AN ABSTRACT OF THE THESIS OF

Silvia E. Benemann for the degree of Master of Science in Botany & Plant Pathology presented on May 31, 2017.

Title: *Phytophthora ramorum* within an Oregon Tanoak Forest: Quantifying Inoculum within Canopy Throughfall vs. Soil Splash

Abstract approved: _____

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Phytophthora ramorum continues to cause extensive mortality of tanoaks in southwestern Oregon. Rain readily washes inoculum down through the canopy, causing new infections on the lower parts of the tree and neighboring host plants. Although this aspect of dispersal is well understood, the relative importance of infested soil and leaf litter as factors contributing to the spread of disease remain unclear. The primary objectives of this study were: (i) to compare the amount of inoculum washed down through the canopy to that splashed up from soil and litter, and (ii) to detect and quantify inoculum in relation to soil depth. Over the course of the 2014-2015 rainy season, rainwater was collected 8 times and soil sampled 3 times from within the Generally Infested Area in Brookings, Oregon. Rainwater, soil, and litter were subject to both qPCR and traditional baiting methods. *P. ramorum* was detected by qPCR more frequently in splash-up from the ground surface than from canopy throughfall. *P. ramorum* was only detected in soil twice via qPCR and was never recovered by baiting. In canopy throughfall water, qPCR also proved to be a more sensitive way to detect the pathogen than baiting with whole rhododendron leaf baits. In the Oregon tanoak forest studied, quantification of *Phytophthora ramorum* was not possible because inoculum

levels were too low and distribution was too uneven for reliable detection with the methods employed.

Phytophthora ramorum within an Oregon Tanoak Forest: Quantifying Inoculum within Canopy Throughfall vs. Soil Splash

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented May 31, 2017

Commencement June 2017

Master of Science thesis of Silvia E. Benemann presented on May 31, 2017.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Silvia E. Benemann, Author

ACKNOWLEDGEMENTS

Support comes in many shapes and sizes.

To all who made this thesis a possibility:

THANK YOU.

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History

Phytophthora is a genus of fungus-like organisms that contains some of the most destructive phytopathogens known in both agricultural and natural systems. The invasive species *Phytophthora ramorum* is no exception. For nearly two decades researchers have been continually challenged to understand its epidemiology and develop effective management strategies.

In the mid 1990's, landowners in Marin and Santa Cruz counties in California began reporting the rapid dying-off of tanoak (*Notholithocarpus densiflorus*) and coast live oak (*Quercus agrifolia)* (Svihra 1999). Soon after, the name 'Sudden Oak Death' was used to describe the phenomenon. Meanwhile *Rhododendron* in both Germany and the Netherlands was being stricken with a twig blight caused by a previously unknown species of *Phytophthora* (Werres et al. 2001). These two seemingly unrelated epidemics were connected by Clive Brasier of the UK Forestry Commission, who suggested that the mystery pathogen that had been plaguing rhododendron in the Europe might in fact be the same as the one causing rapid oak decline in California (Garbelotto and Rizzo 2005). By 2001 DNA from the two pathogens were sequenced and compared, revealing that their ITS regions were an exact match (Garbelotto and Rizzo 2005). This new species was officially described and named *Phytophthora ramorum* S. Werres & A. W. A. M. de Cock (Werres et al. 2001).

The sudden oak death (SOD) epidemic has spread steadily through coastal evergreen forests and tanoak/redwood forests in California. As of 2014, 15 counties in California are infested with *P. ramorum* (California Oak Mortality Task Force – Sudden Oak Death Web Site Home Page n.d.). In 2001, infested areas were detected in tanoak forests just north of the California border in the coastal town of Brookings, Oregon (Goheen et al. 2002). The coastal tanoak forests of northern California and southwestern Oregon have climates suitable for the establishment of *P. ramorum*. Preference for regions with significant rainfall, mild winter temperatures, and suitable hosts has seemingly been restricting its spread to other climate zones.

Different Hosts, Different Symptoms

Phytophthora ramorum is a generalist pathogen. The combination of official hosts and those plants that are associated with *P. ramorum* brings the total number of hosts to over 100 different species (USDA APHIS 2013). As of February 2015 the California Department of Food and Agriculture (CDFA) identified five new hosts in Marin County, California: manzanita (*Arctostaphylos virgate & Arctostaphylos glandulosa*), chinquapin (*Chrysolepsis chrysphylla*), blackberry (*Rubus ursinus*), and chaparral pea (*Pickeringia montana*) (California Oak Mortality Task Force – Sudden Oak Death Web Site Home Page n.d.). These species span a wide range of plant families thereby illustrating how this pathogen's nonspecific nature allows it to be such a challenge when it comes to creating effective disease management strategies.

Depending on the host in question, infection can result in one of three diseases: sudden oak death (SOD), ramorum shoot dieback, or ramorum leaf blight (Davidson

et al. 2003). Each of these diseases causes a variety of symptoms that can vary in severity. Ramorum shoot dieback and leaf blight are diseases associated with multiple tree species, understory plants, and ornamentals. Common examples are rhododendron, Oregon mrytlewood (*Umbellaria californica)*, Douglas-fir (*Pseudotsuga menziesii*), coast redwood (*Seqouia sempivirens*), and species of *Kalmia* and *Pieris* (Davidson et al. 2003).

Sudden oak death affects certain members of the Fagaceae (oak family), most notably coast live oak (*Quercus agrifolia*) and tanoak. Probably one of the most diagnostic characters of the disease is the formation of cankers on the trunk, typically found 1 to 2 m up from the ground. These cankers are dark brown to red in coloration and exude darkly colored sap through the bark of the tree. This 'bleeding canker' will enlarge over time and in later stages will cause the bark to fracture. During early stages of infection the canopy may appear healthy, despite the presence of cankers (Davidson et al. 2003). The infection will move inwards from the bark extending through the cambium and into the secondary phloem and to a lesser extent into the xylem (Rizzo et al. 2002). In tanoak, *P. ramorum* was found in the xylem tissue (sapwood) directly underneath cankers. The obstruction of xylem vessels was deduced to be causing disruption of water transport to the canopy resulting in widespread chlorosis of the canopy that is commonly associated with sudden oak death (Parke et al. 2007). Complete browning of the canopy occurs in a very short period of time, within approximately 2-4 weeks. Although this rapid decline in canopy health may appear as a 'sudden death', the tree can take 2-3 years to fully die from the onset of initial

infection (Davidson et al. 2003). Using models to describe the temporal epidemiology of SOD, Peterson et al. (2015) estimated that 2 years may pass between the time inoculum is introduced and when signs of overstory mortality begin within an infested site.

Biology

Phytophthora spp. are eukaryotic fungal-like organisms in the kingdom Stramenopila within the phylum Oomycota (water molds). Oomycetes are more closely related to brown algae and diatoms than to fungi. Unlike true fungi they produce motile biflagellate zoospores with heterokont flagella, have cell walls made of cellulose and beta (1, 3) glucans, have a diploid vegetative state, and have coenocytic hyphae (Webster and Weber 2007).

Phytophthora ramorum exists as four distinct clonal lineages. In Europe, only the EUI and EU2 lineages are present, whereas in North America the NA1, NA2, and EU1 lineages have all been found (Grünwald et al. 2012; Hayden et al. 2006; Van Poucke et al. 2012;). As a heterothallic species, two compatible mating types (A1 and A2) must be present for sexual recombination to occur (Werres et al. 2001). The North American lineages are of the A2 mating type and the European lineages are of the A1 mating type. The NA1, NA2, and EUI lineages have all been detected in nurseries along the U.S. west coast and into British Columbia (Kliejunas 2010). Until recently only the NA1 lineage (A2 mating type) has been found in forests within California and Oregon (Hansen 2008; Prospero et al. 2007). Due to this spatial

separation of the mating types, there has been minimal risk of sexual reproduction occurring within wild populations. However, in May 2015 a newly symptomatic tree was identified near Pistol River in Curry County, Oregon. Researchers at Oregon State University revealed that the infection was caused by an isolate of the EUI lineage, which is of the A1 mating type, and was likely introduced to the area via infested nursery stock (Grünwald et al. 2015). The introduction of the opposite mating type increases the likelihood of genetic recombination with the pre-existing NA1 populations, which could result in a pathogen with increased virulence and broader host range. In particular, the EUI lineage has been shown to be more virulent in comparison to the NAI lineage as it produces larger lesion areas (Brasier et al. 2006) and produces more sporangia *in planta* (Manter et al. 2010). In 2016, the Oregon Department of Forestry reported that the EUI lineage has now been detected in nearby water drainages, soil, seedlings of Grand fir (*Abies grandis*), and tanoak. These new detections point to a very real possibility for a more virulent strain to emerge. The urgency for vigilant monitoring and continued pathogenicity research cannot be ignored.

Most Oomycetes produce multiple spore types that are associated with either the sexual or asexual state (Webster and Weber 2007*)*. Oogonia and antheridia are the structures associated with sexual reproduction. Sporangia, zoospores, and chlamydospores are the asexual spores that are frequently observed in the environment. These asexual structures are understood to have a role in disease transmission, thus they are considered to be epidemiologically important. Like other

Phytophthora species, *P. ramorum* can be readily dispersed through water, soil, and air (Erwin and Ribiero 1996). Due to the variety of dispersal mechanisms possible, research in this area has become crucial to the development of more targeted and effective management plans.

Disease Cycle and Dispersal

Local dispersal within *P. ramorum* infested forests appears to occur through both a "canopy throughfall" and "splash-up" pathways (Figure 1.1). Infected canopies act as the primary source of inoculum in the "canopy throughfall" pathway. Rain carries sporangia and resultant zoospores down through the canopy, infecting lower parts of the host and susceptible understory plants. In tanoak forests, this results in the development of lethal bole cankers, foliar and twig infection of underlying branches, as well as the infection of adjacent understory hosts such as viburnum, rhododendron, and tanoak saplings (Davidson et al. 2003).

The aerial transmission of sporangia can occur over long distances, if spores manage to disperse above the canopy and associated layer of still air. When airborne, favorable conditions must coincide to allow for the spores to remain viable so that they can germinate and cause new infections (Hansen et al. 2008). This aerial dispersal ability has been observed within Oregon tanoak forests as part of the SOD

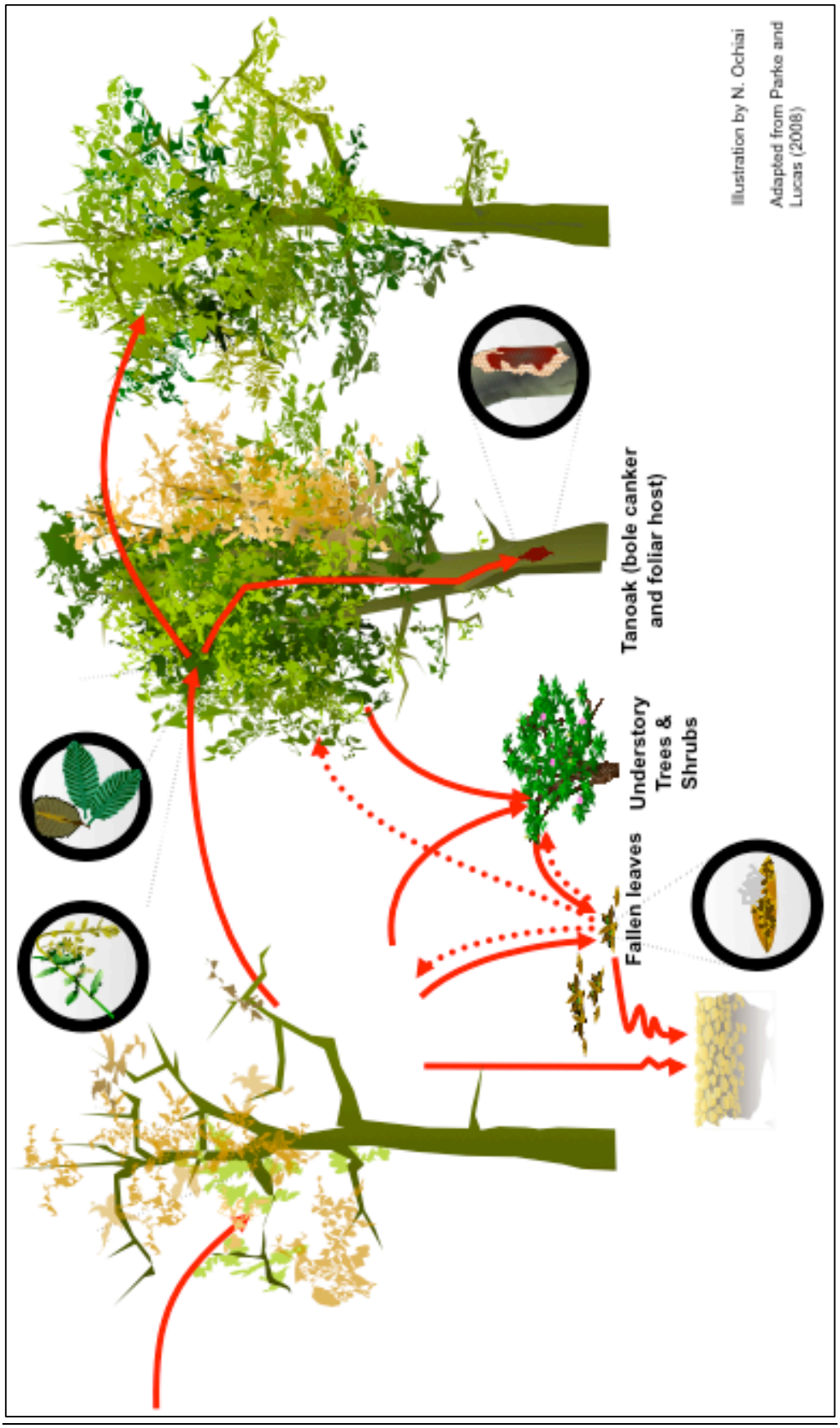

Figure 1.1: Proposed sudden oak death disease cycle of *P. ramorum* in Oregon forests. Solid lines indicate the flow of inoculum within the 'canopy throughfall pathway. Dotted lines indicate the flow of inoculum in the 'splash-up' pathway (Parke and Lucas, 2008).

surveys conducted between 2002 and 2004, which showed that 50% of the newly infected trees documented were within 122 m from infected trees reported the previous year (Hansen et al. 2008). By plotting the distribution of newly infected trees, Hansen et al. showed that long dispersal distances of up to 4 km are possible. Similarly, *P. ramorum* has been observed to spread across Japanese larch (*Larix kaempferi*) plantations in Great Britain over longer distances than would be possible if the pathogen was spreading by the canopy throughfall pathway alone (Brasier and Webber 2010; Hansen et al. 2012).

Another component of the disease cycle is the "splash-up" pathway. The accumulation of infected leaves and build-up of inoculum in the soil, create sources of inoculum for disease to spread over short distances. Rain splashes inoculum from these sources on the soil surface up onto nearby hosts and can re-infect the host above. Over time, decomposing leaves release inoculum into the soil (Figure 1.1). The "splash-up" pathway of the disease cycle is not as well understood and has been the subject of many studies over the past decade, in both nursery and forest systems.

Field studies conducted in Californian mixed evergreen woodlands have provided evidence that infested leaf litter and soil do play roles in spreading disease on a local level. It has been demonstrated that sporangia borne on infected fallen leaves can disperse onto nearby hosts via rain splash, causing new infections (Davidson et al. 2005; Fichtner et al. 2009). Up to one year after burial of artificially infested leaf

disks, *P. ramorum* was recovered from the soil itself suggesting that overtime inoculum can be introduced into the soil profile from buried leaf litter (Fichtner et al. 2009). This soilborne inoculum can then spread infection through rain splash, in a similar fashion as infected leaf litter (Davidson et al. 2005; Fichtner et al. 2009). These studies propose a link between leaves and soil as sources of inoculum that has been further solidified through molecular evidence. A multilocus genotype analysis of populations in soil and foliage revealed genotypic overlap between populations. Specifically, results indicated that genotypes in soil were derived equally from soil and leaf populations from previous years (Eyre et al. 2013), showing that the soilborne populations in part originate from foliage populations.

Much of what is known about the longevity of *P. ramorum* in soil has been driven by the concern within the nursery industry about the risk of disease transmittance through infested soil. In 2013, Vercauteren et al. (2013) buried infected leaf material in both forest soils and potting media to determine the persistence of *P. ramorum*. After 33 months they were able to recover *P. ramorum* from the inoculum buried in potting media. This data extended the known survival period from one year up to nearly three years (Fichtner et al. 2009). The persistence of *P. ramorum* in soil adds to the threat that infested soil is already known to pose. Vercauteren et al. also isolated *P. ramorum* from the roots of asymptomatic plants. This demonstrated the risk of asymptomatic plants harboring the pathogen, and also provided further evidence that infested soil can transmit disease (Vercauteren et al. 2013).

In efforts to treat infested soil, methods such as solarization have been developed. Solarization harnesses solar energy using plastic sheeting that has been laid on the ground. The solar energy heats the soil to temperatures that can kill soilborne pathogens. In a six-week long comparative study of solarized and non-solarized plots conducted in California, *P. ramorum* was found to survive down at a depth of 30 cm on buried leaf disks within non-solarized plots. Within solarized plots, it could not be detected after just 4 weeks at 30 cm (Funahashi and Parke 2016). Such studies provide valuable information on practical ways to manage infested soil to prevent spread of *P. ramorum* within and outside of their nursery.

Sudden oak death in Oregon

Since its first detection in 2001, *P. ramorum* continues to spread throughout the woodlands in Curry County, Oregon. The coastal forests near the city of Brookings are dominated by a mix of Douglas-fir, Western hemlock, Oregon myrtlewood, Pacific rhododendron, huckleberry, and tanoak - to name a few. These species are all known hosts of *P. ramorum* and among them tanoak is the most highly susceptible. Tanoak occurs in dense stands intermixed with other host species, creating pockets of intense disease. Although Oregon myrtlewood is often present in these stands, it is not known to support high levels of sporulation, unlike what has been shown in California where it is considered to be the primary sporulator (Davidson et al. 2005). Thus, tanoak is considered to be the true driver of the epidemic in Oregon. In addition to plenty of host material, mild wet winters and warm dry summers characterize the climate in southwestern Oregon - the exact conditions which *P.*

ramorum is known to prefer (Hansen 2008). Conducive environmental conditions influence the timing of sporulation, and thus influence when new infections are most likely to occur. In Oregon, because of higher annual rainfall, sporulation can occur throughout the rainy season (Hansen 2008), whereas in California sporulation occurs only during the heavy spring rains (Davidson et al. 2005). This combination of favorable environmental conditions and concentration of host material has allowed *P. ramorum* to become well established in this region.

With the goal of eradicating SOD, an interagency consortium consisting of the Oregon Department of Forestry (ODF), Oregon Department of Agriculture (ODA), Oregon State University (OSU), and USDA Forest Service (USDA-FS), was assembled upon the first detection of *P. ramorum* in Oregon. Originally the quarantine area was set at 9 mi^2 to encompass the entire area of infestation. By 2012, the quarantine area had expanded to 202 mi^2 (Kanaskie et al. 2016, *unpublished*). Within this boundary the eradication plan was executed. The plan consisted of using aerial surveys to find new areas of infestation by identifying dead or dying tanoak canopies. Using Global Positioning Systems (GPS) those areas were investigated on the ground. Once a suspected infected tree was found on the ground, samples were taken to verify the presence of *P. ramorum*. If confirmed, the tree and all host plants within 100 m were cut and burned (Kanaskie et al. 2012). The program was carried out for nearly 10 years, costing $11.5 million, and by 2011 obtaining funding to continue eradication efforts proved to be increasingly difficult. Therefore a new plan was drafted, emphasizing early detection as means of slowing the spread of the

disease. In 2012 the Generally Infested Area (GIA) was created within the pre-existing quarantine area. Although within the GIA eradication treatments are no longer required by the state, the surrounding quarantine area continues to receive eradication treatments (Kanaskie et al. 2012).

Despite concentrated efforts, the disease continues to spread and funding continues to be a limiting factor. In 2014, 12 new infestations were found outside the GIA. In early 2015, 21 new infestations were detected. Several of these were found within 1 mile of the quarantine border and one lay just 0.3 miles beyond the border. As of 2016, the quarantine border has been expanded for the 7th time to roughly 515 square miles and the GIA remains at 58 square miles (Kanaskie et al. 2016, *unpublished*).

Impacts of Sudden Oak Death

Although tanoak is not a commercially valuable tree, there are cultural and ecological consequences from its widespread decline. Culturally, the acorns of tanoak have provided a significant food source to indigenous groups (Bowcutt 2015). Ecologically, tanoak provides valuable services within its native range by providing food and habitat for forest fauna (Garbelotto and Rizzo, 2005). They also make associations with as many as 119 different taxa of ectomycorrhizal fungi (EMF) (Bergemann and Garbelotto 2006). EMF play crucial roles in host nutrient acquisition, the transfer of nutrients between adjacent hosts, as well as increasing available carbon through the decomposition of organic matter (Sylvia et al. 2004). Artificial girdling of tanoaks simulates how SOD infection limits the delivery of

photosynthetic exudates to roots. This girdling was shown to result in a significant decrease in the abundance of EMF species that associated with the girdled tree (Bergemann et al. 2013), indicating on a deeper level how the loss of tanoak can affect forest health.

Within Oregon, SOD has had economic impacts on both the timber and nursery industries in the form of quarantine regulations. In response to the concern of shipping infested nursery stock out of state, the USDA-Animal and Plant Health Inspection Service (APHIS) created a certification program for licensed nurseries that ship host material out of state. Similar restrictions and certification requirements have been placed on timber businesses, nurseries, and Christmas tree plantations that located within the quarantine zone in Curry Co., OR.

Tanoak loss also poses problems for landowners. Many of the infested areas within California and Oregon lie at the urban-wildland interface. The accumulation of dying tanoaks in such close proximity to houses presents a serious problem as dead trees not only can cause costly property damage by falling, but can also contribute to the fuel load within the region.

Detection Methods

Part of what has made the management strategy of SOD in Curry County so effective is its emphasis on early detection through frequent monitoring. Due to the dynamic biology of *P. ramorum*, the monitoring program has involved intensive sampling

from water, soil, and host tissue. Depending on the substrate in question, direct or indirect modes of isolation have been used.

Direct isolation involves sampling from symptomatic host material, such as lesions on leaves, stems, or petioles. The tissue sample is plated on selective media and monitored for the growth of diagnostic morphological structures, such as hyphae, sporangia, and chlamydospores. Indirect isolation, or 'baiting', involves placing susceptible host tissue in water or soil that has been flooded with water. Incubation in water induces sporangia to release zoospores, which swim toward the host tissue. After a prescribed number of days, the 'bait' is removed and plated onto selective media. The type of bait used depends on the *Phytophthora* species in question. In practice, rhododendron leaves, D'Anjou pears, and tanoak leaves are commonly used as baits for *P. ramorum*.

Although fairly dependable, each of the aforementioned methods has inherent drawbacks. Direct isolation relies on the presence of symptomatic tissue which risks overlooking a latent infection or obtaining a false negative because the pathogen fails to grow from the tissue (Hüberli et al. 2000; O'Brien et al. 2009). Faster growing organisms can conceal the presence of *Phytophthora.* This can particularly become problematic when indirectly isolating from soil that contains rapidly growing *Pythium* species (O'Brien et al. 2009; Nechwatal et al. 2001). Recovery through baiting depends both on the type of bait used and depends on whether the pathogen is in a biologically active state that will be responsive to sporulation induction. For

example, baiting from soil has proven to be difficult because inducing the sporulation of dormant chlamydospores can be challenging. Additionally, the production of zoospores does not guarantee infection of the bait. When baiting from soils for *Phytophthora cinnamomi*, 16% of the time zoospores were detected in water but the baiting results were negative (Wilson et al. 2000). Traditional culturing methods are not only time consuming but they also require specific expertise to identify a specimen to species using morphological features. Collectively, these issues can limit the number of samples that can be processed.

Molecular detection tools have become increasingly popular due to their sensitivity and ability to deliver fast results. In addition, most molecular tools are very adaptable for high throughput platforms, which greatly increases the number of samples that can be processed at one time. Conventional polymerase chain reaction (cPCR) is the most common DNA-based method that provides relatively fast detection. To date, species-specific primers have been developed for a multitude of *Phytophthora* species (Ersek et al. 1994; Hughes et al. 2006; Martin et al. 2004; Winton and Hansen 2001). Multiplexed PCR protocols are particularity effective as they utilize species-specific primers in tandem with a non-target primer set. This design allows for simultaneous assessment of extraction and PCR efficiency and has been used to detect *P. lateralis* (Winton and Hansen 2001). The drawback of cPCR is the need to visualize the PCR product through gel-electrophoresis which takes additional time and involves the use of a chemical dye, the most common of which is ethidium bromide – a well documented toxin and mutagen (OHSAH MSDS n.d.).

Real-Time or quantitative PCR (qPCR) is a form of PCR that has gained popularity over cPCR for pathogen detection because of its ability to be high-throughput, as it bypasses the need for gel electrophoresis. More importantly it has proven to be more sensitive than cPCR, making it well suited for detecting pathogen DNA in environmental samples, which may occur at very low levels. The most predominantly used platforms of qPCR are SYBR green and TaqMan. SYBR green is a dye that binds to any double-stranded DNA present in the sample, thus amplification of non-targets can lead to false positives. TaqMan utilizes fluorogenic oligonucleotide probes that bind only to the double-stranded DNA of the target of interest (Schena et al. 2013). Although the use of probes is more costly, the higher level of specificity provides some insurance, which is essential when working with samples containing a multitude of closely related organisms. A combined approach using traditional and molecular detection methods should prove to be more effective when detecting the pathogen in environmental samples.

Study Context

Within the framework of the eradicated treatment areas in Oregon, we have gained valuable insight into the epidemiology of *P. ramorum* within tanoak-dominated forests. However, much of what we know about the epidemiological role of soil and litter come from studies conducted in California forests (Davidson et al. 2005; Davidson et al. 2011; Davidson, Patterson, and Rizzo 2008; Fitchner, Lynch, and Rizzo 2007; Eyre et al. 2013). Since no eradication plan was ever enacted in California, the ability to study established infections has always been possible. Yet it

has only been relatively recently with the creation of the GIA, that a similar opportunity has presented itself in Oregon. Having areas no longer receiving eradication treatments means that inoculum levels have had time to build, and are conducive to studies aimed at evaluating the relative importance of understory sources of inoculum, such as soil and litter. In particular, quantitative data on inoculum derived from infested canopies, soil, and litter could provide an estimate of how much these reservoirs contribute to the total inoculum pool within these infested forests. Such information may provide insight to those looking to improve disease management plans aimed at mitigating the small-scale spread of *Phytophthora ramorum*, whether in nurseries or wildlands.

The purpose of this study was to evaluate the relative abundance of soilborne *Phytophthora ramorum* in relation to other sources of inoculum within infested tanoak forests of southwestern Oregon. These data will add to the existing knowledge of the significance of the soilborne phase of *Phytophthora ramorum* as a pathway for the spread of disease on a local level. I approached this study with two objectives. The first objective was to compare the amount of inoculum washed down through the canopy with rainfall to that coming from the soil. Fieldwork was conducted within the GIA in Brookings, Oregon. Over the course of one rainy season, I collected rainwater from six different sites and attempted to quantify the amount of inoculum captured using qPCR. To assess the presence of *P. ramorum* in the environment, leaf baits were set out during collection periods to verify the presence or absence of the pathogen. The second objective was to quantify the

inoculum in relation to soil depth. Few studies have evaluated where in the soil profile *P. ramorum* resides within naturally infested forest soils. At three distinct time periods within the same rainy season, soil cores were collected from each of the six field sites described above. Each column was divided into sections by depth and I attempted to quantify the amount of inoculum captured via qPCR. Soils were also baited to verify the presence or absence of the pathogen.

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Chapter 2: *Phytophthora ramorum* within an Oregon tanoak forest: Quantifying inoculum within canopy throughfall vs. soil splash

Abstract

Phytophthora ramorum continues to cause extensive mortality of tanoaks in southwestern Oregon. Rain washes inoculum down through the canopy, causing new infections on the lower parts of the tree and neighboring host plants. Although this aspect of dispersal is well understood, the relative importance of infested soil and leaf litter as contributing factors to the spread of disease remains unclear. The primary objectives of this study were: (i) to compare the amount of inoculum washed down through the canopy to that splashed up from soil and litter, and (ii) to detect and quantify inoculum in relation to soil depth. Over the course of the 2014-2015 rainy season, rainwater was collected 8 times and soil sampled 3 times, from 5 sites within the Generally Infested Area in Brookings, Oregon. Both sources were subject to qPCR and traditional baiting methods. *P. ramorum* was detected by qPCR more frequently in splash-up from the ground surface than from canopy throughfall. *P. ramorum* was only detected in soil twice by qPCR and was never recovered by baiting. Even though both the canopy and bait buckets captured inoculum carried down in canopy throughfall, detection by baiting was more successful at detecting *P. ramorum* than qPCR. Quantification of *P. ramorum* was not possible because inoculum levels were too low and distribution too uneven for reliable detection with the methods employed.

Introduction

The invasive pathogen, *Phytophthora ramorum*, continues to cause extensive mortality of tanoak **(***Notholithocarpus densiflorus*) populations in southwestern Oregon. As the causal agent of sudden oak death (SOD), *P. ramorum* attacks many of the species found in these mixed woodlands, including Douglas-fir (*Psuedotsuga douglasii*), Pacific rhododendron (*Rhododendron macrophyllum)*, Oregon myrtlewood (*Umbellaria californica*), and evergreen huckleberry (*Vaccinium ovatum*). However tanoak is the most vulnerable host, bearing symptoms such as bleeding bole cankers and extensive canopy dieback which lead to tree mortality within as little as three years (Davidson et al. 2003). Since its first detection in 2001, an aggressive slash and burn eradication program was put in place in to stop the spread of the pathogen (Goheen et al. 2002; Hansen et al. 2008). However, due to the combination of favorable environmental conditions and the dense concentration of hosts, *P. ramorum* has become well established and continues to spread rapidly, presenting an ongoing challenge for the agencies involved in managing the disease and preventing further spread of SOD to new regions.

Like many *Phytophthora* spp., the dissemination of *P. ramorum* depends on sporangia borne on infected host tissue to sporulate and under conducive environmental conditions (Kliejunas 2010). *P. ramorum* is readily spread within infested forests by two pathways. In the “canopy throughfall” pathway, rainfall

carries sporangia and resultant zoospores down through the canopy, initiating infection on lower parts of the host and nearby susceptible understory plants. Alternatively inoculum can travel a "splash-up" pathway, where inoculum is transmitted from infested soil and leaf litter up onto adjacent host material by rain splash. Although there is greater evidence that this localized dispersal appears to be primarily achieved through the "canopy-throughfall" pathway, the overall degree to which these terrestrial sources of inoculum contribute to the initiation of new infections is largely unknown. This information would add to the existing knowledge of transmission biology of *P. ramorum* in natural settings.

Though traditionally classified as an aerially dispersing *Phytophthora* species (Hansen, Reeser, and Sutton 2012), *P. ramorum* has demonstrated the ability to persist in soil over extended periods of time. Chlamydospores are thought to be responsible for this prolonged survival because of their thick walls which allows them to persist through unfavorable environmental conditions (Fichtner, Lynch, and Rizzo 2009). In the United Kingdom, *P. ramorum* was detected in naturally infested garden soils at least two years after the removal of infected hosts (Turner et al. 2008; Elliot et al. 2012). *P. ramorum* was recovered from infected rhododendron leaves in the soil after 33 months (Vercauteren et al. 2013). Within areas subject to eradication treatments in Oregon, infected tanoak sprouts have been found after the removal of surrounding host plants (Goheen et al. 2002), giving evidence that soil splash can be a mechanism for new infections.

Infested leaf litter has also been shown to be a source of inoculum of *P. ramorum.* In California forests, inoculum was shown to be able to travel from infested leaf litter to the aboveground parts of a nearby bay laurel seedlings by way of splash dispersal (Davidson et al. 2005). Once incorporated into the soil the pathogen can survive for up to 49 weeks after burial, even throughout the dry summer months (Fitchner, Lynch, and Rizzo 2007). The transmission of inoculum from buried infected leaf debris to the surrounding bulk soil has been shown in field studies in California woodlands (Fitchner, Lynch, and Rizzo 2007). Through multi-loci gene analysis, Eyre et al. (2013) showed that the genotypes detected in soil were derived equally from soil and leaf populations from previous years. This molecular evidence provides strong support for the idea that soilborne populations are derived in part from foliar populations (Eyre et al. 2013). The fact that propagules in soil and litter are not easily observed is a risk for long distance transmission through the movement of potted host plants within the nursery trade. On a local scale, infested soil and litter on the forest floor are potential sources for new infections.

Most of the studies investigating *Phytophthora ramorum* in soil have relied on baiting techniques. Detection by baiting is prone to false negatives (Williams, Hardy, and O'Brien 2009; Than et al. 2013), as baiting relies on the viability of the pathogen. Viability is defined as the ability to culture the pathogen from a given substrate (Eyre et al. 2013). Traditional culturing methods often lack the sensitivity to detect soilborne pathogens whose viability may be seasonally influenced (Cooke, Schena, and Cacciola 2007; Sanzani et al. 2014). In addition, faster growing organisms can

conceal the presence of the pathogen of interest when culturing samples on media, especially when working with soil and water samples (Sanzani et al. 2014).

To overcome these obstacles, both conventional and quantitative PCR (qPCR) have proven to be effective tools in the field of phytopathology (Schena et al. 2004). In particular, TaqMan qPCR has gained popularity for its sensitivity and specificity, which are essential when screening for pathogens within environmental samples, which may contain small amounts of the pathogen. The fact that qPCR is much faster than conventional PCR makes it ideal for screening plant material for certification programs (Schena et al. 2013).

The combination of both cultural and molecular detection methods can be very informative, as demonstrated by an epidemiological garden study on *Phytophthora ramorum* and *Phytophora kernoviae* in Scotland (Elliot et al. 2012). Two types of unbaited spore traps were used to capture both rainfall and water splashed up from the soil surface. Soil samples were also collected over the course of a two-year study period. The inoculum from the spore traps and soil was quantified using qPCR. In addition, bait plants were used to detect viable inoculum present in the environment. Through quantification, researchers aimed to evaluate the connection between inoculum levels and disease development within these infested gardens. We chose to use this study as a framework to address similar questions related to the soilborne inoculum of *P. ramorum* within the GIA of Curry country, Oregon.

Our study aims to use qPCR to (i) compare the amount of inoculum washed down through the canopy to that splashed up from soil and litter, and (ii) quantify inoculum in relation to soil depth. Traditional baiting techniques were applied to both substrates as an additional approach for detection. Quantitative data on inoculum within infested soil and litter could provide an estimate of how much these reservoirs contribute to the inoculum pool within these infested forests. Such information may inform disease management plans aimed at mitigating the local spread of *Phytophthora ramorum*.

Materials and Methods

Description of Study Location

This study was conducted in the southwestern coastal town of Brookings, Oregon, located approximately ten miles north of the California border. Relative to the rest of the state, the weather is temperate. Winters are generally wet and mild, while summers are warm and dry with early morning fog. The mean maximum temperature is 21° C and the mean minimum temperature is 5°C (Taylor and Hannon 1999; Hansen et al. 2008). Figure 2.1 shows the average total monthly precipitation recorded between 1931 and 2002, showing that the rainy season begins in late fall and lasts through early spring. The greatest amount of rainfall, on average, occurs in winter and spring (Table 2.1).

Figure 2.1: Average total monthly precipitation in Brookings, Oregon from 1931 to 2002.
Source: http://www.wrcc.dri.edu/cgi-bin/cliMAIN.pl?orbroo

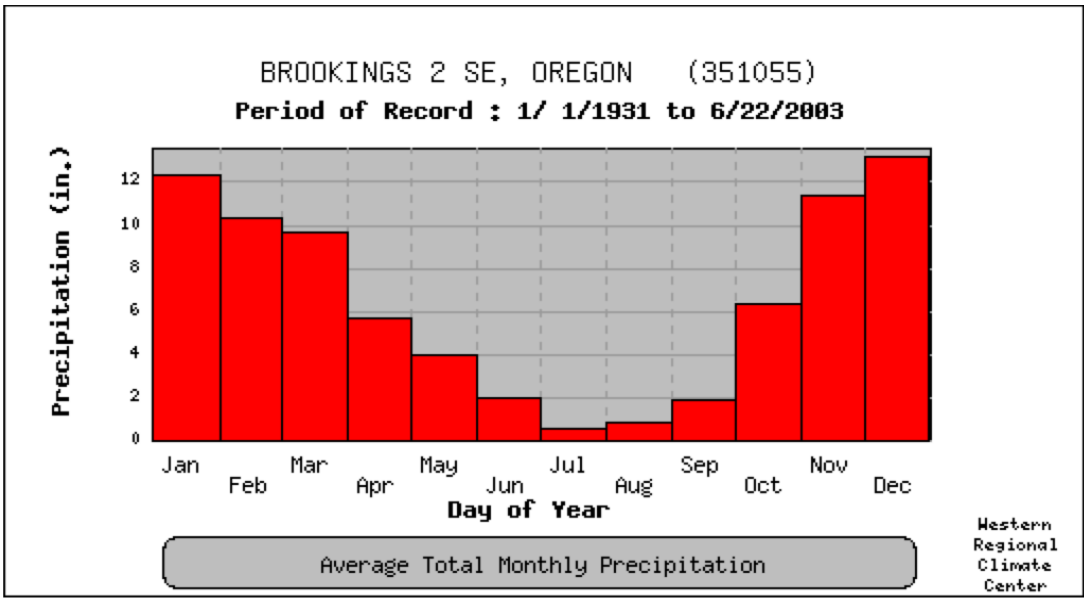


Table 2.1: Seasonal average precipitation levels in Brookings, Oregon from 1931 to 2002. Seasons are defined as: Winter = December, January, February; Spring=March, April, May; Summer= June, July, August; Fall=September, October, November.
Source: http://www.wrcc.dri.edu/cgi-bin/cliMAIN.pl?orbroo

Season	Winter	Spring	Summer	Fall
Precipitation (mm)	302	164	292	816

This region is characterized by mixed evergreen forests that are dominated by tanoak (*Notholithocarpus densiflorus*) and Douglas-fir (*Pseudotsuga menziesii*). Red alder (*Alnus rubra*) and Oregon myrtle (*Umbellularia californica*) are also commonly found within this forest type. Many of the associated species found in the understory

are known hosts of *P. ramorum*. Sword fern (*Polystichum munitum*), evergreen huckleberry (*Vaccinium ovatum*), Pacific rhododendron (*Rhododendron macrophyllum*), salal (*Gaultheria shallon*), and Oregon grape (*Berberis nervosa*) are some of the most common species (Atzet et al. 1996) This combination of a favorable climate and a high concentration of suitable hosts create a conducive environment for the pathogen's proliferation in this region.

Field Work

Site Selection

We chose to conduct this study within the Generally Infested Area (GIA) where eradication practices have ceased since early 2013 (Frankel and Palmieri 2014). Peterson et al. (2014) postulated that low recovery of *P. ramorum* from soils associated with roadways around the North Chetco watershed in Curry County was in part due to eradication treatments that may have reduced inoculum below a detectable level. With nearly two years without eradication treatments in the GIA, we anticipated that inoculum levels would have increased to levels above the detection threshold. Upon consultation with the Oregon Department of Forestry (ODF), five sites were chosen in the woodlands just above the town of Brookings, Oregon (Figure 2.2). Land in this area is under the ownership of U.S. Department of Interior Bureau of Land Management (BLM), South Coast Lumber (SCL), and public and private landowners.

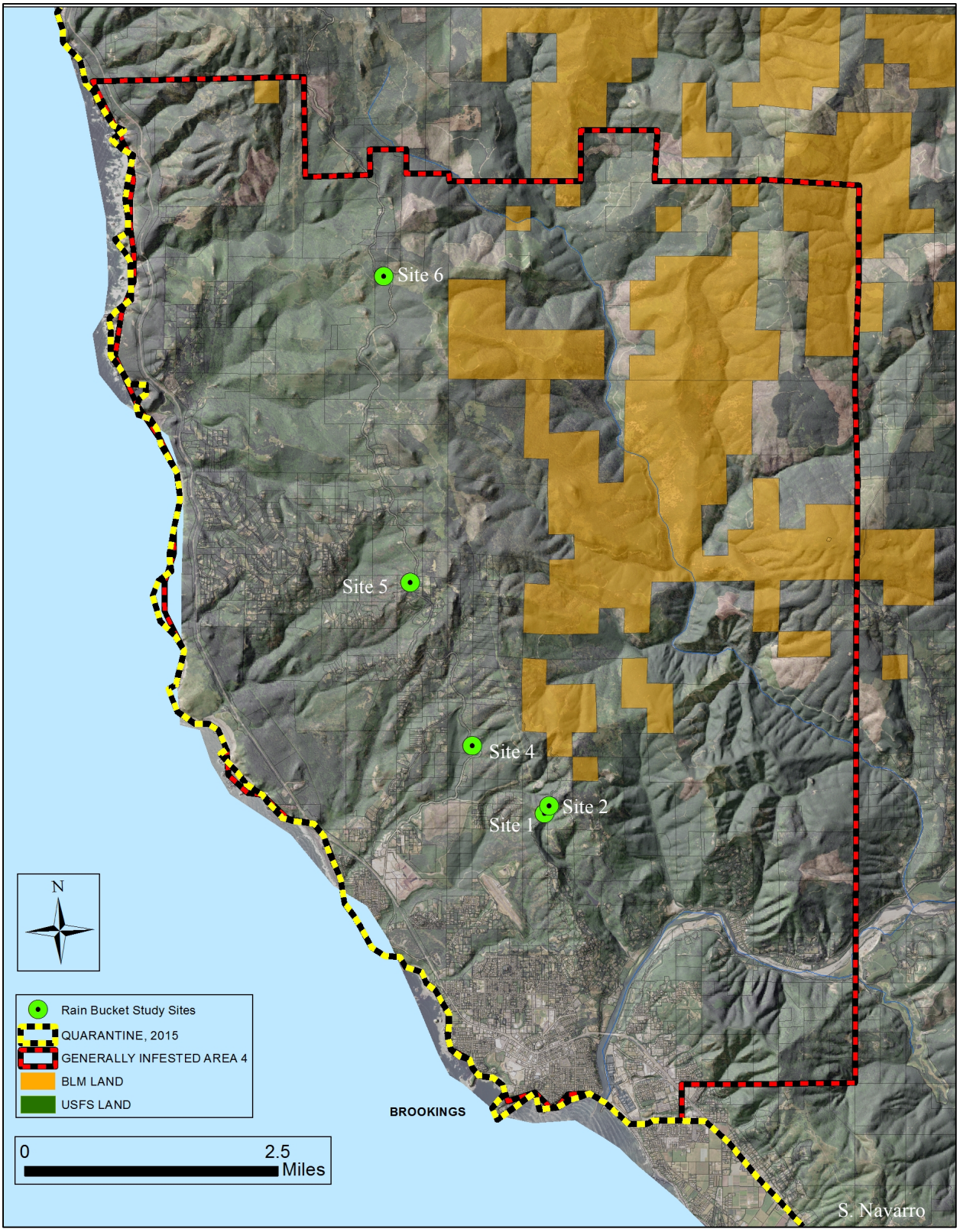


Figure 2.2: Location of five study sites selected within the GIA in Brookings, Oregon.

Plot Design

A schematic diagram of the plot design at each site is shown (Figure 2.3). At each site, a 60 m transect was laid out. Three radial plots were created at 0, 30, and 60 m along that transect. At each of those points, 'candidate trees' within 8 m were flagged and numbered if they fulfilled the following criteria: 1) must be a living tanoak, 2) must be of co-dominant, or average, height, and 3) must be at least 30 m away from any other tree in the neighboring plot. Co-dominant trees were selected as they best represented the canopy within a given stand. From the pool of candidate trees, a 'sampling tree' was randomly selected, from which all samples would be collected. The 30 m-separation criterion was set because it has been demonstrated that sporangia can travel as far as 15 m in a storm event (Davidson et al. , 2005). Setting the sampling trees 30 m apart would create a 15m radius around a single sampling tree, thereby minimizing influences of one sampling tree onto another. This selection process was repeated at each of the five sites, resulting in fifteen sampling trees.

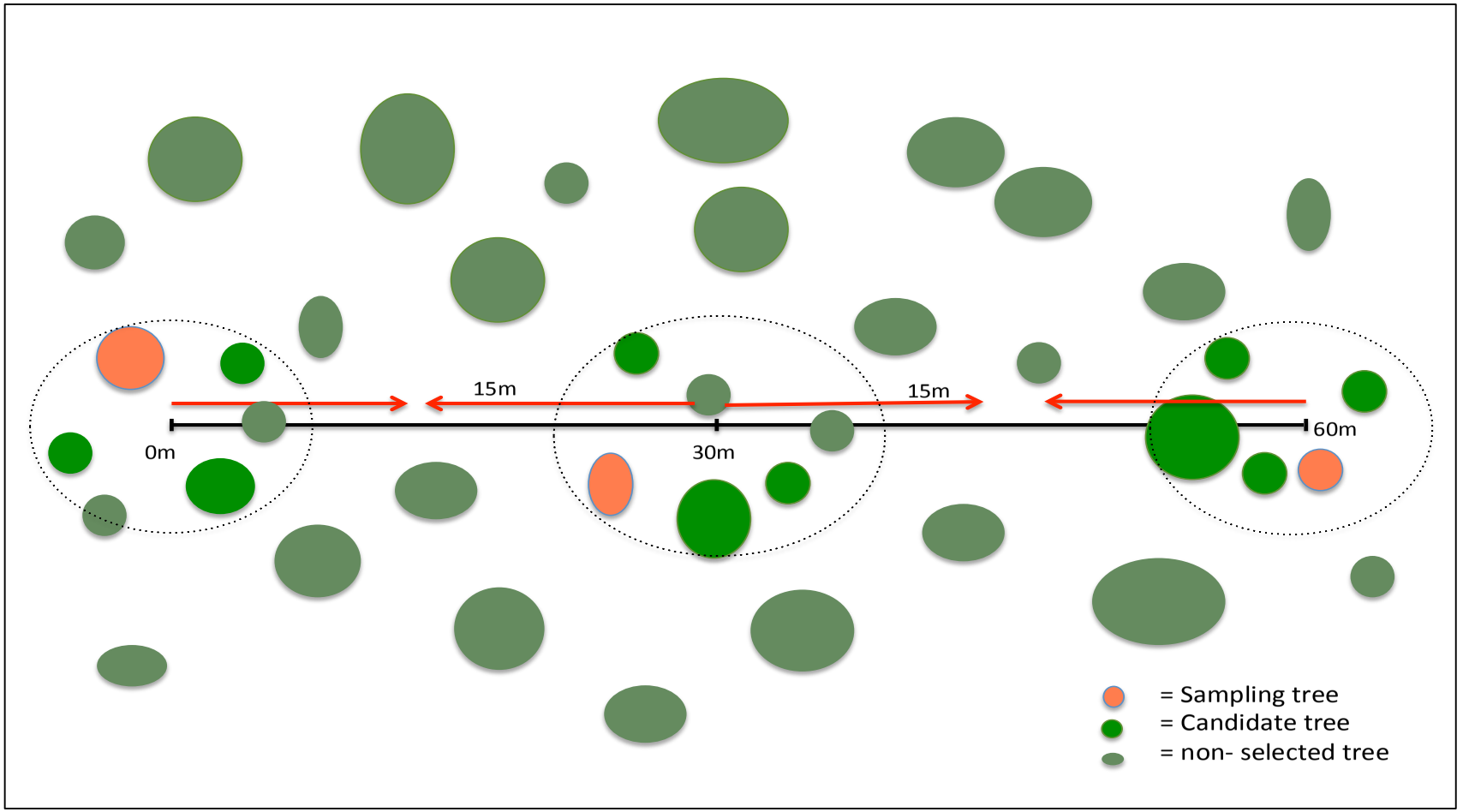
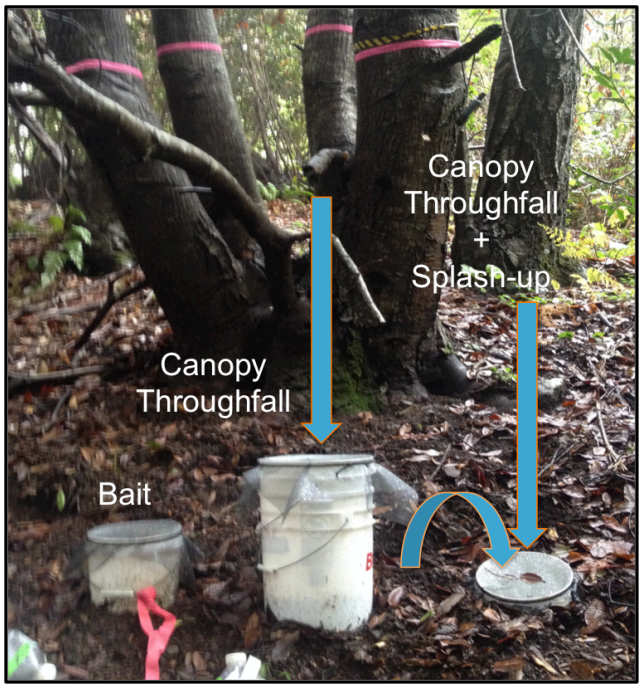


Figure 2.3: Schematic of site layout. Three plots were laid out within a single site. The dotted circles represent the 8m radius that was drawn out around a sampling tree. Variation in circle sizes was done to represent differences in canopy sizes within a stand

Rainwater Collection System

At each of the three sampling trees across all five plots, a tiered bucket system was set up. The tiered bucket system (Elliot et al. 2012) consisted of three buckets, each designed to address a different aim of our study (Figure 2.4).

Figure 2.4: Example of the tiered bucket system set-up under each sampling tree at each of the five plots.



To capture inoculum in canopy through-fall, a five gallon (18.92 L) plastic bucket was set atop the ground surface (canopy bucket). This bucket was nested within an identical bucket to raise the bucket off the ground. We believed this height (59.94 cm) would be high enough to prevent inoculum splashed up from the soil from entering the bucket. Adjacent to the canopy bucket a two-gallon (7.57 L) bucket (ground bucket) was set into the ground, leaving the top 5 cm of the bucket exposed above the ground surface. The ground bucket was intended to capture both inoculum

washed down through the canopy and that splashed-up from the soil surface. We hypothesized that we would be able to quantify the inoculum captured in both buckets, with the difference reflecting the quantity of inoculum derived from soil splash. Both the ground and canopy buckets were left unbaited, so as to not artificially induce inoculum growth or sporulation. The third bucket was used to monitor the presence or absence of *P. ramorum* within each plot over the course of the study in real-time. This 'bait bucket' was baited with three whole *Rhododendron* cv. Grandiflorum leaves. Leaves were brought from the Oregon State University greenhouses to ensure they were free of *Phytophthora* spp. and fungicides that could interfere with their use as baits. All buckets were lined with new plastic bags (W. Sutton, *personal communication*) and covered when it was not raining, using a mesh screen (fiberglass window screen) and plastic sheeting (3.5 mil) secured with a bungee cord. At the onset of rain, the plastic sheet covers were removed, leaving the mesh screen in place to prevent any large debris from falling in the bucket. Weather was monitored to predict rain events using the National Oceanic and Atmospheric Administration website (http://www.noaa.gov/weather). When rain was predicted, an ODF staff member from the Brookings office was contacted to remove the plastic covers. The time between rain onset and cover removal varied depending on staff availability. In practice, 1-2 days may have passed before the covers were removed.

Previous research by Hansen and colleagues in the Brookings area frequently employed the use of baited buckets lined with plastic bags to capture inoculum in rainfall for up to two weeks (W. Sutton, *personal communication)*. However, in our

study we chose to not use leaf baits as we did not want to artificially increase captured inoculum. It was unknown how long propagules in unbaited water would survive; therefore we limited our collected periods to 5 days. The plastic bags used were tested in the lab to check that they contained no compounds that would affect captured inoculum (Appendix A).

Sampling Procedure

Rainwater & Bait Buckets

Rainwater and leaf baits were collected at the same time. Before water was sampled, the depth of the water in the buckets was measured with a meter stick. The water was then mixed well and 1 liter was collected. Using the bucket dimensions, the total volume of water collected in the buckets was calculated. Leaf baits were retrieved and placed in a sealed plastic bag. All samples were stored on ice for transport to Oregon State University (OSU) for processing. Buckets were lined with fresh plastic bags and the bait bucket was filled with 500 mL of fresh deionized water and rhododendron leaves. All buckets were then re-covered until the next collection period. Rainwater and leaf baits were collected over eight different periods between October 2014 and April 2015.

Soil

Soil cores were taken at three time points between November 2014 and June 2015. At one meter from the base of each sampling tree, surface litter was collected and a soil core sample was taken (Hansen and Delatour 1999) using a Par Aide Foot

Extraction hole cutter (Elliot et al. 2012). Four cores were taken around each tree. In the field, extruded cores were cut into the following sections by depth: 0-5 cm, 5-10 cm, and 10-15 cm (Dart et al. 2007; Hansen and Delatour 1999). For each tree, the sections of the same depth, about 2 L in total, were pooled (Hansen and Delatour, 1999). Litter from each plot was also pooled. Litter and soil samples were stored on ice until brought back to OSU, where they were stored at 4°C until further processing. In addition to baiting from each soil depth, gravimetric moisture content was also measured (Appendix D). Approximately 20 g of each soil sample was weighed, dried at 105°C for 48 hours, and then reweighed (Fitchner, Lynch, and Rizzo 2007).

Environmental Monitoring

Average daily precipitation (mm) and average daily maximum and minimum temperatures (°C) were recorded from October 2014 to April 2015 using the RAWS. Ambient temperature was monitored hourly at each site by placing iButton data loggers (Embedded Data Systems, Lawrenceburg, KY) at two plots at each site. Soil temperature was monitored at every plot using iButton data loggers set to record temperature every 120 minutes. These were placed at the surface of the litter layer, at the soil surface just beneath the litter layer (0 cm), and 5 cm and 10 cm below the surface. All data loggers were retrieved and replaced approximately every 2.5 months. Three rounds of data loggers were deployed.

Detection by culturing

Bucket Baits

In the lab, leaf baits were placed inside a plastic crisper box with lid, lined with moist paper towels, and left to incubate at room temperature (18-20°C) for up to seven days to allow for lesion development. Leaves were then removed, rinsed with deionized water, and patted dry. From lesioned areas, ten disks (6 mm) were removed with a standard single hole-punch. If no lesions had developed, disks were punched out from areas near the petiole, rib, and tip. Disks were then plated onto *Phytophthora* selective media PARPH (17g corn meal agar with 200 mg ampicillin, 10 mg rifamycin, 20 mg pimaricin, 66.7 mg terrachlor, 50 mg hymexazol, 1 L water) and stored in the dark for up to 12 days. Plates were checked for colonies with morphological features characteristic of *P. ramorum* after three, seven, and ten days. In most cases, the presence of *P. ramorum* was apparent. In ambiguous cases, a hyphal tip transfer was made onto PAR (corn meal agar with 200 mg ampicillin, 10 mg rifamycin, 20 mg pimaricin, 1 L water) and re-evaluated. The number of infected leaves was totaled across plots and reported as frequency of infected baits per site.

Baiting from soil and leaf litter

Soil baiting was conducted in the lab at room temperature (18-20°C). Soil (500 cm^3) from each sampled depth was placed into a plastic bag (23 x 33 cm). Soil was then flooded with 500 mL of deionized (DI) water and allowed to sit for 48 hours before baiting (W. Sutton, *personal communication*). After flooding, soils were baited with two sachets made from window screen mesh (1.6 x 3.8 cm) each filled with 10 leaf disks excised from *Rhododendron* cv. Grandiflorum grown in a greenhouse on the OSU campus. After 4 days, the sachets were removed, dipped into a 10% bleach

solution, rinsed with DI water, and then patted dry (W. Sutton, *personal communication*). The disks were removed from sachets and plated on PARPH media. Growth was monitored and recorded in the same fashion as the bucket baits described previously. Soil collected from outside the GIA was used for setting up controls (R. Weisse, ODF, *personal communication*). Prior to setting up the controls, these soils were tested using conventional PCR and found to be negative for *P. ramorum*. For the positive controls, artificially infected leaf disks were incorporated into the soil. In the lab, whole tanoak leaves grown on the OSU campus were inoculated using a zoospore suspension of *P. ramorum* (Pr-2107 isolated from tanoak, Curry County, OR, July 2001). The leaves were left to incubate in a crisper box for up to 14 days, or until symptomatic. From lesioned areas, disks (6 mm) were removed with a standard single hole-punch and these were incorporated into the soil by hand. Negative controls had no artificially infected leaf disks added. Controls received the same treatment as field samples. The number of positive leaf disks were added up across plots and reported as frequency of infected baits by soil depth per site.

All collected leaf litter was used for baiting and was treated in the same manner as the soil. For a positive control, whole infected tanoak leaves were used. These were inoculated in the same fashion as the soil inoculum described previously. Uninfected whole tanoak leaves were used as a negative control. Controls received the same treatment as field samples. The number of positive leaf disks were totaled across plots and reported as frequency of infected baits per site.

Quantification

DNA Extraction: Rainwater

Water was filtered twice: first by pouring through a 35 µM nylon mesh and then through a Millipore membrane filter (nitrocellulose, 3.0 µM, SSWP#SSWPO4700) using a vacuum line and a 47 mm magnetic filter funnel (PALL Corporation, New York City, NY) (Figure 2.5). In most cases, a single liter of water was divided into smaller aliquots and each was filtered separately, as filters were prone to clogging due to large quantities of organic debris. Filters were rolled up using sterile forceps, placed into 1.5 mL centrifuge tubes, and frozen at -20 °C until extraction.

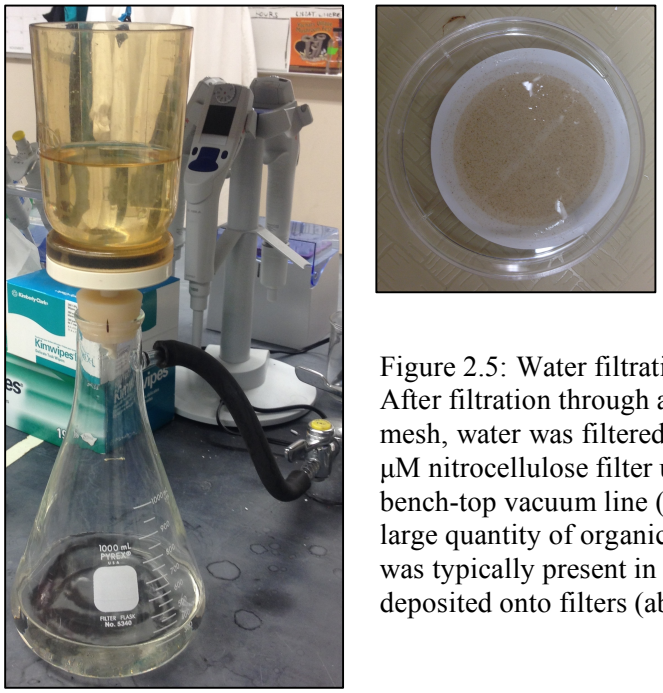


Figure 2.5: Water filtration set-up. After filtration through a 35 µM mesh, water was filtered onto a 3.0 µM nitrocellulose filter using a bench-top vacuum line (right). A large quantity of organic debris was typically present in water and deposited onto filters (above).

DNA was extracted from filters using an extraction kit (Fungal/Bacteria DNA MiniPrep, Zymo Research, Irvine, CA). Typically there were multiple filters per sample therefore the eluted DNA was pooled. One extraction positive control was

included in every set of extractions. These controls were made by adding 10^3 zoospores onto a filter before extraction. DNA samples were stored at -20°C until further use. Samples were organized and processed by time point of collection.

DNA Extraction: Soil

In preparation for DNA extraction, 100 cm^3 of each soil sample was first passed through a 2 mm sieve and then air dried for 2-5 days, depending on the initial wetness of the soil (Ippolito, Schena, and Nigro 2002; Elliot et al. 2015). Once dried, soil was sealed in an airtight bag and stored at room temperature until extraction. A total of 0.6 g of soil per sample was extracted using PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). A single preparation in the PowerSoil kit processes up to only 0.3 g of soil, thus two replicates per soil sample were extracted and the eluted DNA was pooled. One extraction positive control was included in every set of extractions. These controls were made by adding 10^3 zoospores to 0.6 g soil, which were then air dried, before extraction. DNA was stored at -20°C until further use. Samples were organized and processed by time of collection.

qPCR

Both rainwater and soil extracts were subjected to a TaqMan Real-Time PCR (quantitative or qPCR) assay based on the USDA-APHIS protocol as modified by researchers at Washington State University (Rollins, 2016.) for the quantification of zoospores of *Phytophthora ramorum* in water. The assay was performed on an Applied Biosystems 7500 RT-PCR System Thermocycler (Applied Biosystems,

Foster City, CA). Table 2.2 displays the sequences for the primers and probes used. The species-specific primers and probe target the ribosomal ITS region of *P. ramorum* (Martin, Tooley, and Blomquist 2004; Hughes et al. 2006). Samples and standards were spiked with salmon sperm DNA (Cao et al. 2012) to act as an internal positive control (IPC). The internal positive control was detected by the SKETA primers and probe (Rollins et al. 2016). The probe for *P. ramorum* was labeled with a FAM fluorescent dye on the 5' end whereas the SKETA probe was labeled with JOE fluorescent dye on the 5' end.

Table 2.2: Sequences and associated targets of primers and probes.

Assay	Primers and Probes (5'---> 3')		Reporter	Quencher
P. ramorum	Pram-114F	TCATGGCGAGCGCTTGA	FAM	BHQ_1
	Pram 1527-190R	AGTATATTCAGTATTTAGGAATGGGTTTAAAAAGT		
	Pram 1527-134T-probe	TTCGGGTCTGAGCTAGTAG		
Internal Positive Control	SKETA - F	GGTTTCCGCAGCTGGG	JOE	TAMRA
	SKETA22-R	CCGAGCCGTCCTGGTC		
	SKETA-probe	AGTCGCAGGCGGCCACCGT		

Both probes were labeled with non-fluorescent quenchers on the 3' end, BHQ_1 and TAMRA, respectively. Each 25 µL qPCR reaction was comprised of 23 µL of master mix solution and 2 µL sample DNA. The master mix solution contained 2X TaqMan Master Mix, primers and probes for *P. ramorum* and for the IPC, bovine serum albumin (BSA) to reduce inhibitory effects, trehalose to stabilize Taq polymerase, and water (Table 2.3). The following cycling conditions were used: 50°C for 2 min,

95°C for 10 min, 46 cycles of 95°C for 15 s and 58 °C for 15 s, ending with 60 °C for 30 s. Standards were created with genomic DNA of a *P. ramorum* isolate from Brookings, OR (Pr-2107 isolated from tanoak, Curry County, OR, July 2001). Standard curves were generated by creating a 10-fold dilution series ranging from 1ng to 1 x 10^{-6} ng of DNA. Samples and standards were generally run in triplicate. In some cases, samples were only run in duplicate. Each collection of samples was split across two 96-well plates (each plate referred to hereon as 'run 1' and 'run 2') and each run included a non-template control (NTC) and an extraction positive control. The NTC consisted of molecular grade water and the extraction positive controls were those described in the above extraction sections.

Table 2.3: Master mix reagents and amounts used

Reagent	Volume (µL)	Final
TaqMan Master Mix	12.5	1x
Pram F primer (100 µM)	0.0125	50nm
Pram R primer (100 µM)	0.05	200nM
Pram Probe (100 µM)	0.025	100nM
SKETA R (100 µM)	0.025	100nM
SKETA F (100 µM)	0.025	1uM
SKETA Probe (100 µM)	0.025	1uM
Trehalose 50%	2.375	5%
BSA (20 mg/mL)	0.125	
DNA template	*2*	
Salmon DNA	1	
Water (molecular grade)	6.8375	
Total	25	

Data Analysis:

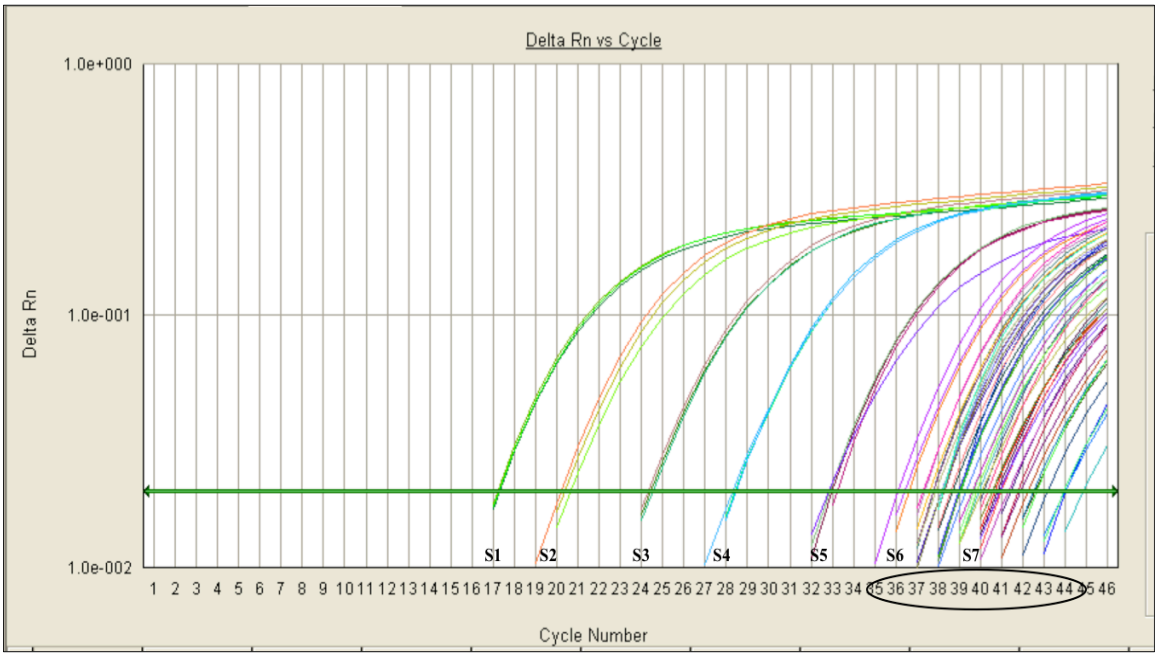
The original objectives of this work were to use qPCR to generate quantitative information on the inoculum of *Phytophthora ramorum* within rainfall, soil, and litter to estimate how much each of these sources contributes to the total inoculum pool within infested tanoak forests. Upon review of the raw data, there were several issues that warranted further consideration as they brought into question the inherent accuracy of the quantitative data collected.

The first issue was that approximately half of the reported quantities from the ABI software appeared to have been linearly extrapolated beyond the estimated range of sensitivity of this assay. This means that many samples were reported to contain less than $1.00E^{-06}$ ng of DNA; quantities smaller than the lowest concentration included in the standard series used. An assay's range of sensitivity (i.e. its upper and lower limits of detection) depends on the range of concentrations used in the standard series tested. Evaluation of the threshold values (Ct) showed that the average Ct of the lowest concentrations included in the standard series were between 37-40 (Table 2.4), whereas many samples had a Ct greater than 40 (Figure 2.6). This indicated that the dataset contained extrapolated quantities and because of the way quantities are calculated (Appendix I), extrapolated values should not be considered accurate because the assay may not be linear outside the range of the standard series tested.

Table 2.4: Average Ct values of the standard series. Each is the average of all the Ct values for a given standard across 16 PCR runs (N=48). Within each run, each standard was run in triplicate.

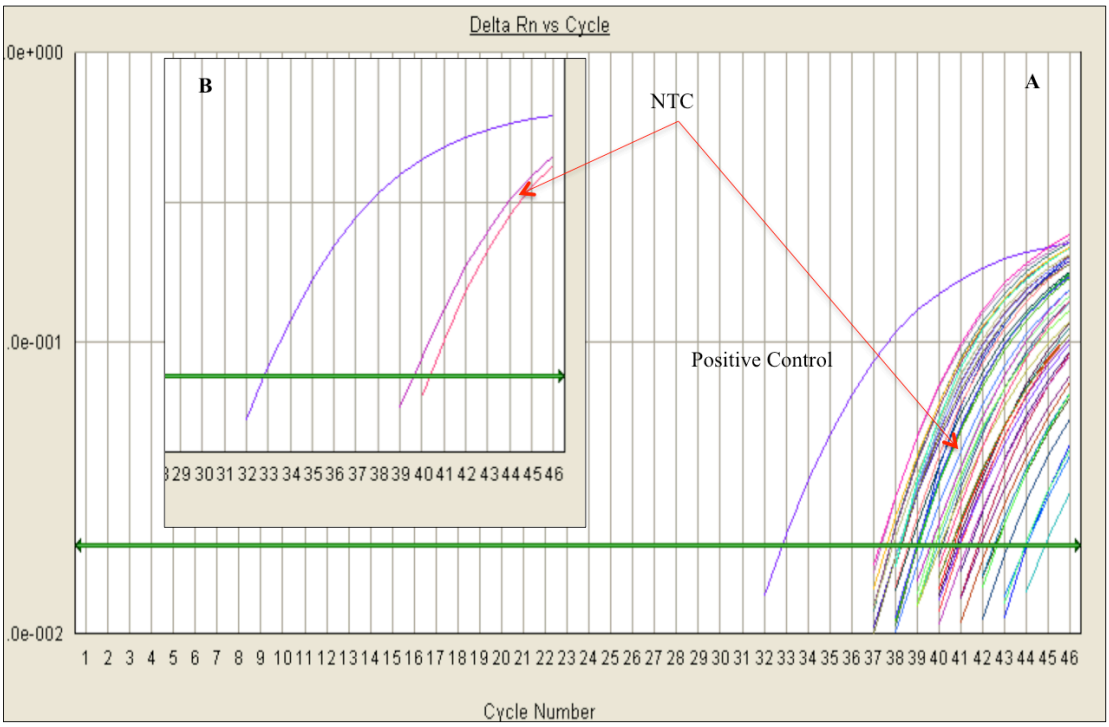
Concentration (ng/uL)	Standard	Average	stdev	Max	Min	Amplitude
1.00E+00	S1	16.7	0.4	17.8	16.1	1.7
1.00E-01	S2	20.3	0.7	24.0	18.9	5.0
1.00E-02	S3	24.8	1.4	30.1	21.7	8.4
1.00E-03	S4	28.8	1.4	31.6	24.6	7.0
1.00E-04	S5	33.7	1.0	36.5	31.8	4.7
1.00E-05	S6	37.3	1.8	41.5	31.6	10.0
1.00E-06	S7	40.5	1.8	43.2	35.5	7.7

Figure 2.6: Example of amplification curves. Standard spectra are labeled S1-S7. The circled Ct values indicate the range of sample Ct values. Roughly half of the sample Ct values lay beyond the lower range of the standard series.



The second issue concerned the non-template controls (NTC). This assay included two types of controls within each 96-well plate of qPCR samples run: an extraction positive control and a NTC. The NTC, which contains water in lieu of a DNA template, should show no signs of amplification. If amplification is observed, the signal should come up much later than the signal of the samples to avoid the interpretation of false positives. For example, a NTC with a Ct that is at least 8 Ct greater than that associated with the expected lower limit of the assay could be accepted (Eurogentex qPCR Guide, PDF). In regards to these data, the lower end of the standard series had on average a Ct 37- 40 (Table 2.4), thus the NTC would need to be as high as Ct 45-48 to be considered acceptable. However, the average Ct of a NTC was 39 (N=32). Although amplification was late in the cycle, the Ct lay within the range of Ct of the samples (Figure 2.7). Therefore because the NTC did not have Ct that were distinctive enough from the Ct of the samples, the control was interpreted to be invalid. As a consequence, the possibility that the data contained multiple false positives should not be ignored. For this reason, how the data would be used and interpreted had to be reevaluated.

Figure 2.7: Example of amplification curves illustrating that the non-template control (NTC) amplified within the same range as samples. A. Amplification curves of samples (Ct 37-45), NTC (Ct 39-40), and positive control (Ct 33). B. Close-up of the NTC spectra. Spectra of the standard series were removed for visual clarity.



For the reasons discussed above, the qPCR data was interpreted as the presence or absence of *P. ramorum* DNA by creating a binary dataset. Due to time constraints, collected leaf litter and soil from spring 2015 were not subject to qPCR analysis.

To begin, all extrapolated data were removed. Since the PCR reaction efficiencies were not consistent across qPCR runs, samples were evaluated by the qPCR run in which they were processed (a sample set). For each sample set, the mean Ct of the lowest concentration in the standard dilution series (S7) was used as a cutoff value (N=3). If a sample had a Ct value greater than this mean Ct, it was removed. A

binomial data set was generated from the remaining samples. To determine whether a sample would be considered as a positive or negative detection, a cutoff point was selected. The Ct of each sample was compared to the mean Ct of the NTC (mean NTC) from the same sample set because of the variability of NTC values between qPCR runs, which ranged from 36.5 to 41.5. The mean NTC was used as a baseline: a sample was assigned a value of 1 if the Ct was greater than the mean NTC (positive detection). A value of 0 was assigned if the Ct was less than the mean NTC (negative detection). A given time period was considered as one collection of samples. The resultant binary dataset included 133 samples.

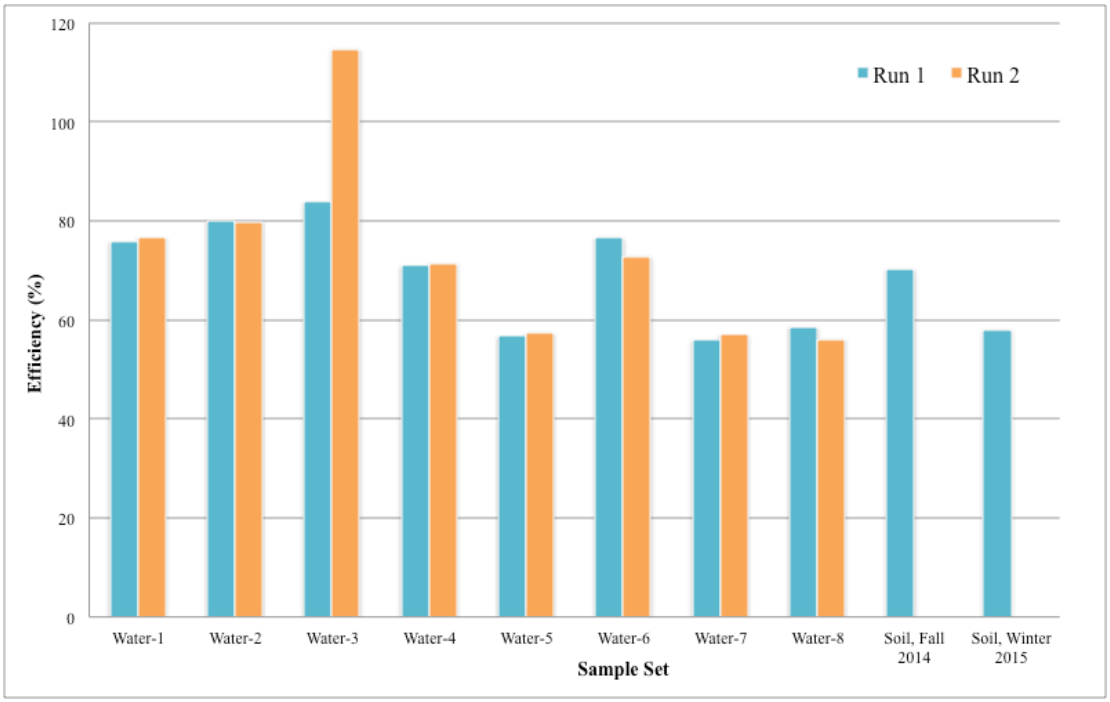
Using this data, we were able to ask what the odds were of having a positive detection of *P. ramorum* in the ground bucket as compared to the canopy bucket. To evaluate the odds of finding a positive detection in either of the two treatment groups (the canopy bucket or ground bucket), the data were put into a general linearized mixed effects (GLMM) model using R studio (R core Team 2015). This model was selected as it has the power to deal with nested fixed and random effects, as well as variables that have non-normal distributions. The fixed effect was the bucket type (treatment). The random effects were the time of collection, plot, site, and the sample set (i.e. run 1 or run 2). There were multiple levels of nested random effects: plots were nested within site and the samples were nested within a qPCR run. The singular assumption of the GLMM model is the assumption of over dispersion, which allows the response measurements to have a finite amount of variability. The assumption of over dispersion was not violated (overdispersion = 0.41)

Lastly, there was an issue of high variability in amplification efficiencies amongst qPCR runs. For data from different PCR runs to be comparable, the amplification efficiency should be consistent. PCR reaction efficiency (E) is influenced by the slope of the standard curve (*m*):

$$E = 10^{(-1/m)} - 1$$

Thus small differences in slope can result in fairly different efficiencies. PCR efficiencies of 90-105% (-3.6>*m*>-3.3) are considered to be acceptable. Our results yielded efficiencies that ranged from 56 to 84%, with one anomaly at 114 % (Figure 2.8). Low efficiencies (<90%) indicate a low number of amplicons of the target was accumulated and measured, which could be due to poor primer design or PCR inhibitors. High efficiencies (>100%) can indicate pipetting errors or the amplification of non-specific products (BioRad, Real-Time PCR Applications Guide, PDF). Therefore, because the efficiencies were variable across PCR runs, data was evaluated by sample set.

Figure 2.8: Comparison of PCR efficiencies across runs. ‘Water’ represents collected rainwater from both canopy and ground buckets. ‘Run’ describes the PCR run, as each collection of samples was split across two PCR runs (with the exception of soil samples).



Results

Detection of *Phytophthora ramorum* by qPCR

Rainwater

Phytophthora ramorum was detected in both the canopy and ground buckets between October 2014 and April 2015. We estimated that the median odds of detecting *P. ramorum* in the ground buckets was significantly greater than in the canopy bucket (z-value = 2.947, p-value = 0.003). Over the duration of the study there were 30 positive detections of *P. ramorum* in the ground buckets compared to 21 in the canopy buckets (Figure 2.9). In general *P. ramorum* was not detected in the canopy and ground buckets within the same site. There were only 10 occasions when this did occur: at site 6 (October 27), at all sites (November 11), at site 2 (November 17 and December 10), and sites 4 and 5 (February 6). The November 11th collection had the greatest number of detections, with 17 in the canopy and ground bucket samples combined. The total amount of rainfall during this collection period was 58.6 mm (Figure 2.10B). The February 27th collection had the lowest amount of rainfall (21.8 mm) and the lowest number of detections - only one in the ground bucket of site 2. Most of the successful detections occurred between October and early February, after which they markedly dropped. Although the March 20th collection did coincide with heavy spring rain event (Figure 2.10B), this did not result in a spike of positive detections in either the ground or canopy buckets.

Figure 2.9: A comparison of the recovery of *Phytophthora ramorum* from different sources over the 8 different collection periods. Both 'Canopy' and 'Ground' reflect the total number of positive detections using qPCR. 'Bait' reflects the total number of positive detections using traditional baiting of canopy flow through. Total bar height is the cumulative number of positives at any given site and time. S= site.

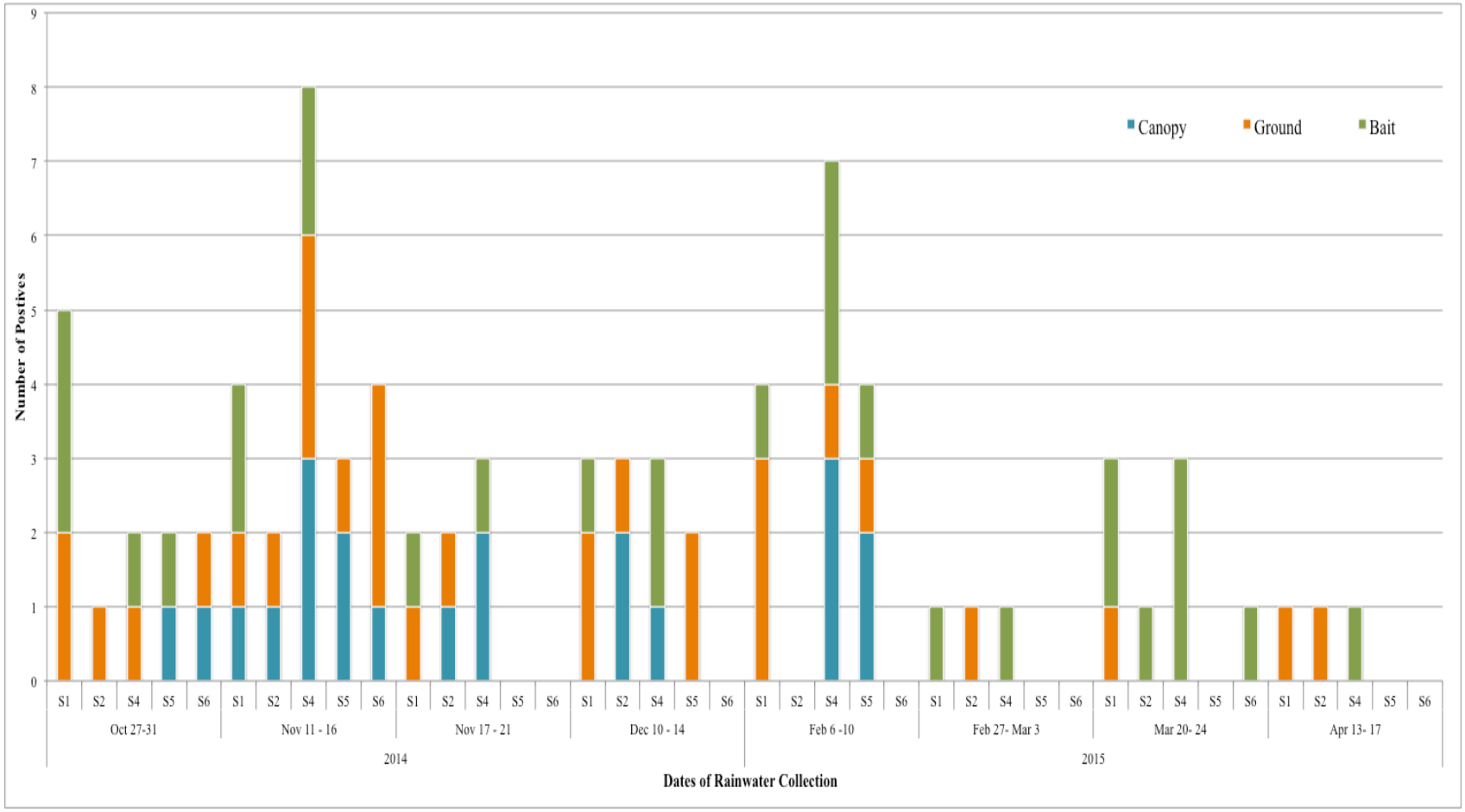


Figure 2.10: Recorded weather per five-day collection periods from October 2014 through April 2015.

A. Mean average temperature and associated mean maximum and minimums.
B. Total rainfall (mm). Reported weather data from the local RAWS in Red Mound, Oregon.

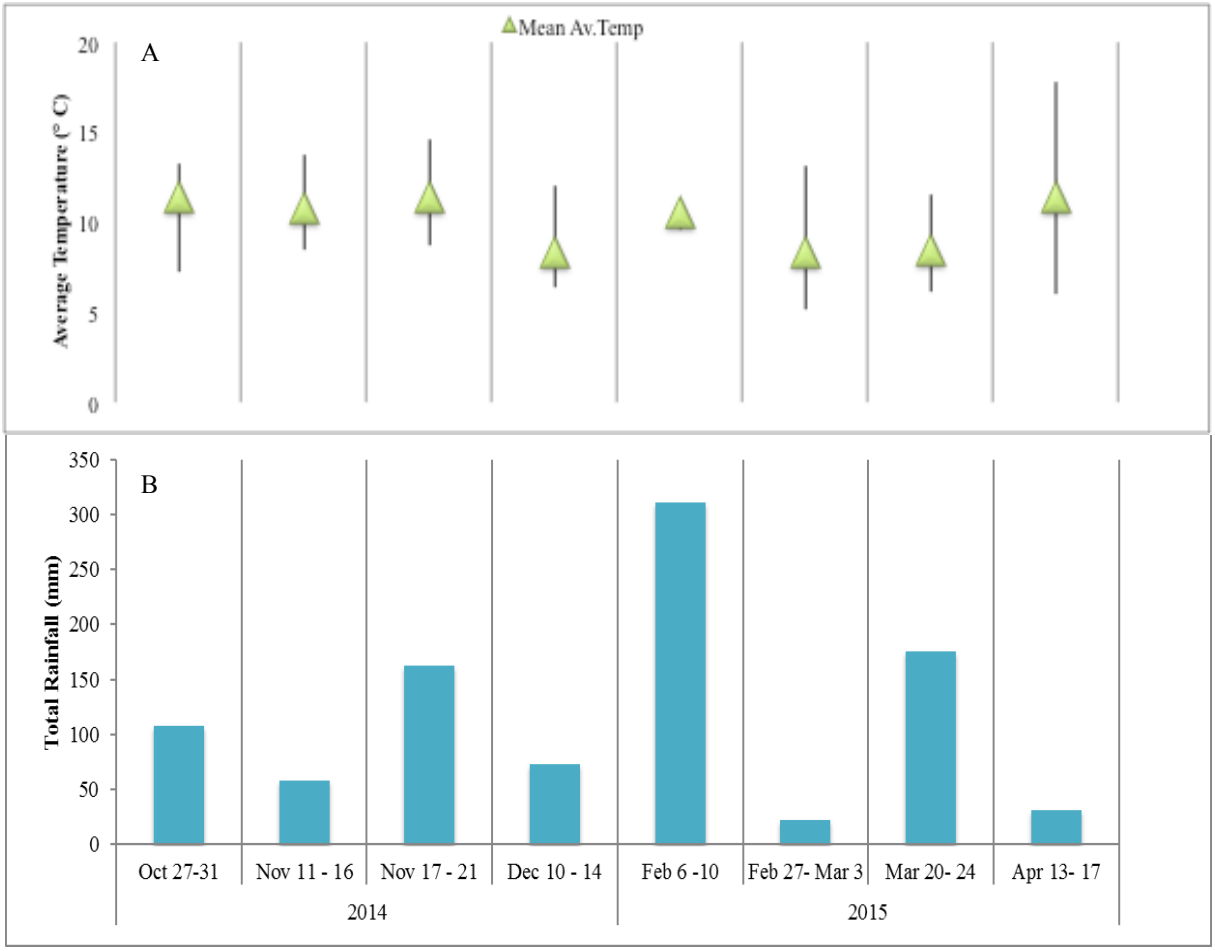
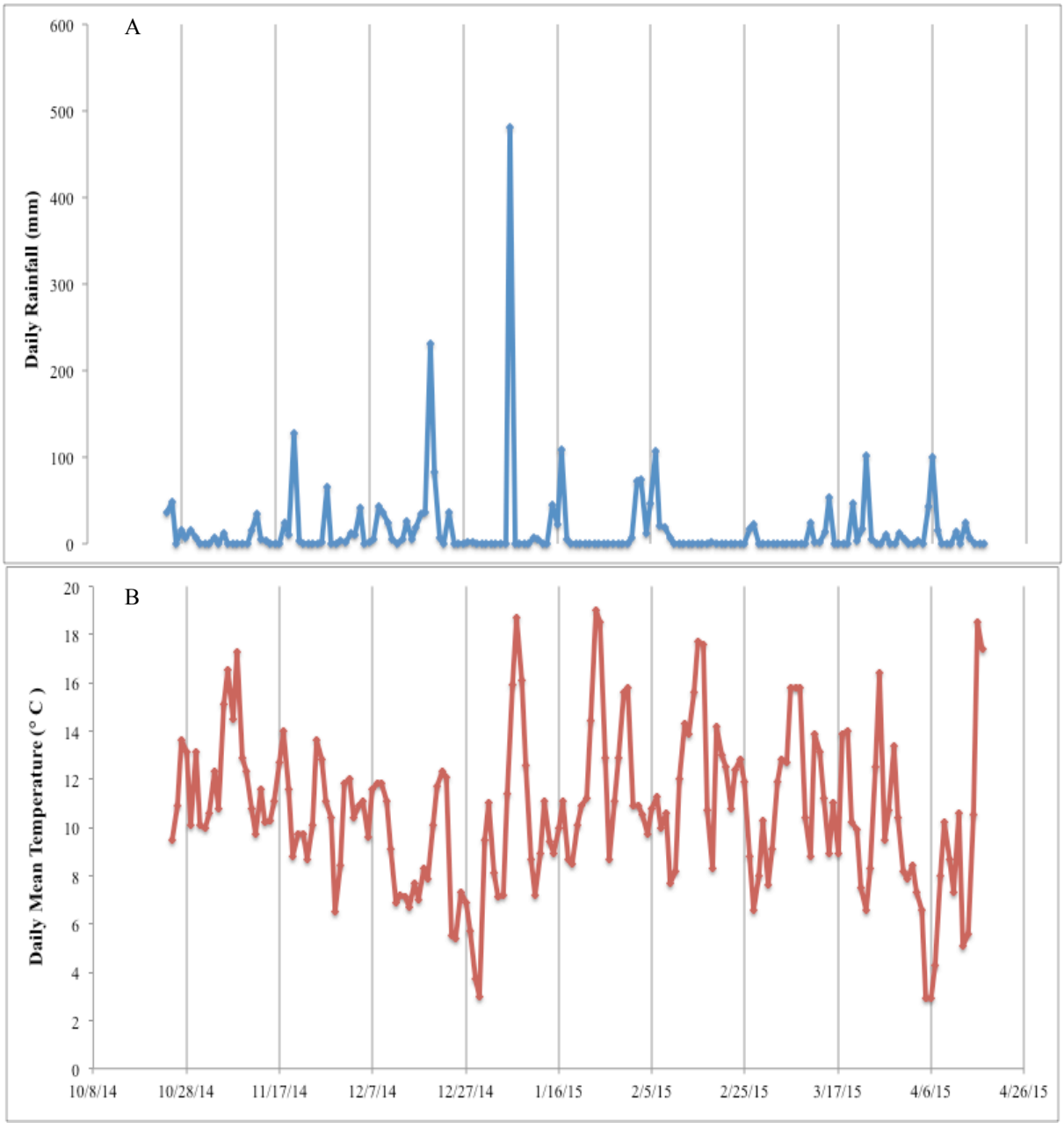


Figure 2.11: Daily rainfall and mean temperature from October 2014 through April 2015 as recorded from the nearest RAWS in Red Mound, Oregon.

A. Daily rainfall (mm) B. Daily mean temperature (°C).



Between October 2014 and May 2015, there was roughly 2.54 m total rainfall recorded. This was greater than the previous year (1.99 m) during this same time frame (Appendix F). Total annual rainfall in 2014 was significantly greater than the previous year, with 3.5 m of total rainfall compared to only 1.87 m (Appendix G). During our study, the average daily rainfall was 15 mm (Figure 11A). There were several larger rain events, with the most extreme being in early January (481 mm). Average daily mean temperature was 10.7 °C (Figure 11B). The dry spring aside, conditions during our study period would seem to be conducive to sporulation events, increasing our likelihood of detection, yet this did not seem to be the case.

Soil

P. ramorum was only detected twice, at sites 2 and 5, both in the February 27th collection period (Table 2.5). Only once was there a positive detection both in the 5 cm soil and ground bucket at the same plot; this was at site 2-plot B in the Feb 27th collection. Due to time constraints all soil samples collected in May 2015 (spring time point), and those at the 10 cm and 15 cm depths from the November and March sampling times, and litter samples were not run through the qPCR assay.

Table 2.5: Summary of detection of *P. ramorum* in soil at the 5 cm depth by qPCR for two collection periods. Numbers indicate the total number of plots with positive detections within a site. A "-" indicates that *P. ramorum* was not detected within that site

Date Collected	Site 1	Site 2	Site 4	Site 5	Site 6
Nov 17, 2014 2014	-	-	-	-	-
Feb 27, 2015	-	1	-	1	-

Detection of *Phytophthora ramorum* by baiting

Rainwater

Successful detection of *P. ramorum* by baiting of collected canopy throughfall occurred 29 out of 40 times over the course of the study period (Figure 2.9). The March 20th collection had the greatest recovery of *P. ramorum*, with 7 positive baits. The previous and following collection periods had the lowest recoveries, with only 2 and 1, positive respectively. Positive recovery from bucket baits varied in magnitude across sites over time. Recovery was the highest at site 4, which had 14 of 120 positive detections. *P. ramorum* was detected only once from sites 2 and 6. These two detections both occurred during the singular heavy rain event in March. The April 11 collection had only one positive detection (site 4) and experienced the second to lowest amount of rainfall (31.5 mm). The period with the greatest amount of rainfall (February 6), with just over 300 mm, had 5 positive detections. Ambient temperatures ranged from 8.3 to 11.4 °C (Figure 2.11A). Recovery was also variable among plots within a site (Appendix C)

Soil and Litter

P. ramorum was never recovered from soil or leaf litter by baiting in any of the three sampling periods (Table 2.6 and Table 2.7). Recovery frequency from the positive controls was 25%, 10%, and 30%. For the winter and spring sampling times, the prepared inoculum used for the positive controls was plated onto PARPH media as a quality control check of the media. Recovery was 40% and 50% for winter and spring respectively, implying that the lack of recovery from baits was not due to insufficient growth media quality. The recovery from the positive controls in the baiting assay of leaf litter was much lower than was seen for the positive controls in the soil baiting.

Table 2.6: Frequency of recovery of *P. ramorum* from soil by baiting. Reported recovery is from 20 leaf disks. Date reported is the time soil was collected.

	Nov. 17, 2014			**Mar. 3, 2015**			**May 15, 2015**		
Site	**5cm**	**10cm**	**15cm**	**5cm**	**10cm**	**15cm**	**5cm**	**10cm**	**15cm**
1	0[a]	0[a]	0[b]	0[ab]	0[a]	0	0[ab]	0	0
2	0	0	0	0[b]	0	0	0	0[b]	0
4	0[b]	0[b]	0	0[b]	0[b]	0[b]	0[ab]	0[b]	0
5	0[b]	0[b]	0[b]	0[ab]	0[ab]	0	0[a]	0[b]	0[a]
6	0	0	0	0[b]	0[b]	0[b]	0[ab]	0[b]	0[a]
Negative Control		0			0			0	
Positive Control		25			10			30	
Control Inoculum[c]		NA			40			50	

[a] Pythiaceous species observed
[b] Other fungal growth observed
[c] 10 of the disks from the inoculum incorporated into the positive soil was plated directly onto PARPH

Table 2.7: Frequency of recovery of *P. ramorum* from leaf litter by baiting. Reported recovery is from 20 leaf disks. Date reported is the time soil was collected.

Site	Nov. 17, 2014	Mar. 3, 2015	May 15, 2015
1	0	0	0[a]
2	0	0[a]	0
4	0[a]	0	0
5	0[a]	0	0
6	0	0	0[a]
Negative Controls	0	0	0
Positive Controls	5	15	15

[a] Other fungal growth observed

Detection of *Phytophthora ramorum:* qPCR vs. Baiting

In general, qPCR and baiting did not yield the same result for any given sample. Results by either method varied across time and by site. Although both the canopy and bait buckets were capturing inoculum carried down in canopy throughfall, detection by baiting was slightly more successful at detecting *P. ramorum* than qPCR (29/40 vs. 21/40 incidences respectively). At times later in the sampling season, when *P. ramorum* was not detected in the canopy bucket, the pathogen was recovered from baits. Over the course of the study *P. ramorum* was detected in both canopy bucket (unbaited) and bait bucket only four times.

Discussion

Detection of *Phytophthora ramorum* by qPCR

Rainwater

Phytophthora ramorum was detected more frequently in the ground buckets than in the canopy buckets. In most cases, for any given plot, *P. ramorum* was detected only in the ground bucket indicating that the captured inoculum was derived from surrounding infested soil and perhaps litter, rather than from canopy throughfall. By placing the two buckets adjacent to one another, we thought the buckets would have at least an equal chance of capturing inoculum during a rain event. However, there were only 5 instances where there was a simultaneous detection in both buckets at the same plot (data not shown).

Rain traps have been a common tool used in many epidemiological studies focused on monitoring the presence of *P. ramorum* in infested forest systems (Davidson et al. 2005; Elliot et al. 2012; Hansen et al. 2008). In particular, our sampling methodology was closely modeled after an epidemiological garden study which also used unbaited rain traps (Elliot et al. 2012). Using qPCR, *P. ramorum* was detected 11 times over the 2-year study from traps left under a sporulating *Magnolia* tree. Of these detections, 4 were in the high level trap ("canopy") and 7 were in the low level trap ("ground"). Their data reported capturing on average 3.5 times the amount of DNA in their ground traps as compared to their canopy traps. The authors attributed the input of inoculum into the ground traps to be from infested soil. Unlike our traps,

which yielded inconsistent detections, Elliot et al. detected *P. ramorum* in both of their trap types 75% of the time. However they did not specify whether or not their two trap types were placed directly adjacent to one another or were just in the same general area. Davidson et al. (2005) also used unbaited traps to quantify inoculum within the bay laurel-tanoak woodlands of northern California. They reported inoculum levels of 0 CFU to very low amounts at the onset of the rainy season, with levels peaking during the warm spring rains. This lag time in sporulation detection was attributed to the time it takes the pathogen to break dormancy after the dry summer season. In Oregon, recovery results from baited rain buckets have shown that detection is possible year round (Hansen et al. 2008). Similarly, our baited buckets detected *P. ramorum* throughout the duration of the study, but the number of detections within both canopy and ground buckets peaked at the start of the rainy season and then dropped off by the end of winter. Between February and April, 41 out of 78 days experienced no rain. The heavy rain event captured during the March 20th collection resulted only in a spike in recovery by baiting, not qPCR.

There are several possibilities as to why our overall detection levels were low. For one, it is possible that captured inoculum and associated DNA was partially degraded by the time rainwater samples were collected and processed, thereby preventing sufficient detection by qPCR. Nucleases released into the environment by dying and lysed organisms can readily degrade DNA they encounter (Schena et al. 2013). Another is that although efforts were made to transport and process water as quickly as possible, the waiting time could have impacted the DNA quality. Water samples

could have been filtered using a hand-pump in the field, followed by storing filters immediately in extraction buffer on ice until they could be frozen. However, it is likely that processing one liter of water using a hand pump would have been problematic; vacuum filtration in the lab often proved to be very difficult using a vacuum pump due to abundant particulates in the water. Lastly, not all the water that was collected in the buckets was taken for processing. Subsamples of 1 L were taken, as it was often not practical to take back all the rainwater that was collected. For example, in early February over 12 L of rainwater was collected in some of the canopy buckets (Appendix H). Thus it is possible there was a severe dilution effect which reduced our ability to fully detect the amount of inoculum present.

Soil

Phytophthora ramorum was only detected twice with qPCR, both at the 5 cm depth during the winter sampling period. Both detections occurred in February, during the period with the least amount of rainfall (21.8 mm). Elliot et al. (2012) also reported measuring the highest levels of soil inoculum at periods of lowest rainfall (50 and 75 mm). They measured the greatest amounts of soil inoculum from under an infected *Magnolia* tree. At this location, inoculum levels persisted over the two-year study period and showed some seasonal variation.

Seasonal variation of soil inoculum has been observed in northern California, where *P. ramorum* was detected over the duration of the rainy season up until one month after the last rain event (Davidson et al. 2005) or as much as three months after the

last rain (Fitchner, Lynch, and Rizzo 2007). Recovery ceased over the dry summer months. Although our study did not span both the wet and dry seasons, we expected detections to be lowest at the onset of the rainy season and continue to rise over the next following months. Further studies would need to be conducted to investigate whether the populations of *P. ramorum* in Oregon forest soils experience the same seasonal patterns that have been observed in California.

In general detection frequency by qPCR was very low and there are several factors that could have contributed to this result. For one, the presence of PCR inhibitors within the DNA extracts cannot be fully discounted. Soil in particular is known to be problematic as it contains multiple compounds, such as polysaccharides and phenolic substances, which can inhibit DNA amplification and ultimately influence assay sensitivity (Schena, Hughes, and Cooke 2006). Inhibitors tend to primarily affect later cycles of amplification, which is problematic when the pathogen is present at very low levels (Sanzani et al. 2014). Additionally, the concentration of inhibitory substances can also vary widely among soil and plant types, and vary over small distances (O'Brien, Williams, and Hardy, 2009; Scala and Kerkhof 2000). For example, the fact that oospores of *Phytophthora capsici* were successfully detected in artificially inoculated soil but not directly from field soil extracts was attributed to inhibitors (Pavón, Babadoost, and Lambert 2007). Another assay for *Phytopthora* 'taxon Agathis' reported that the presence of soil inhibitors lowered their assay sensitivity by tenfold (Than et al. 2013). To account for the presence of inhibitory compounds present in the forest soil extracts tested in this study, a known amount of

P. ramorum DNA could have been added to a dilution series of DNA extracted from *P. ramorum*-free soil. By doing so, changes in PCR efficiency could have been monitored and a positive correlation between Ct values and the amount of DNA added should have been observed.

A large volume of water was processed for extraction, but sample size was a limitation when it came to soil. Most commercially available soil extraction kits are only able to process small amounts of soil. On average they can process less than 1g of soil per sample, with a few up to 10 g. Propagules in soil tend to be non-evenly distributed at small spatial scales, leading to a potentially high degree of variability between samples (O'Brien, Williams, and Hardy, 2009; Sanzani et al. 2014). Although large soil volumes were taken in the field (up to one liter), we were able to only extract a subsample size of 0.75 g. Thus with such small sample sizes, potentially uneven pathogen distribution within soils, and perhaps low inoculum levels to start, it is possible to have missed the pathogen entirely. Some studies have extracted from large sample sizes (up to 250 g) because of the need for large volumes to better address epidemiological questions (Elliot et al. 2015; Than et al. 2013). However both groups utilized methods much costlier than those more commonly available. Thus a balance must be struck between cost and the absolute minimum numbers of samples needed to adequately address the question of interest.

Even though litter samples were not evaluated by qPCR, tanoak leaves can present some potential challenges. Their sclerophyllous nature can prevent sufficient lysing of cell walls during DNA extraction. The leaves also contain relatively high concentrations of phenolics, which are known PCR inhibitors (Osmundson et al. 2013). To get around this, a combination of phenol-chloroform extraction method and a commercial extraction kit (L. Winton, *unpublished*) has been developed. However, this procedure involves extracting from small amounts of tissues (<0.5g), which again leads to the issue of extraction size. Attempts were made to scale up this procedure to process samples up to 1g (1-2 whole leaves), but this was never fully completed. Even at this scale it is questionable whether this would be sufficient to accurately capture and quantify the inoculum in this substrate type.

Detection of *Phytophthora ramorum* by baiting

Rainwater

In Curry County, Oregon, OSU researchers have long used baited rain traps to detect *P. ramorum* and other aerial *Phytophthora* (Hansen et al. 2008; Reeser et al. 2011) using both rhododendron and tanoak leaves as baits. In this study we used both baited and unbaited buckets to address two different aims. Baits amplify the captured inoculum by providing a substrate for growth and further sporulation, whereas leaving some buckets unbaited allowed for the capture of the original inoculum population. Our results showed that baiting proved to be more successful than qPCR in detecting *P. ramorum* in canopy throughfall, though only by a small margin.

Similar differences in sensitivity between recovery from baited and unbaited rain traps were seen in a study that attempted to quantify inoculum levels of *P. ramorum* within coastal redwood forests in Humbolt County, CA. (S. Lucas, M.S. Thesis, Oregon State University). *P. ramorum* was rarely detected in rain traps that were left unbaited. Part way through the study, Lucas deployed baits into the traps. This action resulted in an increase in detections, although recovery was still very low. Elliot et al. (2012) also had lower recovery from rainwater in unbaited traps when compared to recovery from baits. However, Elliot's design used a bait plant in lieu of placing detached leaves in a rainwater trap.

A major question is why our frequency of detection from the canopy throughfall was so much lower than expected. Mapping our study site locations along with known positive trees identified by the ODF since 2010 illustrates that sites 1, 2, and 5 are adjacent to trees previously determined to be positive as early as 2011 (Figure 2.12). Peterson et al. (2015) postulated that there is a two-year delay from when inoculum is introduced to the onset of overstory mortality at a site. This time delay allows sufficient time for newly produced inoculum to spread to nearby locations before the original infested trees cease to actively sporulate. Our study sites and sampling trees were selected during the summer of 2014, thus late stages of disease could explain the lack of detection in canopy throughfall. Decreased sporulation capacity could yield inoculum levels too low to detect by qPCR. In contrast, baiting likely enriched these low inoculum levels present in the canopy throughfall allowing for an increase in successful detections. Choosing live tanoak was a key factor taken into consideration

when selecting our sampling trees. However it is possible that the full extent of canopy dieback was not apparent from the forest floor, as viewing the entire canopy is difficult in these dense forest stands.

Soil & Litter

The lack of detection from both soil and litter is difficult to explain, especially since detections from ground buckets were greater than the canopy buckets, which implied that the input of inoculum into these ground buckets was coming from the surrounding soil and litter. However, the lack of successful detections by baiting is not an absolute indicator that *P. ramorum* was not present in these substrates. Baiting efficacy greatly depends on the viability of the pathogen, where viability is defined as the ability to culture a pathogen from a substrate (Eyre et al. 2013). Chlamydospores are thought to be the primary form of inoculum present in soil and they are embedded within infested litter (Fitchner, Lynch, and Rizzo 2007; Smith and Hansen 2008). In a lab setting, baiting with rhododendron leaves and pears failed to detect free chlamydospores that had been added to the soil (Fitchner, Lynch, and Rizzo 2007).

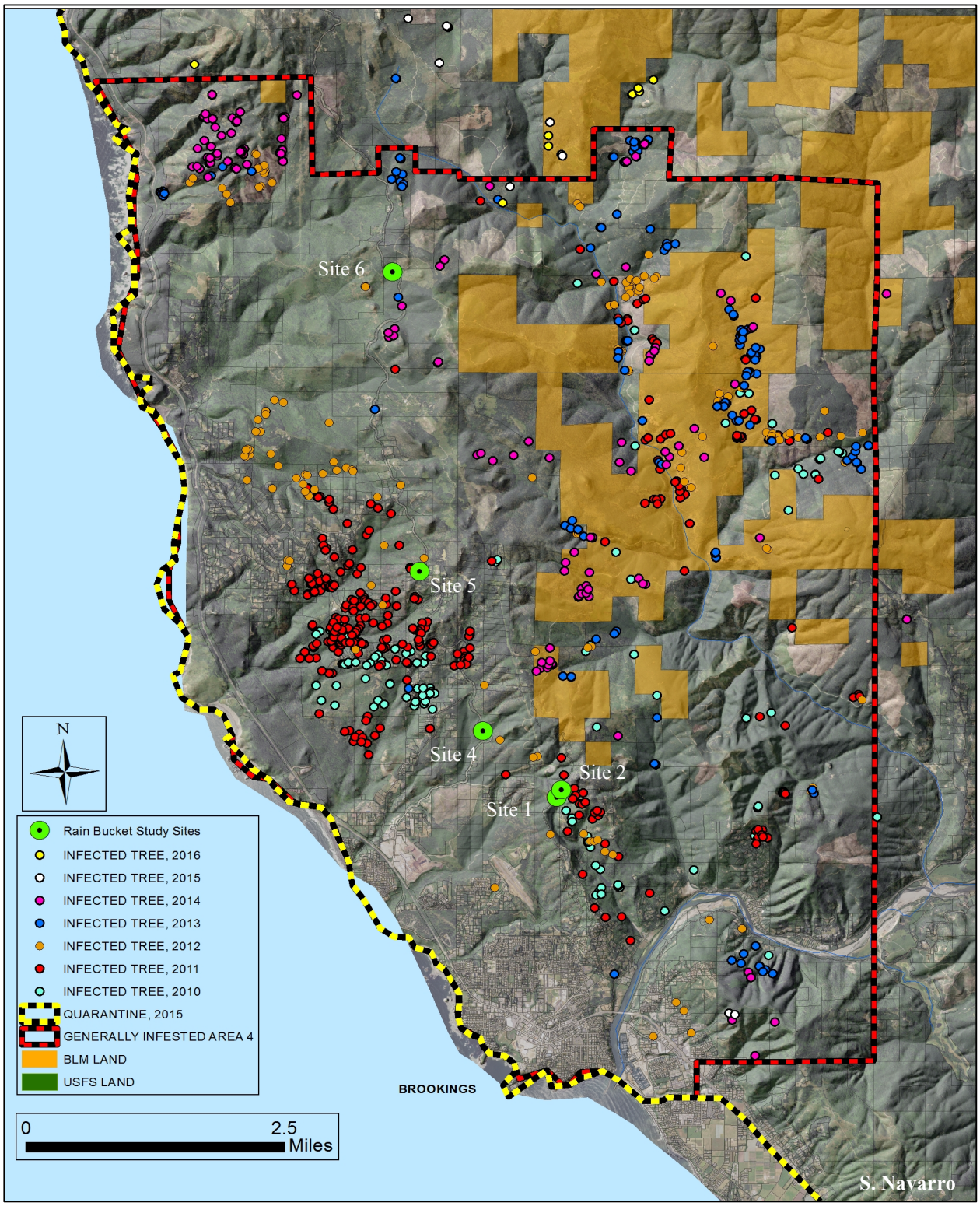


Figure 2.12: Location of five study sites in relation to confirmed positive sites by the Oregon Department of Forestry since 2011.

However, recovery from collected field soil by rhododendron leaf baits was consistently successful**.** Since rhododendron leaves failed to detect chlamydospores in the lab setting, it was presumed that the baits were colonized by zoospores produced by sporangia present in field soils, not chlamydospores. In our study it is possible that baiting from soil was ineffective because the pathogen existed primarily as chlamydospores that were dormant. Inoculum levels of *Phytophthora* increase with the onset of favorable environmental conditions, such as an increase in soil water content (Erwin and Ribiero 1996). For example, recovery from infected leaf baits was shown to increase after the baits were subject to a period of re-hydration (Fitchner, Lynch, and Rizzo 2007). The re-hydration method of baits was not attempted in this study, but perhaps this could have influenced the recovery rates. In addition to moisture, temperature has also been demonstrated to be an important environmental factor that can greatly influence germination because the chlamydospores of *P. ramorum* are able to germinate over a wide range of temperatures (Tooley, Browning, and Leighty 2014). It has been demonstrated that storing infested soil for 30 days at 4°C increases recovery (Tooley and Carras 2011). Soils were stored at 4°C before processing, but not as long as 30 days. Doing so could have influenced recovery rates.

Lastly, it is also possible that the concentration of hymexazol influenced the recovery from both baited soil and litter. Hymexazol is a standard component in selective media commonly used for the detection of *P. ramorum* (Jeffers, Martin, and others

1986). Although it is effective against ubiquitous and fast growing *Pythium* species, it has also been shown to be inhibitory to some *Phytophthora* spp. (Erwin and Ribiero 1996). For SOD surveys in Curry County, the Hansen lab (OSU) lowered the amount of hymexazol to 25 mg/ml so as to not overly suppress *P. ramorum* present in their samples (P. Reeser and W. Sutton, *personal communication*). Parke and Lewis (2007) also reduced the amount of hymexazol in their media due to reported slowed growth of *P. ramorum* in culture (Parke and Lewis 2007). Counter to this, 50 mg/ml is the concentration commonly used. Thus it is possible that the hymexazol concentration used had an inhibitory effect on the outgrowth of *P. ramorum* from baits.

Although we were not able to detect *P. ramorum* within tanoak forest soils within the GIA, this should not be taken to mean that *P. ramorum*, or other forest *Phytophthora* spp., are not found in the soil nor that they are not an important part of this system. It is through the intense survey efforts in post-eradication treatment areas in southwestern Oregon that the diversity of *Phytophthora* spp. present in these forests was revealed (Reeser et al. 2011). Their work spanned over four years and yielded nearly 6,000 soil samples collected from within eradicated areas. From these samples, *P. ramorum* was only recovered 2.5% of the time by baiting. Although a small percentage of the total, it was still the most frequently recovered *Phytophthora* species (Reeser et al. 2011). Roadside puddles have also been baited as an indicator of the presence of *P. ramorum* within soils in the area. In their study, *P. ramorum* was

recovered only 2 of 113 (1.8 %) times from baited puddles. However the authors did note that the eradication treatments might have reduced inoculum to below detectable levels (Peterson, Hansen, and Kanaskie 2014).

A note about qPCR

The qPCR data in this study was interpreted as the presence or absence of *P. ramorum* in the samples tested. As previously discussed, there were several issues that brought into question the inherent accuracy of the quantitative data collected. One of the primary issues was the amplification within the non-template control. Although a definitive cause was never found, the possibility of DNA contamination within one of the PCR reagents cannot be discounted. It is worth mentioning that both labs the PCR work was done in routinely handle *P. ramorum*, both as DNA and in culture. Therefore the reader should keep in mind the issues with the results presented here and some caution should be taken when interpreting the results presented in this study.

Even though qPCR is a valuable diagnostic tool for plant pathogens, there is room for improvement. There is a growing need for transparency among published assays to ensure that results across users are truly comparable (Bustin et al. 2009). This is especially critical for those working in similar systems. For example, there is a tendency to arbitrarily select the threshold value (Ct) (Caraguel et al. 2011; Chandelier, Planchon, and Oger 2010). The interpretation of qPCR results depends

on the Ct, which reflects the particular cycle where the fluorescence signal exceeds a defined background threshold. Therefore unless the assay has been fully evaluated for specificity and sensitivity, the Ct value will be chosen based on empirical data thus creating the opportunity for making false interpretations. It has been suggested that statistical methods be used to determine the exact cutoff for an assay, as this is critical in order to make reliable interpretations (Caraguel et al. 2011; Chandelier, Planchon, and Oger 2010). For example, a receiver operating characteristic (ROC) curve analysis can be used to statistically determine the limit of detection and optimal cycle cutoff (Nutz, Döll, and Karlovsky 2011). Rollins et al. (2016) used this method to determine that a Ct of 38 or greater to be interpreted as a negative result for detecting zoospores of *P. ramorum* in water. In contrast, Hughes et al. (2006) used the cutoff of Ct of 36 or greater to be a negative result for *P. ramorum* in plant material. The authors stated that this cutoff was arbitrarily chosen, as this was 10% below their limit of 40 cycles, which they considered to be enough of a buffer to avoid an interpretation of false negatives. Thus depending on when a cutoff is set, the interpretation of the data can lead to differing results even when the target of interest is the same.

Future Work

In more recent years, digital droplet PCR (ddPCR) has presented itself as a promising technology for the detection and quantification of pathogens within environmental samples. In short, ddPCR uses a water-oil emulsion system to partition DNA samples into thousands of nano-sized droplets and the PCR amplification occurs within each

droplet (Hindson et al. 2011). The major advantages of ddPCR are that it does not rely on the use of a standard curve for calibration and no internal controls are needed (Baker 2012). A main disadvantage to this platform is the cost since it involves expensive proprietary reagents and equipment. Furthermore, the ITS region may not be the best target, which is what many qPCR and PCR primers have been developed to target in plant pathogen assays. However, there are alternatives, such as mitochondrial DNA targets that could be used in the development of a ddPCR assay for the detection and quantification of *Phytophthora* (F. Martin, *personal communication*). As with any new application of a technology, much research and development is still needed for the platform to be applied to the field of plant pathology.

Conclusion

The goal of this work was to evaluate the inoculum levels of *Phytophthora ramorum* contained in rainwater, soil, and leaf litter collected within the GIA in Curry County, Oregon. Although *Phytophthora ramorum* was detected more frequently in the ground buckets than in the canopy buckets using qPCR, the pathogen was never recovered from soil by traditional baiting methods. Even though both the canopy and bait buckets captured inoculum carried down in canopy throughfall, detection by baiting was more successful at detecting *P. ramorum* than qPCR. Lastly, whether by baiting or qPCR, the frequency of detection was inconsistent across sites and within a single site over the duration of the study. These results indicate that inoculum levels may be much lower, and distribution more uneven, than we had believed,

contributing to some of the inconsistencies seen in our results. However, such conclusions cannot be fully supported because of the technical difficulties with the methods used in this study. The foremost recommendation is that the sample size, sample processing, and the qPCR assay used undergo further optimization to generate reliable data that could better address the questions of interest.

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Chapter 3: Concluding Remarks

A primary objective of this study was to gain a sense of how much inoculum is in the soil of tanoak infested forests within the GIA. Much of what we know about *P. ramorum* in forest soils comes from field studies conducted in northern California, where both the combination of different hosts and climate could be significant variables influencing our knowledge on the behavior and epidemiological significance of *Phytophthora ramorum* in soil. This knowledge could be greatly expanded by conducting more long-term studies, similar to work done by Fichtner et al. (2007, 2009), within newly infested areas in Oregon. These kinds of field studies may be critical now that the more aggressive EU1 lineage has been introduced.

Since the first report in the spring of 2015, the EU1 lineage has been detected in soil and streams in Curry County, Oregon near the initial infestation site (S. Navarro, *personal communication*). Although we know sporangia and zoospores are key players in the spread of disease, the full extent of the role of chlamydospores has yet to be understood, especially in soil. We know that chlamydospores of NA1 have lower germination rates relative to the NA2 or EU1 lineages (Tooley, 2014). Specifically, the Oregon NA1 isolate tested was shown to have the lowest germination rates when compared to other isolates of the same lineage (Tooley 2014; Smith, A.L., MS thesis, Oregon State University). Conversely, Tooley et al. showed that the EU1 lineage not only had the highest, but also the most consistent germination rates, when compared to the two North American lineages. Low and

infrequent germination ability could in part explain why recovery of *P. ramorum* from Oregon soils has been low (Peterson, Hansen, and Hulbert 2014; Reeser et al. 2011). It is a growing possibility that a new strain could emerge, which has both increased virulence and produces chlamydospores with greater germination capacity. The introduction of a new strain into the current population could drastically change how sudden oak death behaves, spreads, and ultimately affects our mitigation methods. Thus there is a serious need for new research investigating the potential increased pathogenicity and the epidemiological role of soil populations of *Phytophthora ramorum.*

Appendices

Appendix A: Plastic bag test

Purpose:

Bait buckets were lined with plastic bags to allow for ease of cleaning buckets between collections of rainwater. However, it was unclear whether these plastic bags contained any compounds that could potentially damage captured propagules. Moreover, it was unknown how long captured propagules would remain viable in the absence of leaf baits. The aims of this lab experiment was to:

1) Access whether the plastic bag used would inhibit the germination of captured propagules.

2) Test whether propagules could remain viable up to 5 days in the absence of leaf baits.

Methods:

A 2-gallon plastic bucket was lined with a single plastic bag (BiMart, 8gallon, 0.5 mil). Zoospores were produced by harvesting sporangia harvested from a two week old *P. ramorum* (strain #2027) culture grown on a 1/3 V8 agar (Jeffers 2006) and following the standard lab protocol for zoospore production (Parke lab). Using a hemocytometer, zoospores were counted and a suspension was made to the concentration of 10^3 sporangia /mL of deionized water. 150 mL ($1.5x10^5$ sporangia) of this suspension was then poured into the plastic bag lined bucket. On day 0, two rhododendron leaves were dipped into the suspension and then incubated in a crisper box lined with damp paper towels. The box was kept at room temperature (18 -20°C)

and monitored for up to 7 days, or until lesion development. This process was repeated daily, for up to 5 days.

Results:

All leaves developed lesions within 1 week of incubation (Figure A). Therefore, it did not appear that there were any compounds in the plastic that would be detrimental to the germination of sporangia and released zoospores.

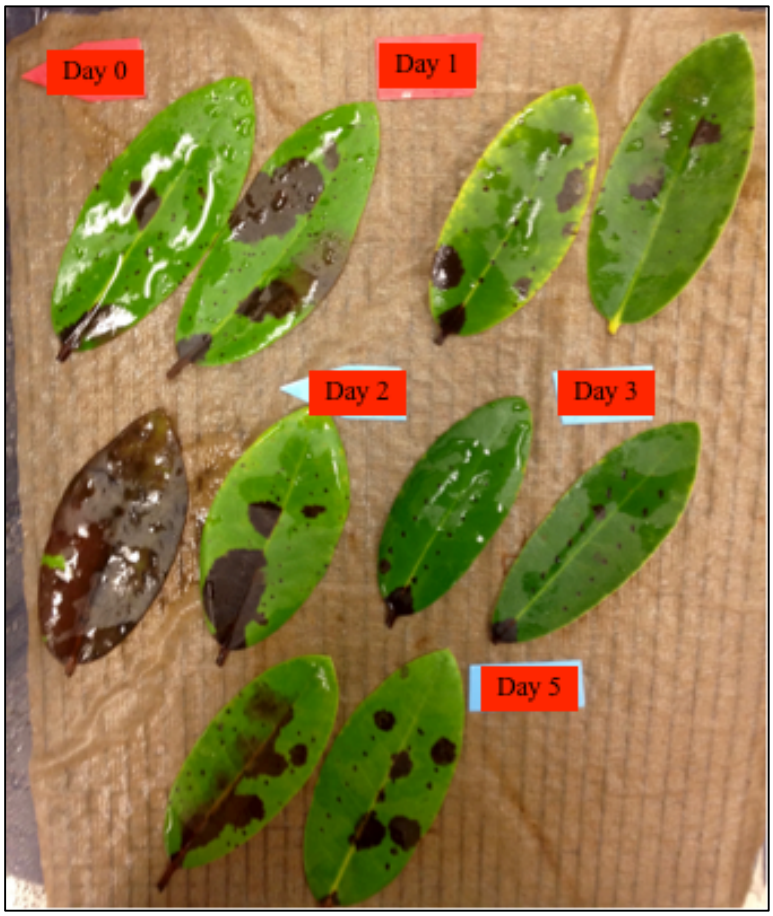


Figure A: Lesion development after dipping in zoospore suspension
Leaves were incubated at room temperature for up to 11 days.
A day 4 sample was not taken.

Appendix B: General description of field sites

Site-plot	Estimated Canopy Cover (%)	Dominant Understory Species	Estimated Ground Cover (%)	General Comments
1A	80	bare/litter	60	Plots A and B very close to road. Plot C further away and downhill from the other two; adjacent to felled ODF known positive tanoak. Dense tanoak stand immediately down hill from site with intermixed red alder. Most of the tanoaks in that stand exhibited strong canopy dieback.
		Huckleberry	40	
		bay	<1	
		fern	<1	
		Rhododendron	<1	
1B	70	Huckleberry	60	
		bare/Litter	40	
		Salal	<1	
		Rhododendron	<1	
		Tanoak saplings	<1	
1C	60	bare/litter	40	
		Salal	35	
		Fern	15	
		Rhododendron	5	
		Huckleberry	5	
		moss	<1	
		grass	<1	
		Tanoak saplings	<1	
2A	80	bare/litter	85	Western hemlock (30%) intermixed within tanoak stands. Very dark understory in plot A which appeared to richer soil than any other plot in study.
	20	Huckleberry	10	
		grass	5	
		Rhododendron	<1	
2B	80	Bare/litter	95	
		Huckleberry	5	
2C	70	bare/litter	80	
	30	Huckleberry	10	
		rhode	10	
		Salal	<1	
		Tanoak saplings	<1	

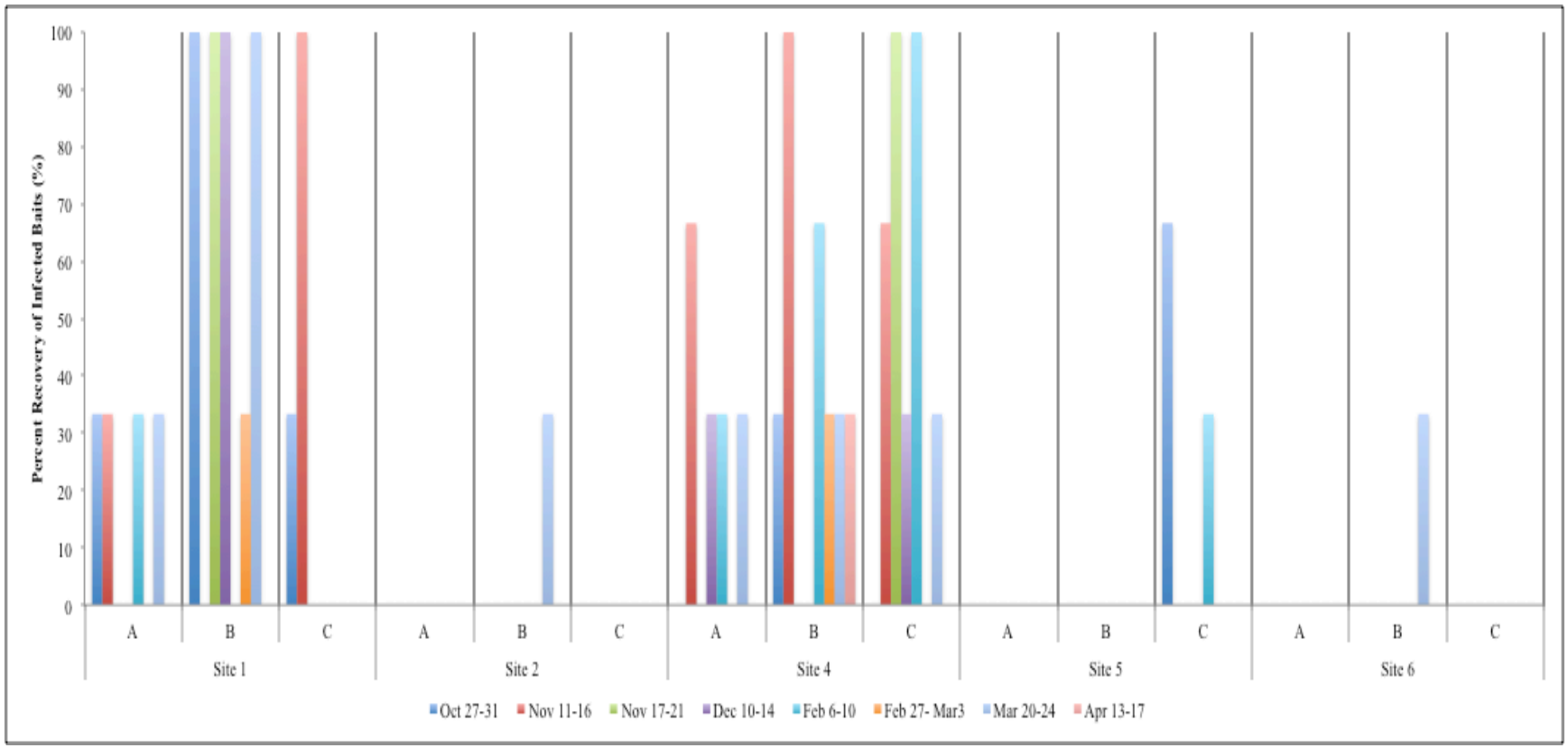
* Continued on next page

Site-plot	Estimated Canopy Cover (%)	Dominant Understory Spp	Estimated Ground Cover (%)	General Comments
4A	70	Huckleberry	60	Plots within this site were aligned along a slope: plot C was at the top, plot B was in the middle, and plot A was at the bottom. Dense stand of tanoak with at least 45% red alder mixed in. Over the duration of the study, this site experienced a fair amount of blow down. Multiple tanoaks around plot C fell.
		bare /litter	25	
		Fern	15	
		Tanoak saplings	>1	
		Rhododendron	>1	
		Alder sapling	>1	
4B	70	bare/litter	60	
		Fern	30	
		Huckleberry	15	
		Salmon berry	5	
		Tanoak saplings	<1	
4C	30	Fern	45	
		bare/litter	40	
		Rhododendron	<1	
		Huckleberry	<1	
		Salmon berry	<1	
		native blackberry	<1	
		violet	<1	

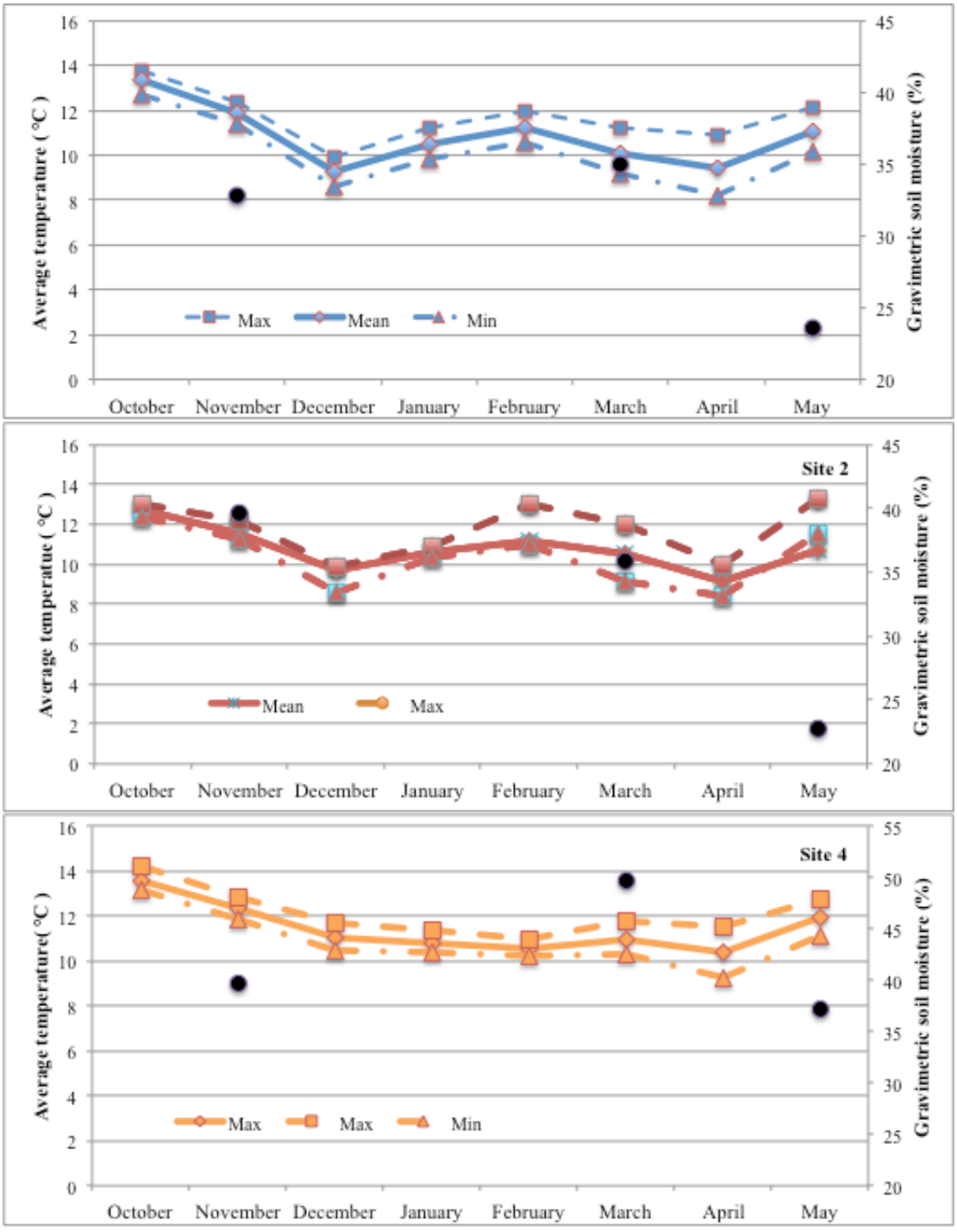
*Continued on next page

Site-plot	Estimated Canopy Cover (%)	Dominant Understory Spp	Estimated Ground Cover (%)	General Comments
5A	65	bare/litter	40	Plot is directly off the side of Carpenterville Rd.. Sampling tree is just downhill from ODF confirmed positive tanoak. Entire site is heavily dominated by tanoak
		Salal	30	
		Rhododendron	20	
		Fern	<1	
		Huckleberry	<1	
		Himalayan Blackberry	<1	
		Fox glove	<1	
		Tanoak saplings	<1	
5B	50	Salal	30	
		Bare/litter	55	
		Salmon berry	15	
		Nootka rose	<1	
		Blackberry	<1	
		Tanoak saplings	<1	
5C	40	Rhododendron	60	
		bare/litter	40	
		Fern	>1	
		Tanoak saplings	<1	
		Miners lettuce	<1	
		Salmon berry	<1	
		Oxalis	<1	
6A	45	bare/litter	70	Plot A sampling tree is a confirmed ODF positive tanoak. Adjacent to it are two small Oregon mrytlewood trees. Entire site is directly adjacent to large stand of Douglas-fir (South Coast Lumber). Very dense understory - darkest site of all.
		Thimble berry	25	
		Fern	5	
		Bay/Oregon Mrytle	<1	
		Nootka rose	<1	
		Wild cucumber	<1	
		Salal	<1	
		bedstraw	<1	
6B	95	bare/litter	90	
		Fern	10	
		Huckleberry	<1	
		Poison oak	<1	
6C	90	Bare/litter	80	
		Himalayan Blackberry	20	
		Fern	5	
		Salmon berry	5	

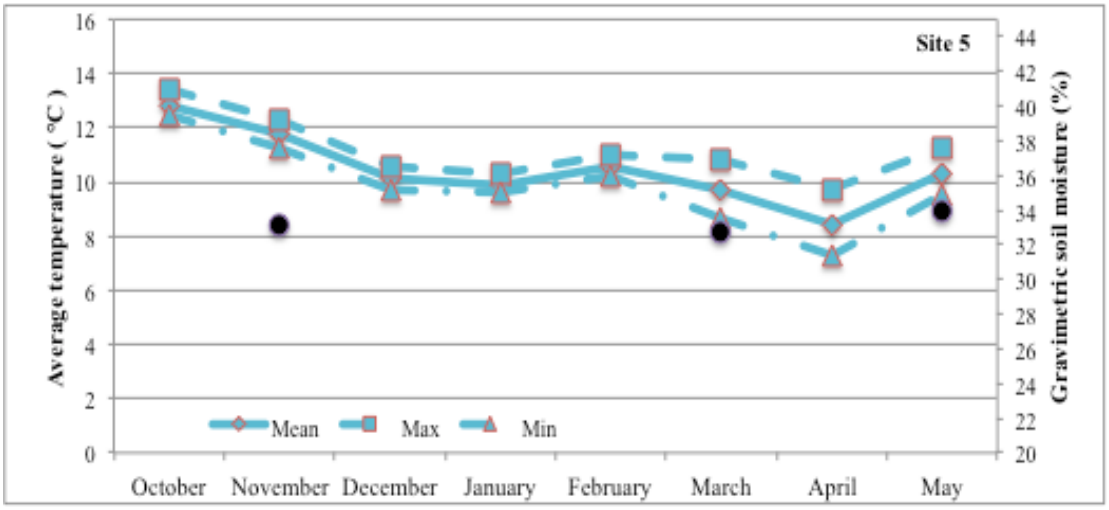
Appendix C: Percent recovery from rainwater baits by plot and site over the 2014 - 2015 collection period.

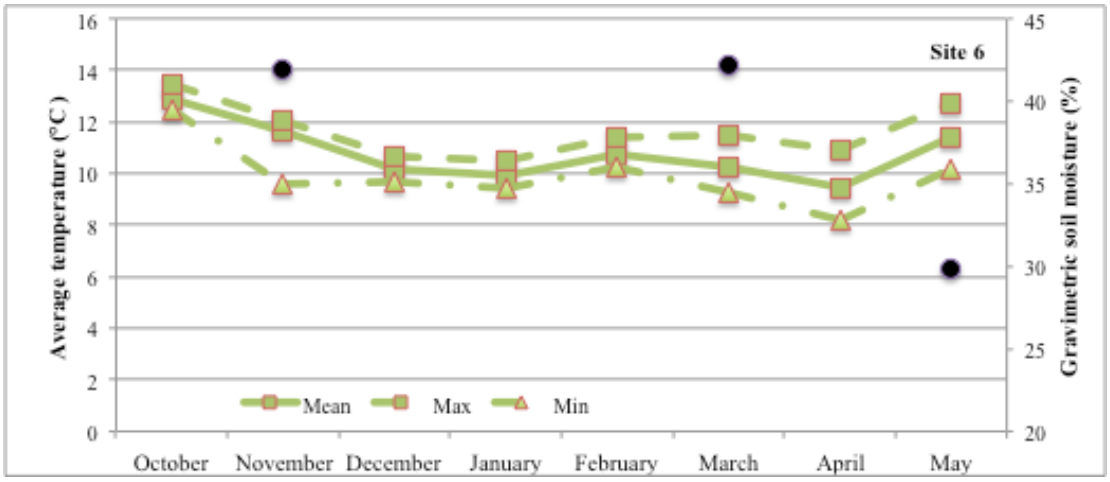


Appendix D: Gravimetric soil content and soil temperature at 5cm over time.



*Continued on next page

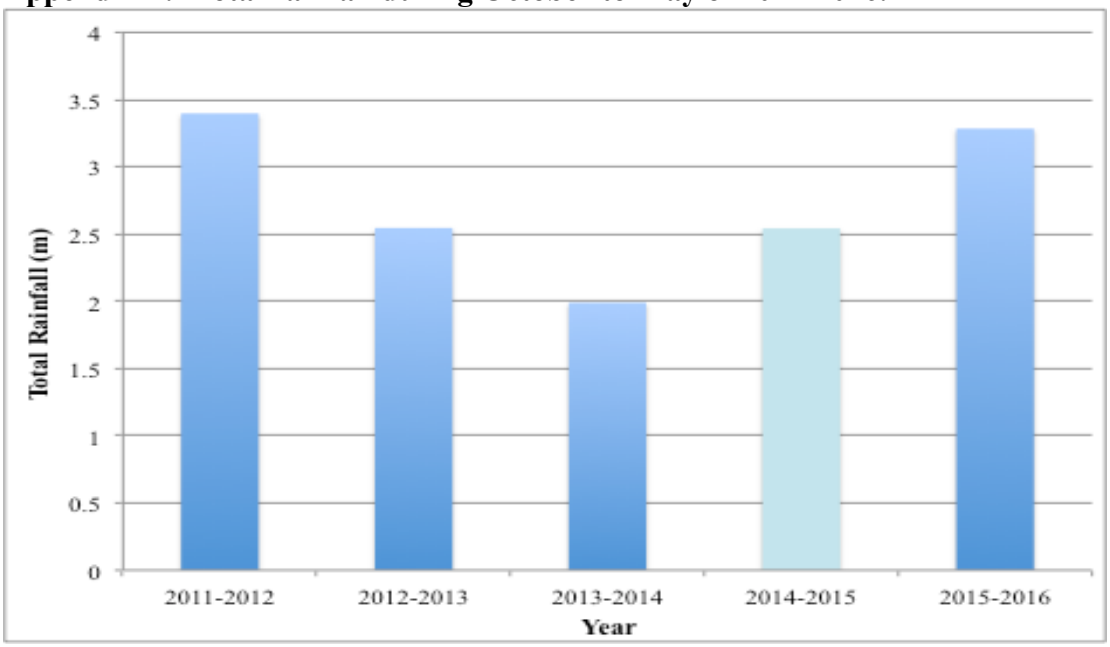

Site 5
Average temperature (°C)
Gravimetric soil moisture (%)
Mean
Max
Min
October
November
December
January
February
March
April
May


Site 6
Average temperature (°C)
Gravimetric soil moisture (%)
Mean
Max
Min
October
November
December
January
February
March
April
May

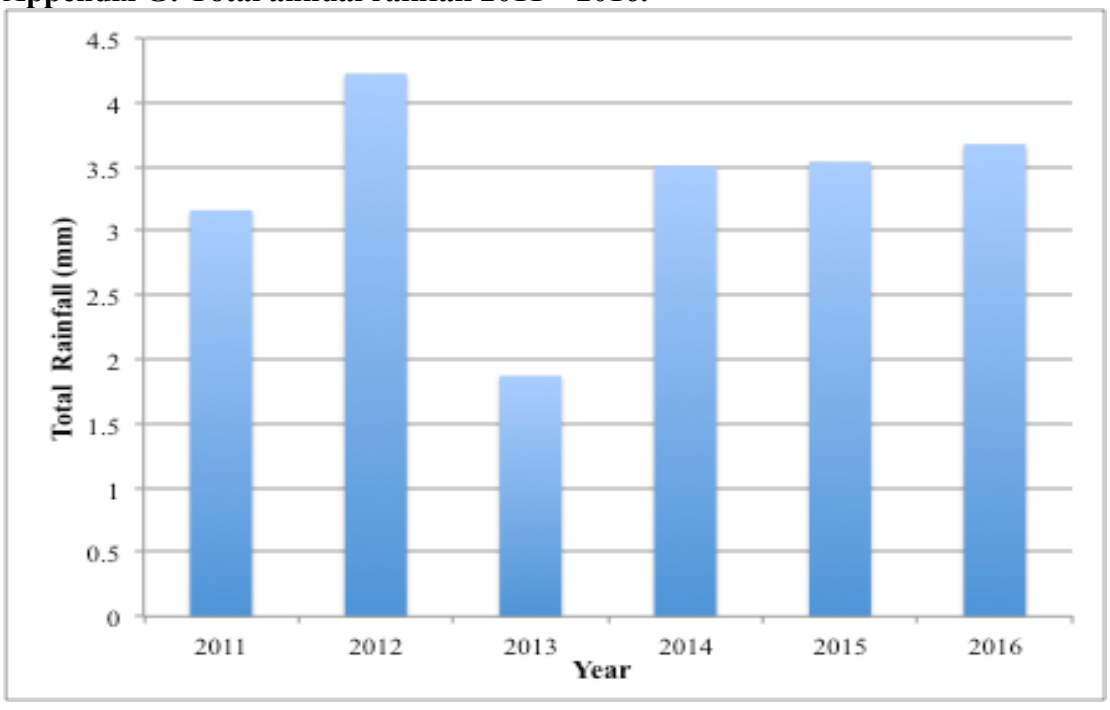
Appendix E: Sequencing

At the start of the study, cultures isolated from leaf baits were sequenced to verify correct identifications were made. Pure cultures were isolated from leaf baits from each of the sites. Isolates were transferred to PAR to obtain pure cultures and were then plated onto cornmeal agar amended with β-sitosterol (corn meal with 20ppm β-sitosterol). DNA was then extracted from pure cultures using a SYNERGY plant DNA extraction kit (OPS Diagnostics, Lebanon, NJ). Eluted DNA was then cleaned using an EXOSAP kit and then sequenced using the Sanger platform (Center for Gene Research and Biocomputing, OSU, Corvallis, OR). Results were analyzed using 4Peaks software (Aalsmere, The Netherlands) and Phytophthora ID (Grünwald et al. 2011).

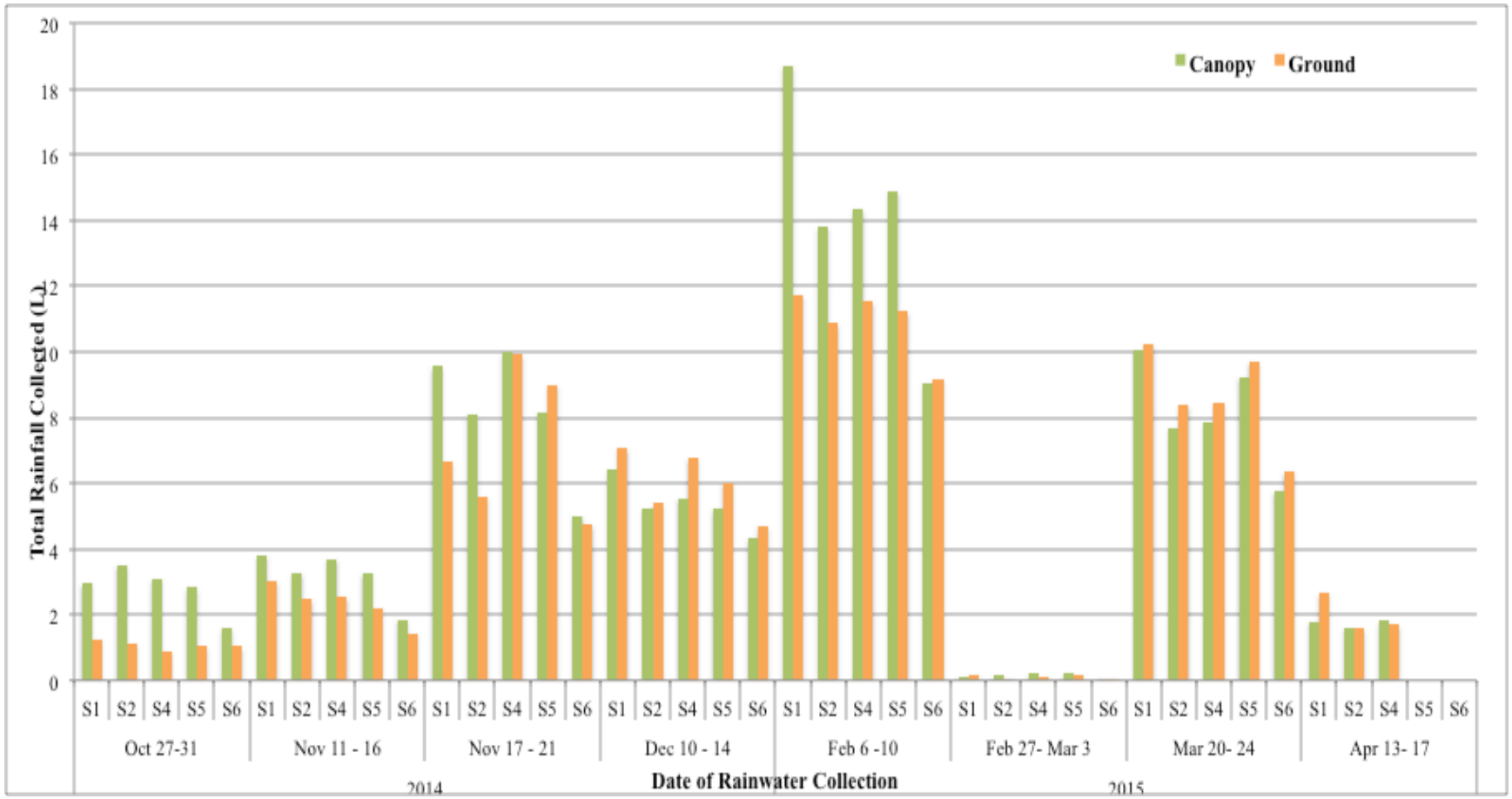
Appendix F: Total rainfall during October to May of 2011-2016.



Appendix G: Total annual rainfall 2011 – 2016.



Appendix H: Total volume of rainwater collected by bucket type.



Appendix I: Quantification using a standard curve

A threshold value (Ct) is the cycle number at which the amplification of the reporter signal is strong enough that it extends well above background fluorescence levels (i.e. above the threshold level). The Ct is determined by the starting amount of template in the sample. A sample with high amount of DNA for example will cross the threshold sooner and therefore have a lower Ct as compared to a sample with a lower amount of DNA. In order to quantify, a standard series is created by serially diluting DNA of the target of interest and run through the PCR assay.

The Ct of the standards are plotted against the log of their defined starting quantities. From this plot, linear regression is used to generate an equation, which can then be used to calculate the quantity of DNA in unknown samples:

$$Ct = m\ (log\ (quantity)) + b$$

$$quantity = 10^{((Ct-b)/m)}$$

where *m* is the slope of the standard curve, *y* is the Ct value of the sample, and b is the intercept of the standard curve. This method should only be used to interpolate quantities of unknown samples because the assay may not be linear outside the range of the standard series. Once outside the linear range, the reaction becomes limited as reagents become used up at later cycles.