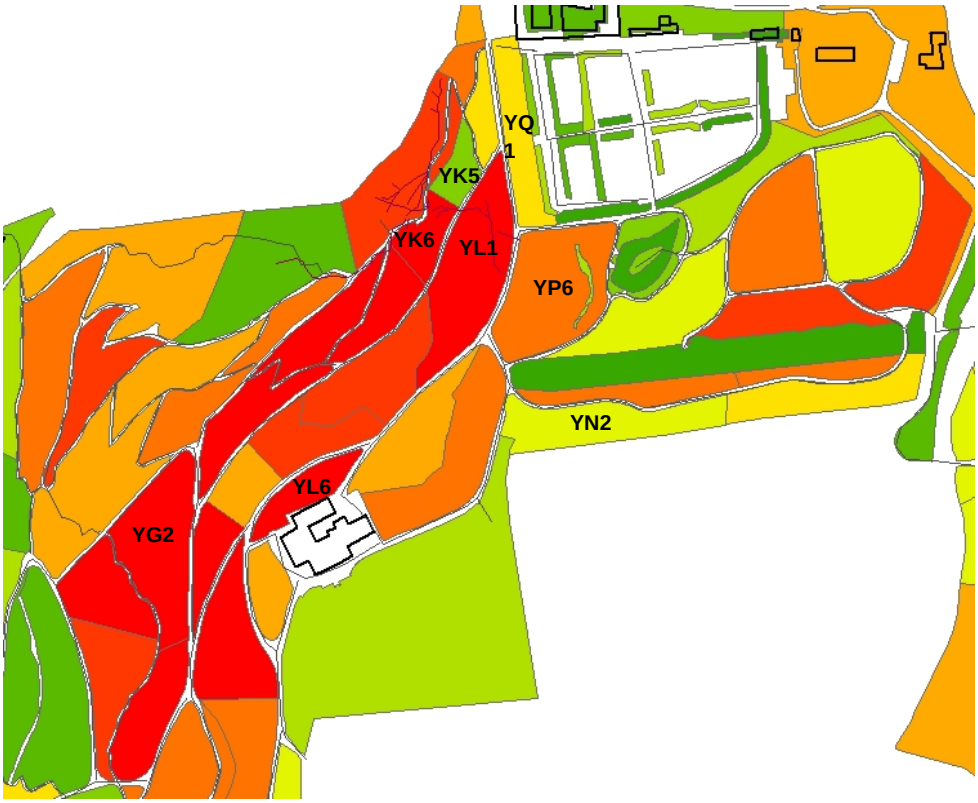


Figure 40: *P. ramorum* host density map in more detail (taken from the bottom left of figure 39. Created using ArcGIS 10

The previously described model framework could not be used to describe the *P. kernoviae* risk at Benmore because very few soil samples contained *P. kernoviae* and the extent of the overall infection at Benmore was restricted to one area. However, a host density map for *P. kernoviae* at Benmore could be produced and is shown in figure 41.

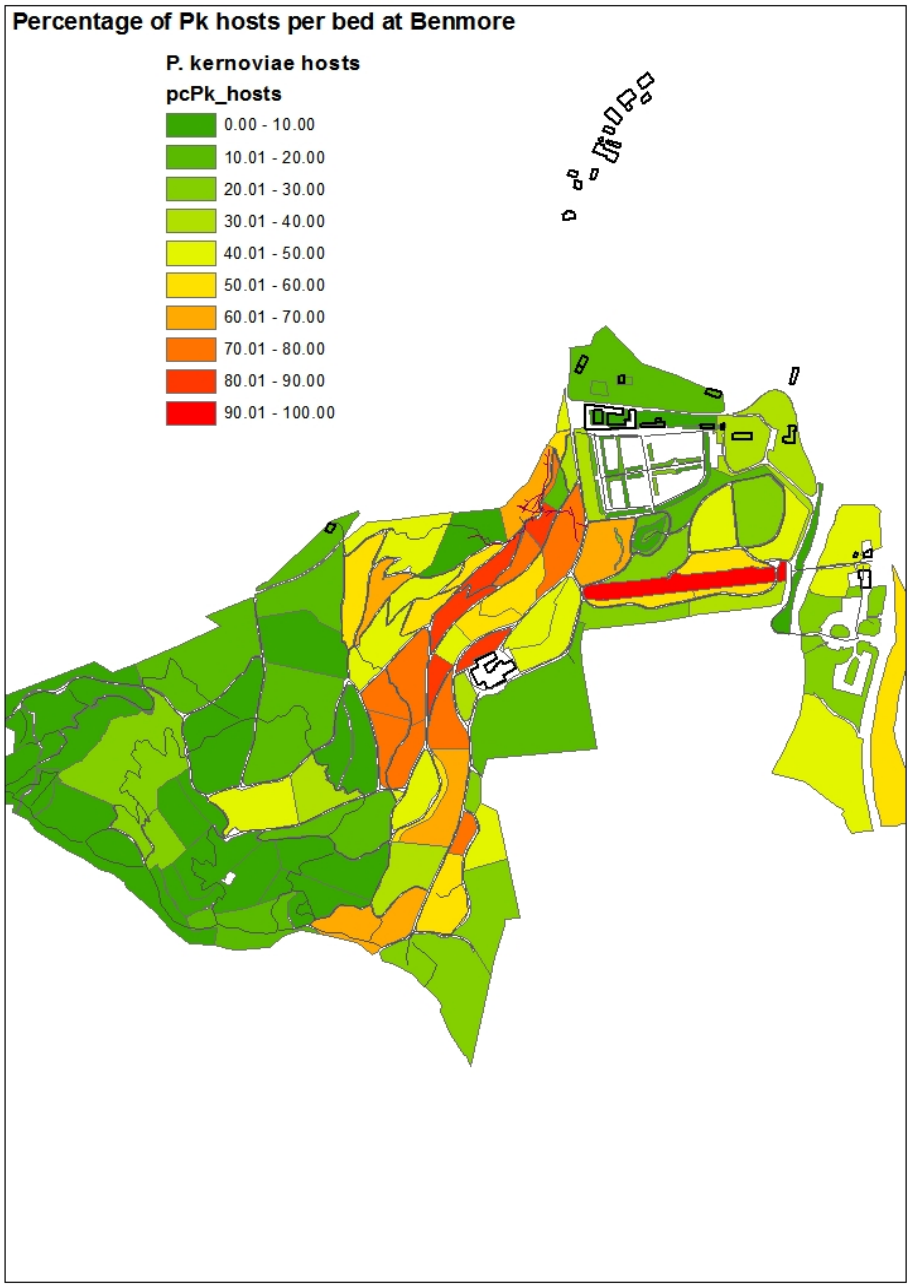


Figure 41: Percentage of *P. kernoviae* host plants per bed at Benmore where red is high density and green is low. Created using ArcGIS 10

Once again, the main *P. kernoviae* infection sits within an area of high host density (highlighted in figure 42). One specific threat to Benmore shown by the host density data is the *Sequoiadendron giganteum* avenue, a major feature of the garden, which is susceptible to *P. kernoviae* and shown to sit in an area of moderately high host density (highlighted in figure 42). There were also a number of beds identified with >76.04% host density where plants have not been found to be infected with *P. kernoviae*, beds YK9 and YL6 for example, these beds are likely to be more susceptible to infection and surveillance should be increased.

Figure 42: *P. kernoviae* host density map with the main infection area highlighted. Created using ArcGIS 10

5.4 Discussion

Large scale *P. ramorum* risk maps have previously been produced for the United States (Fowler, *et al*., 2006; Vennette, 2005), Europe (Meentemeyer, *et al*., 2004; Sansford, *et al*., 2009) and the United Kingdom (Harwood, *et al*., 2009; Sansford, *et al*., 2009). In addition, maps have been produced to the finer resolution of US State (Vaclavik, *et al*., 2010; Meentemeyer, *et al*., 2011; Filipe, *et al*., 2012) and US County (Meentemeyer*, et al*., 2004; Filipe, *et al*., 2009). This is the first risk model for these pathogen species to be produced at the considerably finer garden scale.

The key to creating these fine-scale maps was the availability of accurate plant and GIS map data at the scale of the bed, thanks to intense management by RBGE. Confidence in the risk map is increased because each risk factor map, i.e. proximity and host density, highlighted many of the same beds that contain plants that were actually infected with *P. ramorum*.

As well as providing a quantitative assessment of risk, these maps provide a visual representation of risk which can help garden managers and other stakeholders better understand and manage an outbreak. Plant health inspectors should use the risk prediction map for *P. ramorum* and host density map for *P. kernoviae* to focus surveillance activities and help decide which part of the garden to concentrate on during a visit given that their time in the garden is limited and they cannot cover the whole garden. Beds that have not been sampled but are shown as moderately at risk on the map are the beds where *P. ramorum* surveillance should be increased. The risk is present due to high host density and close proximity to features so the chance of finding a positive soil sample is higher, beds YA2, YA3 and YJ7 for example.

In bed YN2 (see fig. 40 for position of YN2), where two *Osmanthus* shrubs were removed due to infection in 2009, *P. ramorum* was consistently found in soil throughout the study yet the model predicts this bed to be at moderate risk (risk factor 0.51-0.6). The moderate risk score results from the bed not being in close proximity to water and having low host density, however, we know that infection has occurred here. It is a possibility that the *Osmanthus* infections were a result of the introduction of infected mulch, or the planting of infected material and therefore not a consequence of any of the predictor variables. This demonstrates the difficulties of predicting risk in the garden

setting where horticultural practices may be spreading infection rather than other known environmental risk factors. Without a detailed history of the horticultural activities it is difficult to capture such factors.

Host availability is a very important aspect in the epidemiology of any pathogen (Plantegenest, *et al*., 2007). Previous studies have used modelled host density data at various resolutions, for example, the CALVEG database in Californian forests (Meentemeyer, *et al*., 2004; Filipe, *et al*., 2009) is a remotely sensed dataset which has a minimum mapping unit of 1ha so patches of forest under 1ha in size are mistakenly mapped as non-host vegetation like grassland or chaparral. Conversely, this study has utilised much more accurate host density data from the database at RBGE which documents the actual number of hosts in a bed allowing for an accurate count of the individual host plantsin order that a percentage of hosts in each bed could be calculated.

The resulting host density maps for both pathogens can aid in host management at this garden. The beds shown in red on the maps are areas where garden managers should plan to increase the numbers of non-host plant species, and reduce some of the hosts if possible, to lower the risk of that bed becoming infected. These spatial maps are static but they could be re-run, perhaps annually, and then compared with previous maps to assess how management activities such as host clearance have changed the risk predictions to each bed and also allow managers to assess the cost-benefit of such activities.

The significance of proximity to buildings identified in the model was an unexpected result. Most of the buildings at Benmore are used to store horticultural machinery and equipment so this result could be interpreted as the closer a bed is to buildings, the closer to horticultural activity and the higher risk of infection. This study found that infected material was inadvertently moved around Benmore in compost and mulch (see chapter 4) which highlights the risk of *P. ramorum* spread through horticultural activity. Little empirical research has been carried out to identify the role of specific horticultural activities in the spread of *P. ramorum* at a local scale so this study points to the need for more research in this area. Advice on aspects of disease management such as surveillance, quarantine, watering and tool hygiene are available to garden managers

(e.g. DEFRA, 2008) and should be applied to reduce *P. ramorum* infections and onward spread.

This unique approach to assessing disease risk for an individual garden has proved very successful; it will provide the management and gardening team at Benmore with a much clearer understanding of the *P. ramorum* outbreak, the factors leading to the establishment and spread of this pathogen, and will hopefully lead to a reduction in disease. Moreover, the approach taken for risk assessment within Benmore Garden could be readily applied to other sites, such as gardens with plantings of considerable historical or cultural value.

Chapter 6
Discussion

This epidemiological study has provided many useful findings and added to current knowledge of *P. ramorum* and *P. kernoviae* spread in historic gardens. An important finding was that *P. ramorum* infects and may be sporulating on some hosts all year round. This was demonstrated at Benmore under the infected *Magnolia kobus* where inoculum occurred in spore traps in most months of the year, and bait plants became infected throughout the year. This was also the case with the *P. kernoviae* infected *Pieris* at Brodick site 5b. The infection of bait plants by both pathogens during the winter months was unexpected. More work needs to be carried out on this aspect of the disease cycle in order to establish if winter infection occurs from the foliage or bark of nearby infected hosts and/or from infested soil and leaf litter. This finding has important ramifications for current *P. ramorum* infections on larch and other deciduous species as it would suggest that it cannot be assumed that because leaf senescence has occurred on the host that infection of nearby plants cannot take place.

The immediate removal of hosts in gardens after confirmation of infection is a successful strategy to reduce both *P. ramorum* and *P. kernoviae* inoculum in the environment. Inoculum was only regularly found in spore traps where the sporulating host remained at Benmore site 2 and Brodick site 5b. This is important confirmation of this management strategy, however, for research purposes it would be useful if more infected hosts are allowed to remain in the gardens in order that the epidemiology can be studied more effectively using spore traps. This approach has been successfully deployed in Californian forests (e.g. Davidson, *et al*., 2008), it would naturally carry some risk of facilitating spread in gardens, but the information gained could be invaluable.

When *P. ramorum* and *P. kernoviae* inoculum enters the soil it remains there for at least two years as shown at a number of sites at both gardens. This concurs with work carried out in other parts of the UK and further afield that have found that these pathogens persist in soil for at least two years (Turner, *et al*., 2005; Shiskoff, 2007; Widmer, 2011; unpublished work, Alexandra Schlenzig, SASA). The further infection of

hosts from this soil inoculum was also observed here with the infection of bait plants throughout the year at Benmore site 4 in the absence of infected hosts. This has important management implications for the replanting of affected areas because planting host species would leave them at high risk of becoming infected from the soil. In addition, the lower branches of host species already present in an infected area should be removed to prevent infection via soil splash onto lower leaves. This is in line with current DEFRA advice on the removal of leaf litter and the lower branches of susceptible plants (DEFRA, 2008).

It is well known that *P. ramorum* is able to move around sites in watercourses (Davidson, *et al*., 2002; Beales, 2007) and water baiting at Benmore confirmed that this was indeed the case at this garden. *P. kernoviae* was not found with this method in watercourses in either garden which could mean that either baiting is ineffective at detecting *P. kernoviae*, as suggested in some other studies (e.g. CSL, 2008; Turner & Jennings, 2008), or that is was not present in the watercourses at these gardens. Only *Rhododendron* and *Vaccinium* leaves were used for water baiting in this study, it could be the case that leaves from other susceptible species would make more sensitive water baits for *P. kernoviae*.

The discovery of infested soil in unexpected areas of these gardens, and the subsequent finding of *P. ramorum* in compost at Benmore, uncovered one route by which these *Phytophthora* were potentially being moved into new areas of these gardens. It is essential that staff at gardens follow disease management guidelines that are available on aspects of their horticultural work such as tool hygiene, correct composting procedures, surveillance and watering (e.g. DEFRA, 2008).

This study also identified that the use of slot drains can cause inoculum build up in soil. This particular drainage technique is often used in historic gardens to take excess rain water across paths during heavy rain events thereby preventing path erosion. They are often rudimentary open channels made from wood or stone and it was found at Benmore that they have the effect of concentrating inoculum on the downhill side of the drain. It is assumed that inoculum is washed out of infested soil or off the leaves of infected plants during rain, it is then taken across the path in the drain and remains in the soil on the downhill side as the water drains away. This is very difficult to manage, but one solution could be to insert pipes to take the excess water deeper into the

ground as opposed to allowing the water to simply exit the drain onto the surrounding surface soil.

The modelling carried out during this study demonstrates a useful framework by which epidemic processes can be understood, thereby informing disease management. The GEEGLM which described bait plant infection by *P. ramorum* during wet periods with high humidity and a host present supports current understanding of the biological conditions required for infection (Turner, *et al*., 2008; Tooley, *et al*., 2005). The spore trap GLM was less clear in which variables played a significant role in whether inoculum was present in traps. This is perhaps due to the removal of sporulating host plants at the majority of sites leading to the low number of findings in the traps overall, which meant that there was a small dataset for model fitting. The study would perhaps have to take place over a longer period than two years in an environment where the host plants can remain in order to collect enough data for more successful statistical analysis to take place.

The soil modelling was particularly informative, the resulting *P. ramorum* risk map for Benmore is the first time a risk map has been developed at the garden scale. The resulting map will inform managers and plant health officials of the areas of the garden that are more at risk than others due to certain risk factors such as their close proximity to water and high host density. This will lead to more focused surveillance and allow for a better informed management strategy to be put in place at Benmore. It also underlines the importance of investment in technology and keeping up to date plant records so that owners and managers can better understand their garden in this context.

A specific aim of the modelling at the outset of this project was to draw comparisons between the two pathogens and also compare the *P. kernoviae* infections at both gardens because of the contrast in the severity of the infections. It was hoped that answers could be found as to why the infection at Brodick was so severe compared to Benmore. Unfortunately it transpired that a comparison at the statistical level could not be carried out because *P. kernoviae* was not found in the spore traps frequently enough at Brodick, and not at all at Benmore. However, observations could still take place to attempt to understand the difference in *P. kernoviae* progression at the two gardens. The climates at the gardens are similar so perhaps the most significant factor

influencing the speed of spread and the extent of the Brodick infection was the host density in the lower garden, particularly the very high density of *R. ponticum* and other *Rhododendron* species. The horticultural staff simply could not keep up with the clearing effort as more and more plants were identified as infected. Conversely at Benmore the initial *Rhododendron* cultivars that became infected were in a small area that was able to be cleared quickly, there was no *R. ponticum* present and the infected plants were not in direct contact with any more *Rhododendron* plants. The topography of the Benmore *P. kernoviae* site could also have been a factor because there was a wide path below the site and a steep rocky slope above which effectively cut off the infection area from many of the surrounding host plants.

One noteworthy aspect of this study was that the *Magnolia kobus* at Benmore was infected with *P. ramorum* for at least 3 years yet there was very little change in the severity of the observed symptoms. The leaves showed the black-spotting symptoms often associated with *Magnolia* infection but the plant remained vigorous and otherwise healthy, in addition it still flowered annually. If this tree were in a private garden it would be conceivable that these symptoms would remain un-noticed and that an infected tree could take a number of years to die whilst *P. ramorum* sporulated from it onto surrounding plants.

These observations reinforce both the legislation that requires prompt removal of infected plants and the current understanding of the importance of the removal of *R. ponticum* wherever possible whether it is infected or not. Furthermore, garden managers should aim to provide an open environment for plants so that air can pass through, and prevent a large build-up of overcrowded beds that contain host species as was the case with *Rhododendron* at Brodick. Also, once the *R. ponticum* around site 4 at Brodick began re-growing after only partial clearance in 2009, bait plants started to become infected. It is therefore essential that any *R. ponticum* clearance undertaken should be thorough to ensure the plants do not grow back.

The *P. kernoviae* infection on *Vaccinium myrtillus* in Merkland Wood was of particular concern at the start of this study because of the potential impact on heathland biodiversity in Scotland. It is reassuring that, despite the extensive infection at this site, *P. kernoviae* does not appear to have persisted in this environment. The *V. myrtillus* was sprayed with herbicide to remove it in 2009 which reduced inoculum levels to the

point where there were no *P. kernoviae* findings in the spore trap or on bait plants at this site over the two years of this study. There were low levels of inoculum in the soil so it remains to be seen whether *V. myrtillus* becomes infected at this site as it recolonises the area.

As novel exotic *Phytophthora* species continue to be discovered affecting various hosts throughout the UK, the reliable diagnostic and analytical methods developed during this project will continue to be refined so that a better understanding of *Phytophthora* infection and spread in gardens and the wider environment can be gained with the aim of both reducing the frequency and severity of infections.

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