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Assessing spread of *Phytophthoras* in Scottish forests by recreational and harvesting activities using comparative qPCR and metabarcoding techniques

Project Final Report

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Content

1 Executive Summary

This project considers recreation and harvesting activities in the context of tree health and the risk each activity poses in introducing or spreading pathogens in forests. Methods of detecting presence of potential pathogens are also compared.

Pathogens from the genus *Phytophthora* pose some of the greatest threats to tree health and are likely to become increasingly prevalent in the UK as the climate becomes warmer and wetter. An undergraduate project assessed the prevalence of *Phytophthora ramorum* in three public forests in 2016 using a species-specific qPCR assay and baiting for live propagules. The study aimed to determine the level of *P. ramorum* DNA that could be detected on the boot and bike treads of recreational users travelling through two forests that were experiencing *P. ramorum* outbreaks in larch compared to those from a forest with larch free of *P. ramorum*.

This PHC-funded project added new data based on a DNA metabarcoding approach to determine the *Phytophthora* species diversity in the soil samples and sampled material from timber harvesting operations (five active sites in the public forest in Galloway) alongside those of recreational activities (the sites were Kirroughtree, Glentress and Glentrool). We expanded on the previous sampling by using baiting, qPCR and metabarcoding to screen soils from timber harvesting machinery for both *P. ramorum* and a wider range of *Phytophthora* species. Our study demonstrated that the detection methods can distinguish between sites of known infection and those without and confirmed that metabarcoding wasn't as sensitive as qPCR for detecting *P. ramorum* in soil DNA, however, it proved useful in detecting a broader range of *Phytophthora* species in some samples. Quantitative PCR remains a sensitive and reliable method for being able to quickly detect a single-targeted species. Metabarcoding and other meta omics methods are informative but imperfect techniques. Results can depend on methodologies and the reliability of available reference sequences which are important for accurate taxonomic assignments. Improving bioinformatic analysis methods would be an important step in increasing the reliability of this metabarcoding technology in the future.

Metabarcoding detected several other species of *Phytophthora* in all forests. This provides additional insights on samples that had previously only been screened for *P. ramorum* using qPCR. Across the recreational sites, other *Phytophthora* species detected were: (1) *P. cinnamomi* which is a soil-borne species known to have a diverse host range and known to be widely present in GB. This species has been associated with decline of European oak forests; (2) *P. pseudosyringae*, a root and collar rot know to be in GB and often found infecting larch with *P. ramorum*; it's also known to be aggressive on *Nothofagus* species and in GB; (3) *P. gonapodyides* a native species and known minor root rot pathogen; (4) *P. chlamydospora* which can be a pathogen on fruit trees. This species is often detected in streams and soils in GB; (5) *P. austrocedri* is known to be a serious pathogen of juniper in parts of GB; (6) *P. obscura*, distribution uncertain, is known to infect horse chestnut and (7 & 8) *P.* syringae and *P. citrophthora*, which have global distribution and can infect many genera and families.

In addition to the known species, *Phytophthora* sequences were produced where identification to species level was not possible using the ITS1 region. These sequences were shown as a possible complex where the sequence could represent a single species or a number of different species. However, from previous data on species detection in GB, we can infer that the *P. gibbosa/gregata* complex is most likely to be *P. gregata*. Both these species are associated with declining native tree species and *Pinus radiata* in Australia. However no definitive symptoms have been associated with infection by either species and neither are known to cause dieback or mortality on tree species in GB; the wider host range is unknown. Similarly, the *P. europaea/flexuosa/Tyrrhenic*a complex is most likely to be *P. europaea*. This species has been isolated from rhizosphere soils in European oak forests. The *P. crassamura/megasperma* complex is most likely to be *P. megasperma* which is known to be associated with riparian habitats and forests in Europe and has associations with disease outbreaks in horticulture and agriculture.

The results also provided evidence that the diversity of *Phytophthora* species differed substantially between the study forests. Glentrool had the greatest diversity of *Phytophthora* species (10), compared to 8 for Kirroughtree and 5 for Glentress. Only two species were found at Galloway. Two sequences were obtained from one of the recreational sites via metabarcoding. These sequences showed high similarity in each case to *Phytophthora pinifolia* which has had severe impacts on *Pinus radiata* in Chile but is not known to be present in the GB. This is now being investigated as priority with further monitoring at the forest in collaboration with Scottish Forestry. This highlights the value of methods such as metabarcoding with broad pathogen detection, that can alert to the potential presence of new pathogens in our forests.

On the harvester sites, *P. gonapodydies* was the only other *Phytophthora* species detected. A key finding was that harvesting machines arrived on site with soil (and plant debris) already attached to treads from which *P. ramorum* was detected. This indicated that *P. ramorum* has been moved from one forestry site to another via the machinery treads.

We acknowledge that detection of a species via DNA metabarcoding does not necessarily indicate that the organism can cause disease. Soil baiting, if successful, provides evidence that any *phytophthora* detected by molecular methods is viable. In this study, only *P. gonapodyides* was detected in some soil samples recovered from boot treads in Glentress and Kirroughtree, and from some soil samples recovered from harvesters in Kirroughtree. Overall, the method was unreliable in confirming the presence of the different *Phytophthora* species otherwise detected by metabarcoding or qPCR. In future, analysis of RNA with metabarcoding may be an alternative method of providing evidence in relation to the viability of the detected organisms. Further investigation is needed to understand the influence of vegetation communities and site history on pathogen presence, and further methodological improvements should be sought.

This study provides evidence that *P. ramorum* and other *Phytophthora* species can be picked up and moved via boots, bike tyres and harvesters, indicating that pathogens capable of causing tree mortality can potentially be spread by recreational and harvesting activities into new areas; if viable, these pathogens could start new outbreaks. Further work to determine pathogen viability and the potential to start new infections would be valuable in the future. These results underline the importance of good biosecurity practice as advocated by campaigns such as 'Keep it clean'. The recommendations for forestry practitioners would be to ensure boots and machinery are thoroughly cleaned before moving to a different location. Forests used by the public for recreational activities would also benefit from biosecurity messaging around cleaning boots and bike tyres before and after entering the forest.

2 Introduction

Scotland's forests play a vital role in local and national economies through the production of timber and wood fibre products, and through the provision of ecosystem services including recreation and tourism. The Scottish Forestry strategy 2019-2029 has set out its aim to increase integration of traditional forestry with other land-based businesses to increase the benefits provided to health, well-being, quality of life and economic returns. One challenge in this aim is to grow and maintain resilient and healthy forests while balancing this against the needs of different stakeholders who utilise or rely on the forest's natural capital. Globally, outbreaks of new plant diseases have had severe negative impacts on forest species and activities such as recreation and timber harvesting have been identified as needing careful management to minimise the risk of new disease introductions. Many *Phytophthora* species have been found to be pathogenic and invasive on woody plant hosts, and disease outbreaks are frequently associated with human activity and soil disturbance in the area as well as climate change (Jung *et al.* 2018, Riddell *et al.* 2019). For example, *P. ramorum* introduced into the UK is particularly infectious on *Larix* species, and the recent discovery of *P. pluvialis* in the UK has raised concerns for health of two other commercial species, Douglas fir (*Pseudotsuga menziesii*) and Western hemlock *(Tsuga heterophylla)*. The costs of managing and containing a disease outbreak is generally high and sustained; since 2009, *P. ramorum* has spread to many larch stands in western regions of the UK causing high levels of tree mortality and there are continued, rising costs to the management and containment of the disease. Careful assessment and monitoring to identify factors which bring risk to forest health is needed and to inform the development of more biosecure behaviours and policies.

In 2016, an undergraduate project (Penny, 2017: unpublished report) investigated the potential for spread of *P. ramorum* by movement of soil along recreational trails. At three public forests recreational trails, the level of *P. ramorum* inoculum in soil recovered from walking boots and bike treads was measured using a *P. ramorum*-specific qPCR assay and baiting. The qPCR data showed the presence of *P. ramorum* along the trails and suggested that recreational activities had the potential to spread the disease to other, unaffected areas. Here we report the results of a Plant Health Centre-funded project aimed to extend and add further value to the undergraduate project by:

1. **Re-testing soils collected from recreational forests in 2016 using DNA metabarcoding** to investigate *Phytophthora* species diversity on trails in recreational forests and assess the potential risks to tree health from the pathogen species identified. Metabarcoding provides the potential advantage of detecting a broader range of airborne *Phytophthora* in addition to *P. ramorum*. Previous studies have proven the suitability of metabarcoding for assessing *Phytophthora* species diversity in soil samples (Riddell *et al.* 2019) by sequencing a stretch of genomic DNA that is universal to all pathogens but still unique (a barcode) to each species, circumventing the need to develop species-specific assays. Metabarcoding has successfully revealed the presence and diversity of *Phytophthora* species in plant nursery water and root samples and highlighted potential effects of nursery practice on the spread of these diseases. (https://www.forestresearch.gov.uk/research/global-threats-from-phytophthora-spp/).

2. **Comparing detection rates of *P. ramorum* in 2016 samples in metabarcoding data with the species-specific qPCR assay.** Samples from 2016 were re-run with the species-specific qPCR assay to verify the detections of *P. ramorum* and to compare with detections of *P. ramorum* via DNA metabarcoding to determine whether the latter could be successfully used as an alternative to qPCR in disease surveillance.

3. **Comparing *Phytophthora* diversity on active machinery on non-recreational timber sites using samples collected in 2019.** Using the same techniques of baiting, qPCR and metabarcoding we screened soils collected from timber harvesting machinery for both *P. ramorum* and wider phytophthora species. Using the data from both the 2016 undergraduate project and this PHC-funded project, we can make a comparative assessment of the plant health risks of both recreational and harvesting activities and provide evidence to support the 'Keep it clean' campaign, encouraging forest users to take part in biosecurity between locations.

3 Methods

3.1 Sites and sample collection

For assessment of the ability of recreational activities to vector *Phytophthora* pathogens, three sites were selected for sampling in 2016. These sites all form part of the public forest estate and are part of the 7stanes trail network popular with mountain bikers and walkers. The sites were Kirroughtree, Glentress and Glentrool.

- Glentrool was selected as all the larch had recently been felled under SPHN (Statutory Plant Health Notices), within the previous year or in successive years across the area, so we could sample on trails adjacent to an area where infected larch had been removed, based on forest inventory coupe data.
- Kirroughtree had standing, infected larch adjacent to bike/walking trails with infected larch trees extending branches over the bike trails in places. Shed, discoloured larch needles from infected trees were present on the ground. Transects were selected that were adjoining standing infected larch.
- Glentress was selected due to absence of SPHNs/known infection at the time of sampling.

Aside from larch, other tree species were noted at all recreational sites in close proximity to the transects, including Sitka spruce (*Picea sitchensis*), Norway spruce (*Picea abies*), Douglas fir (*Pseudotsuga menziesii*), pine species including *Pinus sylvestris*, ash (*Fraxinus excelsior*), rowan (*Sorbus acuparia*) and species of willow, hemlock, birch, rhododendron, *Vaccinium* and heather.

Sampling was carried out in 2016 as part of a final year student project, across two sampling periods, June, and October, in Glentrool and Kirroughtree to coincide with typical sporulation periods for *P. ramorum*. Sampling was carried out in June only for Glentress owing to time constraints. Soil and plant material was collected from boots and bike tyres after movement along a 100m transect of a recreational trail. Three field replicates were collected from each transect for both boot and bike investigations, five transects were sampled at each site. Transects were selected based upon their proximity to standing infected or recently felled infected larch at the infected sites of Glentrool and Kirroughtree. For each transect, 1 field replicate represented the 100m transect being walked or cycled once and soil and plant material were collected from the bike and boot treads. Material was collected into a ziplock bag using clean gloves and a sterile spatula. Treads were thoroughly cleaned between sampling by removing any remaining material with water, followed by applying Propellar (a disinfectant widely used in forestry to prevent the spread of fungal pathogens including *P. ramorum*) for 5 minutes and then a final rinse. This process was repeated twice more to provide a total of three field replicates per transect for both walking and cycling investigations. For each sampling of a transect a dry weight average of <20g of soil was collected from bike tyres, <20g and around 23g from boot treads.

Sampling of harvesting sites was carried out in June/July 2019 at five active sites in the public forest in Galloway. The exact locations are anonymised as they are all in the *P. ramorum* infection Management Zone and were subject to SPHNs at the time. The infection Management Zone is an area in Galloway severely affected by *P. ramorum* and movement of affected trees is regulated to restrict introduction of the disease to other areas beyond this area. The Management Zone is now a sub-division of a newly designated 'Risk Reduction Zone' after revision to the *P. ramorum* management strategy in 2021 (Scottish Forestry publication 2021 a & b). Soil was sampled from treads of either harvester or forwarder machinery.

(a) Soil samples (termed field samples) were initially taken from four treads on harvester/forwarder machinery on arrival at a site (i.e., pre-harvest activity). Operators had confirmed that these harvester machines had arrived on site still containing soil and plant material combined with material from the current site during

offloading. The pre-harvest soil sample was collected into a ziplock bag using clean gloves and a sterile spatula. The treads were then decontaminated with clean water and ethanol. Three separate pre-harvest field samples were taken for each of the 5 sites.

(b) The same four treads were then resampled on the site on the same day following around 3 to 4 hours of timber harvesting activity (i.e. post-harvest). Three separate post-harvest field samples were taken for each of the 5 sites.

For each sampling, a dry weight average of 1kg of soil was collected from the harvester treads. Soil from the field samples were used for both DNA extraction and soil baiting (refer to section 3.2).

Once collected, soil samples were stored at 4°C for a maximum of 48 hrs before baiting for viable pathogens. On return to the laboratory, samples destined for DNA extraction were immediately placed in an oven at 55°C to dry, which took around 24 hours (hrs).

3.2 Soil baiting for Phytophthora *species*

Phytophthora baiting was used to identify live and viable inoculum in the samples. Soil and associated plant debris collected from harvester/forwarder and recreational tracks were placed into sterile containers and flooded with sterile water before floating visually non-diseased 'bait' rhododendron leaves, collected from plants within the grounds of the research station (NRS), on the surface. Leaves were monitored for up to one week to identify the presence of lesions. As the amount of soil and plant material collected from bike tyres and walking boots was much smaller, these samples were baited by boring a 12mm diameter by 2cm deep hole into a surface-sterilised granny smith apple. The hole was filled with either the entire sample where the samples were <10ml in volume or 10ml of sample if >10ml. The bore hole was sealed with Sellotape and incubated at room temperature. Lesions from both the rhododendron and apple baits were isolated and plated onto *Phytophthora*-selective media (SMA; amended as per Brasier *et al.*, 2005) and incubated at 17°C. Subsequent cultures that resembled the morphology of *Phytophthora*, i.e. branched, aseptate, hyaline, sometimes coralloid mycelia, were sub-cultured onto V8 media and grown at 17°C before carrying out DNA extraction to identify the pathogen by Sanger sequencing of the ITS1 region using primers ITS4 and Ph2 (Scibetta *et al.* 2012).

3.3 DNA extraction

To maximise the chance of capturing rare sequences of rare *Phytophthoras*, two duplicate DNA extractions were made from each field replicate, totalling 60 extractions from harvester/forwarder treads, 120 extractions total from Kirroughtree and 120 at Glentrool (which includes for each, 60 extractions from boots and 60 extractions from bike treads collected over June and Oct) and 60 at Glentress (which includes 30 extractions from boots and 30 extractions from bike treads collected over June only). Dried soils were ground up in a Retsch MM300 mixer mill using the 50ml canisters. Samples were run through twice at 25Hz for one minute each time. DNA was extracted from 2 x 250 mg subsamples of milled soil per sample using the PowerSoil® DNA extraction kit (Qiagen, USA) as per instructions.

3.4 qPCR assay to test for presence of P. ramorum

For recreational samples from Glentress, Glentrool and Kirroughtree, duplicate DNA extractions were pooled by field replicate for qPCR analysis. In contrast, duplicate soil DNA extractions of field replicates from harvesting machinery in 2019 were tested individually for qPCR analyses due to different approaches of the field sampling.

Each DNA sample was tested for the presence of *P. ramorum* using the assay developed by Schena *et al.* (2006), targeting ITS1 of the nuclear ribosomal RNA gene, using TaqMan chemistry. DNA samples were run in triplicate (technical replicates) on 96-well plates using 2µl of DNA, 2x Environmental Taq (Applied Biosystems, USA), and primers as per Schena *et*

al. (2006) in a 25µl reaction. Standards, created from DNA of pure *P. ramorum* culture, were run on each plate ranging from 2ng to 2pg in a 1 in 10 dilution series as well as a minimum of two wells for negative controls. Samples with just one positive amplification out of three technical replicates were counted as negative for *P. ramorum*. In some instances, one positive in three technical replicates can be the result of a random DNA cross contamination event during qPCR set-up and should be discarded. A minimum of one field replicate out of three was counted as a positive result for *P. ramorum*.

3.5 DNA metabarcode sequencing: nested PCR, indexing and metabarcode sequencing of oomycete-positive samples

DNA pools of individual transect, or machinery field replicates were created prior to carrying out DNA metabarcode analysis. To amplify oomycete sequences, including the *Phytophthoras*, all 180 DNA pools were processed individually through a nested PCR that enriches for a 250bp region of the ribosomal RNA (rRNA) internal transcribed spacer (ITS1) gene (Scibetta *et al.* 2012). Each sample was run through the nested PCR in duplicate to maximise the chance of amplifying rare sequences, and the results were combined after bioinformatic analysis. The nested PCR primers are designed to capture *Phytophthora* species but will also amplify some closely related oomycetes such as downy mildews. Samples that produced an amplicon in the nested PCR were oomycete-positive and were identified by gel electrophoresis on a 1% agarose gel. Oomycete positive PCR reactions were prepared for sequencing following the protocols described for 16S metagenomic sequencing library preparation (Illumina, 2013) and each uniquely indexed to assign reads back to each sample.

All DNA libraries were quantified, normalised and pooled for paired-end (2x250bp) sequencing on an Illumina flowcell using the MiSeq v.2 500bp standard kit at the James Hutton Institute (JHI), Dundee. Standard DNA control mixes containing four synthetic sequences (designed by D. Cooke, JHI, Dundee) at known concentrations were also run through metabarcoding, indexing and sequencing to calibrate the plate and check for cross-contamination between samples.

After quality control and de-multiplexing, FASTQ files for sample reads were exported for bioinformatic analysis. Sequence data were processed using the bioinformatics software 'THAPBI PICT' (Cock *et al.* 2023). THAPBI PICT performed quality trimming and merged paired reads, removed primers and collated unique sequences with a minimum sample abundance of 50 reads. The unique sequences were matched to a species in the THAPBI PICT tool's curated ITS1 database using the default classifier, which requires perfect matches or at most a one base pair difference. Any sequences not matching the curated database, but which matched a broader set of sequences downloaded from NCBI database based on 2bp difference, were reported to genus only. If a sequence did not match anything within a 2bp difference, then it was marked as 'unknown'. For the purposes of this report, the most abundant unknown sequences were run through BLASTn (Altschul *et al.* 1990) in GenBank using default parameters to identify the closest genus in the GenBank nt database.

3.6 Statistical analysis

All data cleaning and analyses were performed in RStudio using R Version 4.2.1 with figures created using ggplot2 (Wickham, 2016) and plotly (Sievert, 2020). Prior to analysis any samples which did not result in the detection of any species were removed from the analyses. If a species was detected in either of the technical replicates then that was considered to be proof of a positive sample. Binary presence/absence data were analysed using a Jaccard dissimmilarity matrix in the vegan package (Oksanen *et al.*, 2018). Permanova was used to test whether there were significant differences in the dissimilarity matrices based on site (Glentress, Glentrool, Kirroughtree), season (spring/autumn), or sample type (boot/bike). The harvesting sites were not used in the clustering analysis as only a single species was detected across all samples. Significant predictors were then analysed using the pairwise adonis function in the EcolUtils package (Salazar, 2023) with 1000 permutations. Species scores were added to the ordination using the metaMDS function in the vegan package. To

calculate the proportion of reads attributed to each species in each sample the mean number of reads across the replicates was calculated and then the total reads per sample summed. The proportion of reads represents the proportion within each sample.

4 Results

4.1 *Sampling effort*

Three recreational sites (Glentrool, Glentress and Kirroughtree) were sampled in June 2016. This sampling involved taking 3 replicate boot tread samples and 3 replicate bike tyre samples in each of 5 transects resulting in a total of 15 boot tread samples and 15 bike type samples per site. A total of 30 samples were collected at each site (90 across all sites). This sampling was repeated in October 2016 for Glentrool and Kirroughtree only. A total of 30 samples were collected at each site (60 across all sites) (Table 1).

In addition, sampling of harvester and forwarder treads was carried out on 5 active sites in a public forest, Galloway, in June/July 2019. For each site, 3 replicate soil (field) samples were collected from treads pre-harvest activity and post-timber harvest activity (Table 2).

4.2 *Baiting for* Phytophthora *species*

Only one *Phytophthora* species, *P. gonapodyides,* was successfully baited from soils at Glentress, Kirroughtree and two harvesting sites in the Galloway Forest. *Pythium* and *Elongisporangium* species were also detected (Tables 3 to 6).

4.3 *Metabarcoding detection of* P. ramorum *and other* Phytophthora *species*

A total of 130 samples were tested using the nested PCR (Scibetta *et al.* 2012). Electrophoresis of the PCR products identified 80 PCR reactions that were positive for the presence of oomycetes and were subsequently indexed for metabarcoding. Twenty-nine of these were DNA samples from soil on boots, 19 from harvester treads and 32 from bike tyres. Twenty-two samples came from Glentrool, 29 from Kirroughtree, 10 from Glentress and 19 from the five harvesting sites in the Management Zone. After sequencing and processing sample data through the THABIPICT pipeline, 78 samples returned sequences. Thirty-six samples contained oomycete sequences from boot and bike samples and 12 from harvesting machines. Two libraries from a Kirroughtree sample failed to return any data. The number of reads returned from the samples varied from 298 to 58,242.

Phytophthora species diversity varied across the sites. The diversity of species is shown in Tables 3 to 6 and summarised in Table 7:

- Kirroughtree had 8 *Phytophthoras* detected which included 6 known species, 1 complex and 1 unknown species. All 8 were detected by metabarcoding.
- Glentrool had 10 *Phytophthoras* detected which included 8 known species, 1 complex and 1 unknown species. All 10 were detected by metabarcoding.
- Glentress hads 5 *Phytophthoras* detected which included 4 known species and 1 unknown species. All 5 were detected by metabarcoding.
- The Galloway Forest site had 2 known *Phytophthora* species detected. Only *P. ramorum* was detected by metabarcoding, while *P. gonapodyides* was detected by soil baiting only.

Some *Phytophthora* species cannot be differentiated using the ITS1 locus and are listed in the results represented with (*), highlighting a possible larger complex of species that each sequence might represent. In total, 10 *Phytophthora* known species and 3 complexes were found from 6 taxonomic clades (2a, 3a, 6b, 7a, 8c, 8d) (Table 10). Unknown *Phytophthora* sequences were detected. These were sequences that did not match the curated database but matched a broader set of sequences downloaded from NCBI database based on a 2-base pair difference. These could only be reported to genus. Whether these were the same species reoccurring at the different sites or completely different species could not be ascertained without further investigation.

4.4 *P. ramorum detections based on qPCR and metabarcoding*

From the recreational site samples, *P. ramorum* was detected by metabarcoding in 6 samples from Glentrool and 5 samples from Kirroughtree, compared with 12 and 19 detections of *P. ramorum,* respectively, using qPCR (Tables 3 to 5). There were no detections of *P. ramorum* using either method from soil samples collected from transects along recreational trails at Glentress.

From harvesting site samples, *P. ramorum* was the only *Phytophthora species* found on harvesting machinery with detections in ten metabarcoded samples from samples at sites 3 and 5, compared to 19 detections in harvesting site soils from sites 2,3,4 and 5 using the species-specific qPCR assay. Soil from harvesters also had a low frequency of sequences with similarity to a *Pythium*.

4.5 *Other Phytophthoras detected by metabarcoding*

Other *Phytophthora* and fungal species identified are represented in Figure 1 along with the diversity of *Phytophthora* species and complexes found across all sites in Table 7. *Phytophthora* and fungal distribution, species clades and pathogenicity are summarised in Table 10. Only *P. ramorum* was detected in the harvesting sites so all other species detected were found in soils sampled at recreational sites.

4.6 *Analysis of species diversity*

Differences in the *Phytophthora* species composition based on the results of the permanova are shown in

Table 8. Based on 95% confidence intervals, there is a highly significant difference in species composition according to site (P=0.01). Season and sample type were not significant (P=0.82 and 0.63 respectively). Table9 shows the pairwise comparisons and significant differences based on 95% confidence intervals for species differences between recreational sites. The species composition at Glentrool is significantly different to Kirroughtree and Glentress (P = 0.037 for both). There was no significant difference between Kirroughtree and Glentress (P = 0.104)

Table 1: Sampling effort across 3 sites showing the number of replicate soil samples collected from boots and bike tyres. Samples were collected June and October 2016 (Glentress = June only)

	Glentrool		Glentress	Kirroughtree	
Transect	**June 2016**	**Oct 2016**	**June 2016**	**June 2016**	**Oct 2016**
1	3 boots	3 boots	3 boots	3 boots	3 boots
	3 tyres	3 tyres	3 tyres	3 tyres	3 tyres
2	3 boots	3 boots	3 boots	3 boots	3 boots
	3 tyres	3 tyres	3 tyres	3 tyres	3 tyres
3	3 boots	3 boots	3 boots	3 boots	3 boots
	3 tyres	3 tyres	3 tyres	3 tyres	3 tyres
4	3 boots	3 boots	3 boots	3 boots	3 boots
	3 tyres	3 tyres	3 tyres	3 tyres	3 tyres
5	3 boots	3 boots	3 boots	3 boots	3 boots
	3 tyres	3 tyres	3 tyres	3 tyres	3 tyres

Table 2. Sampling effort at Galloway Forest showing the number of soil samples collected from harvester machinery treads at 5 active harvest sites

	Galloway Forest	
Site	**Pre-harvest**	**Post harvest**
1	S1 treads S2 treads S3 treads	S1 treads S2 treads S3 treads
2	S1 treads S2 treads S3 treads	S1 treads S2 treads S3 treads
3	S1 treads S2 treads S3 treads	S1 treads S2 treads S3 treads
4	S1 treads S2 treads S3 treads	S1 treads S2 treads S3 treads
5	S1 treads S2 treads S3 treads	S1 treads S2 treads S3 treads

Tables 3 to 6: Summary of sample collection at recreational and harvester sites and testing results for baiting, metabarcoding and qPCR.

- For recreational sites, samples were collected June and October 2016 (Glentress = June 2016 only); two DNA extractions were performed per replicate sample and DNA extractions for each sample replicate were pooled for qPCR.
- For harvester sites, samples were collected in June/July 2019; two DNA extractions were performed per replicate sample and each DNA sample was tested separately for qPCR.
- ND = nothing detected.
- *Phytophthora sp*. (unknown) = a sequence that did not match the curated database but matched a broader set of sequences downloaded from NCBI database based on 2bp difference, so was reported to genus only.
- (*) = species that could not be differentiated using the ITS1 locus are listed as a larger complex of species that each sequence might represent. The complex is highlighted in green.

Table 3: Phytophthora and other fungal species detected by different testing methods at Glentroll recreational site

	Soil baiting using apples	**Metabarcoding**			**Q PCR (+/-)**	
	Pooled sample	**Boots x 3 samples**	**Tyres x 3 samples**	**No. *Phytophthora* spp. identified**	**Boots x 3 samples**	**Tyres x 3 samples**
June 2016						
Transect 1	ND	*Phytophthora cinnamomi*	*Phytophthora ramorum*	2 known	+	+
Transect 2	ND	*Phytophthora ramorum* *Phytophthora* sp. (unknown)	*Phytophthora chlamydospore* *Phytophthora pseudosyringae* *Phytophthora gonapodyides*	4 known 1 unknown	+	+
Transect 3	*Elongisporangium undulatum*	*Phytophthora ramorum* *Phytophthora* sp. (unknown) *Phytophthora gibbosa/ gregata**	ND	1 known 1 unknown 1 complex	+	+
Transect 4	ND	*Phytophthora europaea/ flexuosa/tyrrhenica**	ND	1 complex	-	-
Transect 5	ND	*Phytophthora austrocedri* *Phytophthora citrophthora*	*Phytophthora cinnamomi*	3 known	-	-
Oct 2016						
Transect 1	ND	*Phytophthora europaea/ flexuosa/tyrrhenica**	ND	1 complex	+	-
Transect 2	ND	ND	ND	0	-	-
Transect 3	*Elongisporangium undulatum*	ND	ND	0	+	-
Transect 4	ND	*Phytophthora ramorum* *Bremia* sp. (unknown)	ND	1 known	+	+
Transect 5	ND	ND	ND	0	+	+

Table 4: Phytophthora *and other fungal species detected by different testing methods at Glentress recreational site*

	Soil baiting using apples	**Metabarcoding**			**Q PCR (+/-)**	
	Pooled sample	**Boots x 3 samples**	**Tyres x 3 samples**	**No. *Phytophthora* spp. identified**	**Boots x 3 Samples**	**Tyres x 3 samples**
June 2016						
Transect 1	*Pythium anandrum*	*Phytophthora citrophthora*	*Phytophthora gonapodyides* *Phytophthora syringae* *Phytophthora pseudosyringae*	4 known	-	-
Transect 2	ND	ND	*Phytophthora* sp. (unknown)	1 unknown	-	-
Transect 3	*Phytophthora gonapodyides* *Pythium senticosum* *Elongisporangium undulatum*	ND	ND	0	-	-
Transect 4	*Phytophthora gonapodyides*	*Phytophthora pseudosyringae*	*Phytophthora pseudosyringae* *Phytophthora* sp. (unknown)	2 known 1 unknown	-	-
Transect 5	ND	ND	*Phytophthora gonapodyides* *Phytophthora* sp. (unknown)	1 Known 1 unknown	-	-

Table 5. Phytophthora *and other fungal species detected by different testing methods at Kirroughtree recreational site*

	Soil baiting using apples	**Metabarcoding**			**Q PCR (+/-)**	
	Pooled sample	**Boots x 3 samples**	**Tyres x 3 samples**	**No. *Phytophthora* spp. identified**	**Boots x 3 Samples**	**Tyres x 3 samples**
June 2016						
Transect 1	ND	*Globisporangium* sp.(unknown)	ND	0	+	+
Transect 2	*Elongisporangium undulatum*	*Phytophthora pseudosyringae*	ND	1 known	+	+
Transect 3	ND	ND	ND	0	+	-
Transect 4	ND	*Phytophthora cinnamomi* *Phytophthora gonapodyides*	ND	2 known	+	+
Transect 5	ND	*Phytophthora cinnamomi* *Phytophthora syringae*	ND	2 known	+	+
Oct 2016						
Transect 1	ND	ND	*Phytophthora gonapodyides* *Phytophthora syringae* *Phytophthora obscura*	3 known	+	+
Transect 2	*Elongisporangium undulatum* *Pythium senticosum*	*Phytophthora ramorum,* *Phytophthora cinnamomi* *Phytophthora* sp. (unknown) *Phytophthora crassamura/ megasperma**	*Phytophthora ramorum,* *Phytophthora cinnamomi* *Phytophthora pseudosyringae* *Phytophthora crassamura / megasperma**	3 known 1 unknown 1 complex	+	+
Transect 3	*Elongisporangium undulatum*	*Phytophthora ramorum,* *Phytophthora crassamura/ megasperma**	ND	1 known 1 complex	+	+
Transect 4	*Phytophthora gonapodyides*	*Phytophthora ramorum*	*Phytophthora gonapodyides* *Phytophthora cinnamomi* *Phytophthora* sp. (unknown)	3 known 1 unknown	+	+
Transect 5	*Pythium balticum*	*Phytophthora gonapodyides* *Phytophthora pseudosyringae*	*Phytophthora pseudosyringae*	2 known	+	+

Table 6: Phytophthora and other fungal species detected by different testing methods at Galloway harvester site

	Soil baiting using water baits and leaves	**Metabarcoding**		**Q PCR (+/-)**	
	Pooled sample	**Tread samples x 3**	**No. *Phytophthora* spp. identified**	**Pre-harvest**	**Post-harvest**
June/ July 2019					
Site 1	*Pyhium balticum* *Phytophthora gonapodyides* *Elongisporangium undulatum*	ND	I known	+	-
Site 2	ND	ND	0	-	+
Site 3	*Elongisporangium undulatum*	*Phytophthora ramorum*	1 known	+	+
Site 4	*Elongisporangium undulatum*	ND	0	+	+
Site 5	*Phytophthora gonapodyides,* *Elongisporangium undulatum*	*Phytophthora ramorum*	2 known	+	+

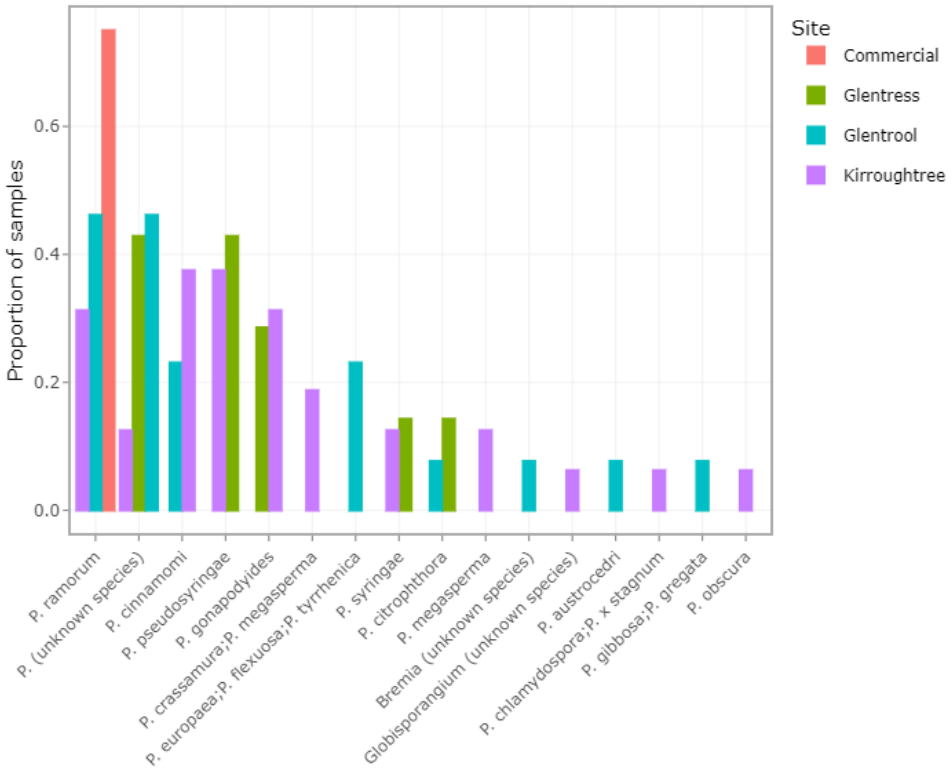

Figure 1 Phytophthora *species and other fungal genera identified by metabarcoding at each site*

Table 7 Phytophthora species identified by metabarcoding and baiting at each site. (*) represents complexes.

Site (and type)	**Glentrool**	**Glentress**	**Kirroughtree**	**Galloway**	**Total no. sites for detection**
***Phytophthora* species or complex**	**Recreational**	**Recreational**	**Recreational**	**Active Harvest**	
P. ramorum	X		X	X	**3**
P. cinnamomi	X		X		**2**
P. Pseudosyringae	X	X	X		**3**
P. gonapodydies	X	X	X	X	**4**
*P.crassamura/megasperma**			X		**1**
*P. europaea/ flexuosa/tyrrhenica**	X				**1**
P. syringae		X	X		**2**
P. citrophthora	X	X			**2**
P. austrocedri	X				**1**
P. chlamydospore	X				**1**
*P. gibbosa/gregata**	X				**1**
P. obscura			X		**1**
Phytophthora sp. (unknown)	X	X	X		**3**
Total no. species or complexes detected	**10**	**5**	**8**	**2**	

Table 8 Permanova results testing main effects of site, sample season (spring vs autumn) and sample type (bike vs boots) (1000 permutations). The results are based on a significance level of 0.05 (95% confidence levels). A P value of lower than 0.05 is significant.

Variable	Df	SumOfSqs	R2	F	P value	Significance
SITE	2	1.61	0.12	2.08	0.01**	Highly significant
TYPE	1	0.30	0.02	0.78	0.63	Not significant
SEASON	1	0.22	0.02	0.57	0.82	Not significant
Residual	29	11.19	0.84			
Total	33	13.37	1.00			

Table 9 Pairwise comparisons for all sites using permutational MANOVA between Glentrool (G), Kirroughtree (K) and Glentress (T). The results are based on a significance level of 0.05 (95% confidence levels). A P value of lower than 0.05 is significant.

Combination	SumsOfSqs	MeanSqs	F.Model	R2	P value
G <-> K	0.974	0.974	2.557	0.090	0.037* Significant
G <-> T	0.856	0.856	2.328	0.120	0.037* Significant
K <-> T	0.637	0.637	1.671	0.081	0.104 Not significant

*Table 10 Summary of pathogenicity, host and clade data for all oomycete species detected in this study by baiting, metabarcoding and qPCR. * = species that could not be differentiated using the ITS1 locus and are listed as a larger complex of species that each sequence might represent*

Species and complex	**Pathogenic status**	**Known hosts**	**Known distribtuion**	***Phytophthora* clade**
P. citrophthora	Yes	Mainly Citrus, Cacao, also *Nothofagus macrocarpa* in Chile, horse chestnut (*Aesculus hippocastanum*) in Turkey, but affects 88 genera in 51 families	Yes, global distribution	2a
P. pseudosyringae	Yes	Root and collar rot; a moderately aggressive pathogen of various *Quercus* spp., *Larix* spp., *Alnus*, and *Fagus* species. Aggressive on *Nothofagus obliqua* and *Nothofagus alpina* in UK.	Yes, Europe inc. UK, North America, South America (Chile)	3a
*P. gibbosa/gregata** *Most likely to be P. gregata based on UK detection data*	*P. gibbosa* Uncertain	*P. gibbosa*: *Acacia pycnantha*, *Xanthorrhoea gracilis,* Grevillea sp.	Associated with Western Australia	6b
	P. gregata Uncertain	*P. gregata*: no known hosts, identified in soils	Associated with Western Australia and occasionally detected in metabarcoding studies. Has been isolated in Scotland from nursery waste piles. (Schiffer-Forsyth *et al.*, 2023)	
P. gonapodyides	No, a minor pathogen root rot	13 genera in 11 families, including *Malus* spp. (Rosaceae); possibly also on Pinaceae seedlings including *Pseudotsuga menziesii* (Douglas fir), *Abies* spp., and *Tsuga mertensiana* (mountain hemlock)	Yes: native	
P. chlamydospora	Can be	*Fagus*, *Prunus* genera e.g., almond, cherry, Walnut (*Juglans regia*), Postharvest Fruit Rot on Apples and Pears.	*P. chlamydospore*: yes, native Frequently baited from streams and soils in the UK	
*P. crassamura/ megasperma** *Most likely to be P. megasperma based on UK detection data*	*P. crassamura* Uncertain, maybe opportunistic	*P. crassamura*: *Juniperus phoenicea* (Cupressaceae), *Picea abies* (Pinaceae), *Castanea sativa* (Fagaceae).	No, but present in Europe	
	P. megasperma Yes	On Fabaceae, isolates previously referred to as *P. megasperma* are now considered to be three distinct species.	Yes – native and widespread throughout Europe, USA (CA), Bulgaria, Taiwan.	

Species and complex	Pathogenic status	Known hosts	Known distribtuion	*Phytophthora* clade
			Baited from nursery waste piles in UK and soils in the UK (Schiffer-Forsyth *et al.*, 2023)	
P. cinnamomi	Yes, aggressive pathogen	Many, of note: *Abies*, *Castanea, Chamaecyparis lawsoniana*, *Fagus sylvatica, Quercus* spp. Citrus sp., *Pinus echinata* (USA), *Quercus* spp.	Yes, also present in Australia, South Africa, Europe, and USA	7a
*P. europaea /flexuosa/ Tyrrhenica** *Most likely to be P. europaea based on UK detection data*	*P. europaea* Uncertain	Associated with European oak forests; weakly aggressive to seedlings, isolated from necrotic lesion on alder root	No, but present in Europe (France, Germany), North America (USA) Frequently detected by metabarcoding in wider environment and UK nurseries	
	P. flexuosa Uncertain	*Fagus hayatae* (Fagaceae) (Asia)	No, but present in Asia	
	P. tyrrhenica uncertain	*Quercus ilex*, *Q. suber* (Fagaceae)	No, but present in Europe (Italy)	
P. ramorum	Yes	At least 26 genera in 17 families, including Rhododendron (Ericaceae) and *Quercus* spp. (Fagaceae) and Larix spp. (Larch)	Yes, Europe inc. UK, North America (Canada, USA: WA, OR, CA)	8c
P. austrocedri	Yes	UK: Lawson cypress (*Chamaecyparis lawsoniana*), Nootka cypress (*Chamaecyparis nootkatensis*), Common juniper (*Juniperus communis*). South America: Chilean cedar (*Austrocedrus chilensis*)	Yes	8d
P. obscura	Yes	*Aesculus hippocastanum* (Sapindaceae), *Pieris* sp., *Kalmia latifolia* (Ericaceae)	No, present in North America (USA: OR), Europe (Germany)	
P. syringae	Yes	29 genera in 14 families, including *Fagus sylvatica*, *Syringa vulgaris* (Oleaceae) and Rosaceae	Yes. Also found in Africa (Morocco, South, Africa), Australia, Asia (Korea) Europe (Italy), North America (Canada, USA), South America (Argentina, Brazil, Chile)	

Species and complex	**Pathogenic status**	**Known hosts**	**Known distribtuion**	***Phytophthora* clade**
Elongisporangium undulatum	Yes, often aggressive and causes root rot in trees	Christmas tree plantations of *Abies procera* and *Pseudotsuga menziesii*. Other studies report infection of *Picea sitchensis*, *Picea abies, Pinus contorta* and *Pinus sylvestris*	Limited reports from Ireland, Germany, Finland and USA	n/a
Pythium balticum	*Pythium* species often cause seed and root rot of plants and damping-off of seedlings	Unknown	Unknown	n/a
Pythium senticosum	*Pythium* species often cause seed and root rot of plants and damping-off of seedlings	Unknown	Originally isolated from temperate forest soil in Japan	n/a
Bremia (not to species level)	Yes, often	Many species of plants, and particular problem for crops, causing downy mildew	Yes	n/a
Globisporangium (not to species level)	Yes, often	Many species of plants, affecting roots	Yes	n/a

5 Discussion

5.1 Methodological comparisons – comparison of qPCR and DNA metabarcoding

We consistently found that the detection rate of *P. ramorum* using metabarcoding is lower than the detection rate found using qPCR (Table 1). It has previously been determined that the lowest minimum detection threshold for metabarcoding is one attogram (ag) (Cooke D., pers comm March 2018), which is five orders of magnitude greater in terms of detection limit compared to the species-specific qPCR primers in this project where detection goes down to 100 fentograms (Schena *et al.,* 2012). This shows that the nested PCR in the metabarcoding protocol has the potential to detect much lower levels *of P. ramorum* DNA than the species-specific qPCR assay. However, a previous PHC project (Riddell *et al.,* 2020) has suggested that a high abundance of other non-*Phytophthora sequences* in the metabarcoded DNA sample may reduce this detection rate as they come to dominate the amplicon pool during the PCRs and a similar interaction may have occurred in this project.

Species detected include the regulated pathogen *P. austrocedri* which has caused widespread mortality in native juniper (*Juniperus communis)* populations throughout Great Britain and has been seen to severely impact some cypress species in amenity and garden plantings (Green *et al.* 2016). Juniper is a keystone and pioneer species supporting a wide array of wildlife. Recreation could increase the risk of spread of *P. austrocedri* to disease-free, fragmented juniper populations and threatens the success of conservation and restoration plantings. After *P. ramorum,* the most frequently occurring species detected across all sites were detections of *P. pseudosyringae* in eight samples and *P. cinnamomi,* and *P. gonapodyid*es in six samples. *Phytophthora pseudosyringae* has been found frequently in Britain infecting several important UK species including *Nothofagus* spp., *Fagus sylvatica* (Scanu & Webber, 2012), *Larix kaempferi* (J. Webber and A. Harris, Forest Research, UK, pers comm 2022) and *Castanea sativa* in Italy (Scanu *et al.,* 2010). *Phytophthora cinnamomi* has a very broad global host range, including many woody species and has been associated with decline of European oak forests (Jung *et al.,* 2018). *Phytophthora gonapodyides* is associated with aquatic habitats and has been suggested to degrade plant debris (Brasier, *et al.*, 2003) but has caused root and collar rots and aerial cankers in Europe during extremely wet periods (Jung et al 2011). Other species detected in the soils include *P. chlamydospora(*), P. x stagnum(*); P. crassamura(*), P. megasperma(*)*; *P. europaea(*), P. flexuosa(*), P. tyrrhenica(*), P. obscura*; *P. syringae*, many of which are frequently detected in the wider environment and nursery settings. A potential close relation of another aggressive *Phytophthora* was also detected *(*see below). *Phytophthora chlamydospora* and *P. megasperma* are aquatic species frequently baited from nurseries, gardens and the wider landscape (Landa *et al.* 2021) and would seem the species most likely to be present from their respective species complexes. *Phytophthora chlamydospora* is cosmopolitan with a broad host range (Cooke 2015). *Phytophthora megaspema* is associated with riparian habitats and forests in Europe and also has associations with disease outbreaks in horticulture and agriculture in cases of waterlogging (Brasier *et al.*, 2003). *Phytophthora europaea* would seem the most likely member of its species complex to be present in our study since it has been isolated from rhizophere soils in European oak forests (Jung *et al.,* 2002). It is regularly detected in metabarcoding studies in Scotland, but the associated organism has not been isolated into culture in the UK (Riddell *et al.,* 2019). Both *P. obscura* and *P. syringae* are closely related soilborne species, also closely related to *P. austrocedri* in clade 8d. They cause root rots, cankers and foliar/shoot blights in ornamental and forest tree species (Grunwald et al 2012). *Phytophthora syringae* is considered common in Britain, causing disease on a wide range of woody and non-woody hosts (Cooke 2015).

The single detection of *P. gibbosa(*)/P. gregata(*)* and two detections of *P. citrophthora* are interesting. These *Phytophthora*s are rarely detected by metabarcoding in the UK; *Phytophthora gibbosa(*)/P. gretata(*)* are associated in Australia with declining native tree species and *Pinus radiata*. No definitive symptoms have been associated with infection by

either species and neither are known to cause dieback or mortality on host species and the wider host range is unknown (Jung *et al.,* 2011 and references within). Schiffer-Forsyth *et al* (2023) recently reported successful baiting of *P. gregata* from nursery waste, and detections of the species have been rare, to date. *Phytophthora citrophthora* was first detected in citrus trees in the USA but is cosmopolitan, with a wide host range and has previously been detected in nursery settings (Prigallo 2015) and restoration plantings (Gyeltshen *et al.,* 2020). A report from Chile noted *P. citrophthora* as causing dieback of *Nothofagus macrocarpa* (Valencia et al 2011).

A proportion of sequences were returned as 'unknown *Phytophthoras*', encompassing barcodes with high similarity to sequences in our database but lacking verifiable, published evidence of assignment to species level. These unknowns were put through NCBI ntBLAST and often the sequences were closely related to *Pythium* and *Peronospora* species. Some sequences had low similarity (<95%) to known *Phytophthora* species such as *P. boehmeriae*. Two sequences from one of the recreational forests showed high similarity (several base pairs differences) in each case to *Phytophthora pinifolia* which has had severe impacts on *Pinus radiata* in Chile but is not known to be present in the UK. This finding is now being investigated further with Scottish Forestry. Should the finding be validated, it potentially has major implications for forest health and management at that.

One variable that has not been considered in this project is the ground flora beneath the tree canopy. Studies have shown that other plant species can often serve as a reservoir for infection of tree host species and harbour pathogens with the potential to shift to other hosts (Gyeltshen 2020). Further investigation of this variable is therefore warranted to understand the risk these hosts pose to plant health and site management.

5.2 Comparison of findings from baiting and metabarcoding

The results from baiting and metabarcoding contrast in terms of the number of *Phytophthora* species identified. Specifically, a higher number of *Phytophthora* species was detected in the soil samples using metabarcoding than baiting. One possible explanation for this difference is that the pathogens detected by metabarcoding were either not viable, i.e., dead or were not able to infect live plant material. Baiting at both the recreational and harvesting sites only identified *P. gonapodyides*, which is fast-growing and adapted for aquatic environments, producing motile, asexual zoospores that can swim to new hosts, so it is unsurprising that this species occurred so frequently with baiting. However, other species identified by metabarcoding, such as *P. syringae, P. ramorum and P. cinnamomi,* produce resting structures (oospores, chlamydospores, hyphal aggregations) to endure inhospitable environments and these species can consequently be slower to colonise host material (La Spada *et al.,* 2022). Another complication is that baiting was carried out using apples for soils from the recreational sites which may not be the most suitable host tissue to bait out the variety of *Phytophthora* species identified using metabarcoding. Many studies have found differences in the species recovered from a sample using baiting and metabarcoding. Recent findings report spore viability, the growth rate of lesions, timing of sporangial production and zoospore release, competition with other *Phytophthora* and choice of bait leaves can all affect the species recovered by baiting (Sarker *et al* 2021; La Spada *et al.,* 2022). Additionally, some *Phytophthora* species are regarded as unculturable and so the ability to detect them must rely on molecular methods (Català *et al.,* 2017).

5.3 Testing for presence of P. ramorum with qPCR

From 209 DNA samples tested using the qPCR assay, 65% (136 samples) were found to contain *P. ramorum* DNA. Thirty- six samples of the 209 tested were counted as negative, with two of the three replicates giving low Ct values below the threshold of detection (<37) meaning that *P. ramorum* levels were very low and barely detectable in the sample. Such low Ct values can be problematic as they are undiscernible from low levels of DNA contamination in a sample. One potential solution is to trial the use of digital PCR (dPCR) that provides absolute

quantification of low levels of DNA and could increase confidence in detecting low levels of *P. ramorum*.

5.4 Detection associated with activities

Phytophthora ramorum DNA was detected in samples of soils collected at Kirroughtree and Glentrool along recreational trails in both sample periods. From the recreational soil samples, *P. ramorum* was detected in 12 samples from Glentrool and 19 samples from Kirroughtree compared to 6 and 5 occurrences respectively, in the metabarcoding data. As no significant difference was found in the fungal species composition detected on boot tread and bike tyres, this shows that boots are an equally effective vector as bike tyres with respect to their risk of transferring infectious propagules to new locations.

For four of the Galloway harvesting sites, *P. ramorum* DNA was detected in soil from harvesting machinery treads by qPCR in both pre-harvesting (sites, 1, 3, 4 and 5) and post-harvesting samples (2, 3, 4, and 5) indicating that *P. ramorum* was present in soil on machinery treads before it commenced harvesting, either picked up during offloading of the vehicle onto the site, or carried in the treads from a previous use. The fact that *P. ramorum* was detected in harvestor treads both pre and post-harvest, shows that infectious propagules can be picked up and moved via these vehicles.

Metabarcoding detected only half of the *P. ramorum*-positive soil samples identified by qPCR. There are several possibilities that explain this discrepancy, such as the effect of other highly abundant sequences dominating the sequencing data which, when unbalanced enough, has previously been found to reduce the chance to capture low abundance targets (Riddell *et al.*, 2020). There is also the possible effect of the differences in DNA pooling strategies used for qPCR and metabarcoding; for recreational site samples qPCR was carried out on individual field replicates on each transect, while metabarcoding was carried out on a pool of the three field replicates for each transect. This potentially diluted low abundance sequences and left them below accepted thresholds for inclusion in the data. However, recovery in commercial samples was still only 52.63%. Metabarcoding is five times more sensitive than qPCR but may still not be enough to counter dilution effects present with the recreational site samples.

6 Conclusions

Our project set out to compare the efficiency of baiting, qPCR and DNA metabarcoding for the detection and surveillance of the important statutory pathogen, *P. ramorum* in recreational and harvesting sites. We built upon a previous student project by combining those results with new sampling and methodology in this PHC-funded project. We also sought to examine the risks to tree health created by recreational site activities and movement of harvesting machinery between sites by assessing *Phytophthora* diversity in the forest soil on the treads of boots, bikes and machinery.

Using qPCR, we were able to detect *P. ramorum* DNA in soil collected from boot treads and bike tyres at recreational forests in Kirroughtree, where bike and walking trails ran beneath diseased larch, and Glentrool where trails ran through areas where *P. ramorum*-infected larch had recently been felled. In contrast, there were no detections of *P. ramorum* DNA by qPCR or metabarcoding on bike or boot treads in samples from Glentress collected in 2016 when no outbreaks of *P. ramorum* had been recorded. As expected, harvesting machinery working to remove diseased larch in the Galloway Management Zone had a high frequency of *P. ramorum* in soil accumulated in the machine treads. These findings highlight the risk of material (soil, needles, fine branches) containing *P. ramorum (*and possibly other pathogenic fungal or *Phytophthora* species*)* to be moved out of affected areas where there are diseased trees, and even out of areas where diseased trees have been felled and removed. Movement of infectious propagules to new locations may result in new infections and outbreaks.

The results from baiting were not reliable enough to confirm whether the pathogens detected were viable. Further studies would be valuable to determine whether infectious propagules transferred to a new location could drive new infections. For this type of study, an alternative method of determining pathogen viability could be evaluated, for example analysis of RNA with metabarcoding might be an option.

We have shown the value of metabarcoding to detect both *P. ramorum* and other *Phytophthora* species in a single assay. The metabarcoding also highlighted the presence of other aggressive *Phytophthora* species such as *P. austrocedri* at Glentrool as well as finding two sequences of another potentially new *Phytophthora* species for the UK. Several pathogenic *Phytophthora* species known to affect UK tree species were found, such as *P. cinnamomi*, as well as identifications of relatively rare species for the UK, *P. gregata(*) / P. gibbosa(*)* and *P. citrophthora* at Glentrool, making this site particularly diverse in *Phytophthora* species compared to Kirroughtree and Glentress. *Phytophthora gonapodyides* was common to all sites, and *P. pseudosyringae* was common amongst all three recreational sites. In contrast, only *P. ramorum* and *P. gonapodidyes* were detected on the non-recreational harvesting sites sampled in Galloway. It is not possible to easily explain the differences in species diversity, but we can only speculate as to possible reasons. The differences found in *Phytophthora* diversity may be linked to the activities on these sites based on the understanding, from this study, that pathogens can be carried on shoes treads or via bike tyres and potentially be moved to new sites/locations. It is important to point out that the recreational sites also hold a higher diversity of host tree species than harvesting sites (predominantly larch plantations) and these may serve as hosts for a greater variety of pathogens. It's possible that new species of *Phytophthora* or other pathogens are being introduced via traded planting material (Green *et al.,* 2021). The recreational sites contained both commercial species such as Sitka spruce and Scots pine, as well as native rowans and birch, and understorey plant species of *Vaccinium*, ferns and heather. Having a greater understanding of UK hosts for *Phytophthora* species would be beneficial to understand how the composition of the vegetation communities may influence the reservoirs for plant pathogens.

Collectively, our findings highlight the possible contribution of both recreational and harvesting activities in the movement of established and novel *Phytophthora* diseases within our forests and the wider environment. Considering the results, recommendations for forestry

practitioners would be to ensure boots and machinery are thoroughly cleaned before moving to different site/location. Forests used by the public for recreational activities would also benefit from biosecurity messaging around cleaning boots and bike types before and after entering the forest. The results show the importance and relevance of campaigns such as 'Keep it clean' to help raise biosecurity awareness to both the public and forestry workers.

Suggestions for further work

- Viability of species present. Future soil analyses could test for the presence of RNA as well as DNA to investigate the potential viability of soilborne *Phytophthora* species identified by metabarcoding and assess the risk of soil movements to unaffected areas causing new disease outbreaks.
- Baiting protocols. Except for *P. gonapodyides*, we were unable to bait *Phytophthora* from our soil samples even when qPCR or metabarcoding indicated they were present. Future studies could work to optimise the baiting protocol to improve recovery of viable pathogen species.
- New digital PCR methods. Digital PCR could be trialled as an alternative to qPCR as a more reliable way to detect low levels of pathogen DNA.
- Greater research is needed into the 'unknown *Phytophthora'* which has high similarity to a known *Phytophthora* species damaging a forest species in South America. Specifically, it would be beneficial to obtain a baiting methodology for the closely related *Phytophthora* species from South America as this would enable us to maximise the chances of obtaining a live isolate of the 'unknown *Phytophthora'* species and determine whether viable propagules are present in the forest. We would also be able to sequence the 'unknown *Phytophthora'* more extensively and elucidate its relationship to known *Phytophthora* species.
- Vegetation communities. Further work could investigate the presence of Phytophthoras in understorey plants to ascertain their potential role as reservoirs for pathogens and also examine the risks associated with particular tree/vegetation combinations.

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