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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: PHO414

2. Project title:
   P. ramorum/P. kernoviae: Development of post-eradication strategies for management/treatment of contaminated substrates and inoculum at outbreak sites

3. Contractor organisation(s):
   Central Science Laboratory
   Sand Hutton
   York
   YO41 1LZ

4. Total Defra project costs (agreed fixed price): £ 117,057

5. Project: start date ..... 01 April 2006
   end date ..... 31 March 2008

6. It is Defra's intention to publish this form.
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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

A number of strategies have been used for the eradication of the quarantine pathogens *P. ramorum* and *P. kernoviae*, in both nurseries and the wider environment. However, there remain a significant number of nurseries and gardens in which residual inoculum of these pathogens can still be detected following eradication, or where recurrent outbreaks continue to occur. The main sources of residual inoculum and their significance in terms of risk of new outbreaks have yet to be quantified. The proposed project aimed to investigate and identify sources of residual inoculum at selected nursery and garden/woodland outbreak sites where differing levels of eradication action had been taken, and to assess outbreak management scenarios for prevention of re-infection. Experimental work also measured levels of inoculum dispersal and disease development in ongoing outbreaks in order to monitor rates of spread of infection on host plants and determine levels of spore dispersal in relation to host plant, pathogen species, environmental conditions and level of disease management.

Initial experimental work within the project focussed on the further development of new sensitive diagnostic methodologies using TaqMan® PCR for quantification of pathogen inoculum in a range of substrates. These techniques were fully validated and protocols developed for routine detection and quantification of low levels of contamination of *P. ramorum* or *P. kernoviae* in soil or measurement of spore loads in water samples collected during the monitoring work. Overall, the use of quantitative methodologies proved highly successful and the technique has facilitated measurement of residual contamination at eradicated outbreaks and allowed measurement of the frequency and quantity of dispersal of the two pathogens over time. Baiting methods were used alongside the TaqMan PCR for validation and to check pathogen viability. Baiting methods were shown to more effective for detection of contamination in large water bodies, as the bait is exposed to potential inoculum for several days whereas direct PCR involves testing a very small sub-sample taken at one point in time. Conversely, direct PCR was found to be highly sensitive and reliable for testing soil samples and rainwater samples from traps, generating quantitative data on levels of contamination.

Monitoring of residual contamination following eradication was carried out on four selected nurseries, a managed garden and a woodland site. Monitoring on the nurseries detected either

zero contamination or extremely low levels of residual inoculum present (all local to the original infection) following removal of infected plants. Results suggest that the policy of early removal of inoculum sources (infected plants) on nurseries is very effective in preventing significant contamination of the wider environment hence minimising risks of further outbreaks. However, results also show that contamination can persist for at least a year following an outbreak.

Within the eradicated garden site, monitoring was carried out in soil, watercourses and along a gravel path where previous monitoring had shown there to be inoculum present. Monitoring in watercourses showed that inoculum of *P. ramorum* was still detectable 3 years after the original outbreak and over two years after the last plant became infected, although levels still showed an overall downward trend. It was reported previously that inoculum generally decreased significantly in watercourses during the summer months. However, monitoring in 2006 demonstrated that these trends could be significantly affected by rainfall events. Hence levels in the summer were high when monitoring was conducted following a heavy thunderstorm and were lower in the autumn following several weeks of prolonged rainfall. However, no new plant infections occurred in the vicinity of the watercourses and there was no evidence from the monitoring that the contamination present posed a significant risk for re-infection of the site. Monitoring of a gravel pathway known to be contaminated by *P. ramorum* (Defra report PH0195) detected levels of contamination that were several thousand times higher than in soils sampled from areas where infected plants had previously been located. Results indicated that inoculum could accumulate on paths via run-off and highlighted the need to consider pathways as potential sources of inoculum spread on infected sites. However, no new plant infections occurred in the vicinity of the infected path and there was no evidence from the monitoring that the contamination present posed a significant risk for re-infection of the site. Monitoring of soils in areas where previous infections had occurred showed that little or no inoculum remained in the soil following thorough removal of the infected plants.

Monitoring of soil contamination in woodland in which all rhododendron had been removed showed that limited residual inoculum was present and that the action taken had been very successful in containing the disease. Only one test using the traditional bait test was positive whereas quantitative PCR showed that very low levels of inoculum were present in a few areas, either where previous plant infections had been particularly severe or in areas where subsequent contamination had occurred usually through re-infection of re-growth on stumps. Frequency of infection of regrowth declined following retreatment with herbicide and no new tree infections have occurred.

All monitoring data clearly demonstrated that the strategy of removal of the infected plant and associated leaf litter is highly effective in controlling disease spread and substrate contamination by both *P. ramorum* and *P. kernoviae*, especially when carried out quickly and effectively. Removal of root and stump material as part of the eradication action was shown to increase the effectiveness of eradication still further and in a number of cases resulted in complete eradication of the pathogen from the local environment. Ongoing treatment of regrowth was also observed to be effective in reducing residual inoculum over time. Pruning and subsequent fungicide application was shown to be relatively ineffective in controlling disease spread in a large rhododendron plant in Cornwall. Re-infection of the plants occurred and levels of contamination in leaf litter and soil remained high. It is possible that repeated targeted fungicide treatment might have resulted in better control but would be unlikely to be completely effective on such a large plant. Tests on the use of Panacide M to decontaminate a pathway heavily infected with *P. ramorum* showed that although the treatment initially appeared to eradicate the inoculum from the path surface, within 2 months levels of inoculum had returned to previous levels. The treatments used may not have penetrated sufficiently to achieve effective control or recontamination could have occurred from external sources during rainfall. Use of other treatments, i.e. fungicides which have been shown to be effective in laboratory tests (Defra report PH0308), may be more effective but would need testing in outbreak situations.

Experimental work also measured levels of inoculum dispersal and disease development in ongoing outbreaks in order to monitor rates of spread of infection on host plants and determine levels of spore dispersal in relation to host plant, pathogen species and environmental conditions. Monitoring in an ongoing outbreak of both *P. ramorum* and *P. kernoviae* in a second

managed garden showed that localised splash dispersal of both pathogens occurred throughout the year, peaking during the autumn and winter. Wind-driven spore dispersal during rainfall was most commonly detected in the early autumn and winter months. Quantitative data on spore dispersal indicate that the epidemic of *P. kernoviae* at the garden site was more severe than that of *P. ramorum* despite the fact that the two pathogens were first detected at a similar time ((28/01/2005 and 06/05/2005 respectively). Based on monitoring between May 2006 and March 2008 (18 months), the data indicate that dispersal events for *P. kernoviae* occured more frequently than those for *P. ramorum*. Long distance dispersal of both pathogens, which coincided with a period of wet, windy weather, was first confirmed in December 2006 at a distance of >50 m from infected plants. These records were obtained near to heavily infected sites and again illustrate the increased risk of infection and spread if sources of inoculum are allowed to remain and contribute to epidemic development and escalating inoculum dispersal. Spore trapping was also demonstrated to be a highly effective technique for detecting hot spots of inoculum dispersal to assist targeting of management actions. Overall, data indicate that eradication can have a significant impact on spore dispersal levels. Throughout the monitoring period, levels of dispersal in the eradicated woodland area were less than 100 spores/250 mL of rainwater. This compared with a peak of over 400 spores/250 mL of water in traps placed nearer to an infected area and up to 50,000 spores/250 mL in traps placed near to an infected host.

Monitoring plant infection caused by *P. kernoviae* and disease progress showed that the most significant increases in disease spread within the host plants occurred following the new flush of leaves in September, rather than following the April flush. Data from the rain trap monitoring indicated a peak of spore dispersal of *P. kernoviae* which began in late summer/early autumn which would correlate well with the new infections observed in the late autumn. Results indicate greater susceptibility of rhododendron to *P. kernoviae* in the autumn but more work is needed to confirm this as the spore monitoring data cover a very limited time scale. Comparison of frequency of disease spread with seasonal anomaly meteorological data did not indicate any consistent relationship between weather conditions and disease development. More frequent monitoring and finer detailed weather data may clarify this and assist in further quantifying factors important in driving disease spread.

Report authors: Turner JA, Jennings P, Thorp G, Lockley DL, Taylor MC

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

Introduction

Considerable progress has been achieved in developing strategies for the eradication of *P. ramorum* and *P. kernoviae* in both nurseries and the wider environment. These strategies have largely focussed on the removal of infected host material and contaminated leaf/plant debris. Previous monitoring in managed gardens, carried out between 2003 and 2006, has demonstrated that when eradication action has been taken promptly, and vigilance maintained on treating/removing re-growth of the host, it has been possible to prevent further infection of host plants. However, analysis has also shown that the pathogens can persist in the environment, particularly in soil, pathways and watercourses, and evidence is starting to accumulate that residual root and stump material (remaining in the ground following eradication) may also harbour the pathogen (Defra project reports PH0195, PH0318). On affected nurseries there has been similar success, with the majority of nurseries being able to eliminate the pathogen from the site (Defra statistics, 2008).

However, there remain a significant number of nurseries and gardens on which residual inoculum of *P. ramorum* and/or *P. kernoviae* can still be detected following eradication or where recurrent outbreaks continue to occur. The main sources of residual and their significance in terms of risk of new outbreaks occurring have yet to be quantified.

Objectives

The proposed project aimed to investigate and identify sources of residual inoculum at selected nursery and garden/woodland outbreak sites where differing levels of eradication action had been taken and to assess outbreak management scenarios for prevention of re-infection. Experimental work also measured levels of inoculum dispersal and disease development in ongoing outbreaks in order to monitor rates of spread of infection on host plants and determine levels of spore dispersal in relation to host plant, pathogen species, environmental conditions and level of disease management.

Scientific objectives

1. To implement new methodologies for monitoring *P. ramorum* and *P. kernoviae* through the adoption of techniques developed in previous research projects

2. To investigate sources and levels of residual inoculum at sites where eradication action has been taken for *P. ramorum* and/or *P. kernoviae*

3. To review and evaluate control strategies for effectiveness in nursery and natural environments

4. To categorize inoculum levels at sites where action has been taken by comparison with inoculum levels of *P. ramorum* and *P. kernoviae* at a location heavily infected by both pathogens

5. To monitor the sporulation potential and spatial and temporal aspects of infection development on rhododendrons infected with *P. ramorum* and *P. kernoviae* and compare and contrast seasonal effects on disease development and spread.

All objectives set out in the contract have been met in full.

Materials and Methods

1. New methodologies for monitoring *P. ramorum* and *P. kernoviae*

Recently completed research projects have started to develop new diagnostic approaches for the detection and quantification of *P. ramorum/P. kernoviae* inoculum associated with outbreaks on nurseries and in the natural environment. These projects have examined the accuracy and effectiveness of techniques such as filtering for extraction of inoculum from water (Defra project PH0195 and HDC project HNS134) and modified PCR for extraction and quantification of inoculum of *P. kernoviae* in soil (Defra project PH0318). Traditional methods such as baiting and isolation, and methods such as LFD and PCR tests, have been used widely to date but only give a qualitative result in terms of presence or absence of disease. In analysing the scale of contamination following eradication or the significance of inoculum dispersal at an outbreak site, it has become increasingly important to measure the levels of inoculum present.

This project aimed to develop, validate and implement quantitative diagnostic techniques to monitor inoculum loads in soil, litter and in a range of substrates on nurseries, in watercourses and in the air (trapped in rain traps and aerial spore traps), to provide a mechanism to measure accurately and routinely, for the first time, the relative levels of contamination at a range of spatial and temporal scales. Contaminated samples (water, soil, compost, gravel, leaf litter) were collected from a range of locations to develop the protocols for use of water filtration, DNA extraction and quantitative PCR methods in routine monitoring of residual contamination at nursery and garden/woodland outbreak sites.

The method used for extraction of DNA from soil utilises the natural affinity of DNA to bind to silica in the presence of chaotrophic salts, as described by Boom *et al* (1990). This natural property is combined with a magnetic particle extraction stage, to produce high quality DNA extracts. Soil samples were homogenised and a 4 g sub sample placed in 2 or 3 volumes of soil extraction buffer. Each sample was beaten on a Kelco grinding mill for 2 min, decanted into 2 x 2 ml tubes and spun for 3 min at 2000 g on a Sigma 4 centrifuge. Supernatent (500 µl) was transferred to a tube containing 250ul of Buffer B (Promega, Z Z3191) and the extraction completed using a Wizard® Magnetic DNA Purification System for Food (Promega, FF3750) following the manufacturers protocol in combination with a Kingfisher ML magnetic particle processor (Thermo Electron Corporation). Samples were eluted into 200 µl of molecular grade water and stored at –35°C until required. Firstly, known numbers of spores of *P. ramorum* were added to sterilised soil. DNA was then extracted from these samples and tested for the presence of *P. ramorum* using TaqMan® PCR. The resultant Ct (cycle threshold) values were plotted against the original number of spores added to each sample to examine and calibrate levels of detection. Previous validation work has shown that the soil type did not influence the efficacy of the DNA extraction (J. Woodhall, Fera Pers. Comm.). The methods used to produce a calibration curve for quantification of *P. ramorum* in soil were as those described in Defra project report PH0318 (for detection *P. kernoviae*).

To produce calibration curves for detection of *P. kernoviae* and *P. ramorum* in water, 250 mL volumes of water were baited with 0, 10, 100, 1000 and 10000 sporangia and each volume filtered through Durapore® membrane filters (5 µm). The DNA was extracted from the filters using a NucleoSpin® kit (according to the manufacturer's protocols) and a TaqMan® PCR analysis carried out.

Following full validation, these methodologies were implemented for routine testing of samples from disease outbreaks. Each test was run against a full set of positive and negative controls such that a calibration curve was generated for each test run undertaken. All samples were tested for both *P. ramorum* and *P. kernoviae* using three replicates for each sample.

2. Investigation of sources and levels of residual inoculum at sites where eradication action has been taken for *P. ramorum* and/or *P. kernoviae*

Eradication action has now been taken at a large number of outbreaks, both on nursery and in large gardens/woodlands. Monitoring work has demonstrated that this action has been largely effective in preventing new plant infections especially if action was taken early in the outbreak and was followed up by regular inspection. However, monitoring also shows that residual inoculum can often be detected in the soil and, although so far this has diminished over time, there is still concern that this inoculum could give rise to further outbreaks in the future. Indeed, there is some evidence that this might occur, as there are a number of nurseries that have suffered recurrent outbreaks possibly as a result of re-infection from these residual sources.

Monitoring data (Defra projects PH0318 & PH0195) indicate that the two diseases differ in their ability to survive in the natural environment. It has been shown that *P. ramorum* has been able to survive in the UK environment for at least the last two years and that chlamydospores can survive considerable extremes of temperature and pH. Comparison with results from monitoring *P. kernoviae* indicates that this pathogen may be less able to survive, possibly due to an inability to produce oospores under UK conditions. Certainly, work in two woodlands in Cornwall indicates that eradication action, involving complete removal of the susceptible foliar rhododendron host, has been a very effective strategy.

The project investigated sources of residual inoculum on a range of nursery and natural outbreak sites and measured levels of inoculum persisting following eradication action. Techniques outlined and developed under objective 1 were used to establish baseline levels of inoculum on four nurseries, a large managed garden and two woodland sites. Further monitoring examined changes in inoculum levels over time and aimed to measure the effects of environmental factors and management practice.

(a) Monitoring of residual inoculum on nurseries

Four nursery sites were selected in 1) Cornwall, 2) North Yorkshire, 3) Lancashire and 4) South Yorkshire for monitoring of residual inoculum following outbreak eradication. At each site, samples were taken from areas where infected plants had been standing, and from silt traps, irrigation channels, lagoons etc to determine the extent of any residual contamination. All samples were analysed using both rhododendron leaf baiting and quantitative PCR tests.

(b) Detection of residual inoculum at an eradicated outbreak of *P. ramorum* in a managed garden in south east England

This managed garden site was monitored extensively over the period 2003-2005 and results reported in Defra project report PH0195. Eradication action was taken on the site during 2002/2003 and subsequently inspected regularly for new outbreaks. No further outbreaks were recorded on the site up to the start of the monitoring work in this project in 2006. Ongoing monitoring of the site in this project focused specifically on areas where residual inoculum had been previously shown to be present: in the watercourses and two areas of pathway/runoff.

(i) Monitoring of water courses (P. ramorum)
Seasonal monitoring of the watercourse at a *P. ramorum* outbreak in a garden in the south-east of England was carried out in the summer and autumn of 2006. Water baits were deployed into the two tributaries feeding into the north end of the gardens water system; previous results (Defra project PH0195) had shown these to be heavily contaminated with *P. ramorum*. Baits were deployed in positions 1-34 as described in PH0195, in addition baits were placed at 10 m intervals between bait points 23 and 34 (Figure 1). This more frequent baiting was used to measure the potential distance of downstream dispersal of *P. ramorum* inoculum.

Each bait consisted of a piece of muslin cloth containing cut pieces of rhododendron leaves and two pieces of polystyrene packaging material tied onto a piece of string approximately 1 m in length. Baits were deployed in the watercourse overnight before being plated onto agar, incubated and examined for the presence of *P. ramorum*. All baits were sealed in separate bags at the time of retrieval and care taken to avoid cross contamination. In addition, a 500 mL water sample was collected at each bait

point as the water bait was being collected. All water samples were filtered through Durapore® membrane filters (5μm). The DNA was then extracted and a TaqMan® PCR analysis carried out to quantify the levels of *P. ramorum* present in each sample.

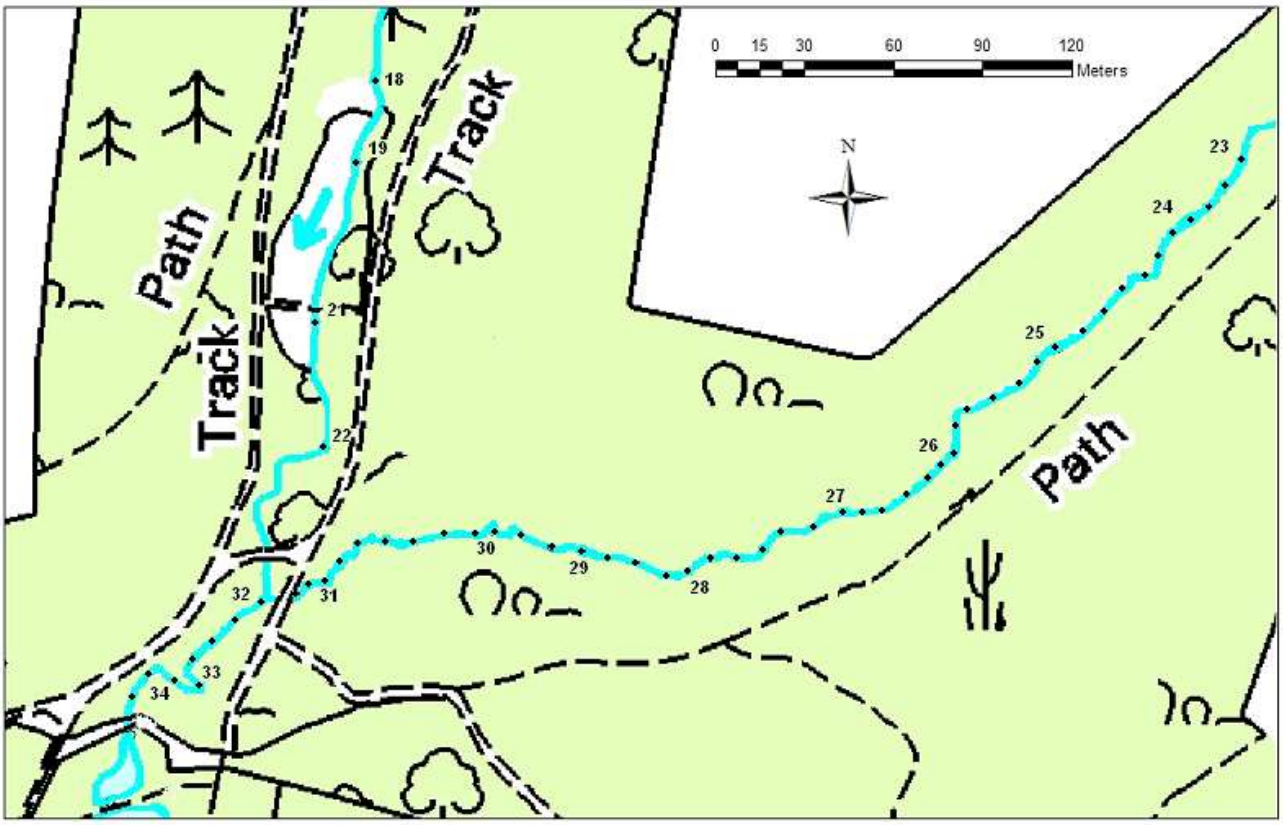


Figure 1. Water bait locations in tributaries feeding into north end of the *Phytophthora ramorum* outbreak site in the SE of England. The black dots indicate bait location; some sample points also match locations tested as part of PH0195 (points 1-17 not included on map).

ii) Monitoring of residual inoculum in pathways

A gravel pathway in a managed garden had previously shown to have consistently high levels of residual contamination even after eradication action had been taken on all infected plants (detailed in Defra report PH0195 (site 4)). Sampling of this path continued in summer 2006 to determine whether residual levels of inoculum had reduced over time. Soil/gravel samples were taken during the summer of 2006 from within a sampling grid as shown in Figure 2. Samples of the soil/gravel substrate were tested using a rhododendron leaf bait test and levels of *P. ramorum* DNA were measured using quantitative TaqMan® PCR analysis. Routine monitoring of the path ceased once the effects of disinfectant treatments applied to the path had been assessed in January 2007 (see Objective 3).

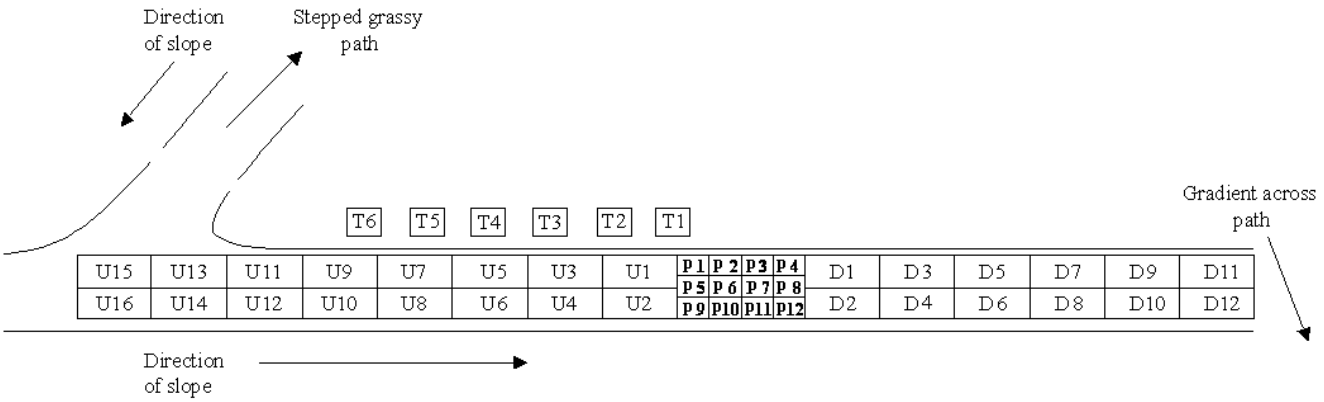


Figure 2. Sampling plan for the path contaminated by *P. ramorum.* Arrows indicate downward direction of run-off.

A second area of runoff, along the slope below the path was also tested for contamination. This area had previously also shown consistent levels of contamination. This area was not a true pathway and, being a sparsely grassed soil substrate, provided a contrast with the monitoring described above on a gravel substrate.

(c) Detection of residual inoculum in soil at an eradicated outbreak of *P. kernoviae* in woodlands in Cornwall

These woodlands had previously been affected by a severe outbreak of *P. kernoviae* and were cleared of all rhododendron and the leaf litter removed during eradication action in November 2004 (see Defra report PH0318). The two woodlands had been continuously monitored since October 2004 to obtain baseline information on levels of plant infection and litter soil contamination before and after eradication action was implemented. Results showed that the action was largely successful but there were still a small number of locations where the pathogen could be detected near to stumps where re-growth occurred subsequently became infected with *P. kernoviae*. Spore trapping using rain-traps showed that inoculum was dispersed from these sources during rainfall. Following removal of re-growth the levels of spore dispersal declined again but the pathogen was still detectable in the bark of the stumps and in the soil in the root zone.

In order to continue monitoring of residual contamination of these sites following clearance, soil samples were collected in October 2006 and June 2007 from marked locations across both woodlands (total 262 samples). Samples were tested using rhododendron leaf baits and quantitative TaqMan® PCR. Sample points were mapped onto the pre-eradication disease maps of the woods which were produced for project PH0318. Quantitative PCR analysis was continued on soil samples collected from a limited number of locations during June and September 2007 to determine whether soil inoculum loads changed over time.

The stumps and the soil surrounding the root area were also sampled to determine whether residual inoculum was present in the plant tissue and to quantify contamination in the surrounding soil. This aimed to assess the role of stumps and roots in supporting the survival of *P. kernoviae* in outbreak sites following eradication.

In a nearby wood, action had also been taken to control a *P. kernoviae* outbreak. This involved removal of rhododendrons along a path used by the public. Unlike the situation in the two woodlands above, the leaf litter at this site was left in situ. Samples were taken from this site on two occasions (October 2006 and June 2007) to measure whether there were any difference in levels of residual contamination compared to the first two woodlands which could be attributable to the difference in management action.

3. Evaluation of control strategies for management of residual contamination of *P. ramorum* and *P. kernoviae* in nursery and natural environments

Considerable progress has been made in developing strategies for the eradication of *P. ramorum* and *P. kernoviae* causing foliar infection on susceptible hosts. However, the main focus in terms of implementation has been on control of plant infection rather than treatment of residual contamination. A review of previous research work and data generated by other experimental work within this project was undertaken to identify proven strategies for management of residual contamination following eradication action. In addition, some additional experimental work was undertaken to further assess potential management strategies. A number of nurseries and large gardens which had problems with residual contamination or recurrent disease outbreaks were selected for study. Visits were made to assess and quantify sources of contamination and then treatments or cultural/management strategies were applied to attempt to eradicate the inoculum present. These included pruning, fungicide treatment and removal of contaminated debris, implementation of cultural actions during low risk periods for dispersal and disinfection of substrates. Priority substrates to be treated will include pathways and standing areas. Monitoring continued post-treatment to evaluate the success of the strategies implemented.

(a) Effectiveness of control strategies on nurseries

Monitoring of residual inoculum levels on nurseries in 2006/07 indicated either zero levels of contamination or extremely low levels of inoculum present. No sites had sufficient levels of contamination in a wide enough area to warrant further experimental work on control strategies to be undertaken.

(b) Effectiveness of control strategies in garden/woodland environments

Data from monitoring of residual contamination at all natural outbreak sites in this study were evaluated to examine the efficacy of the eradication and containment strategies implemented at each site. In addition, a range of specific control strategies were evaluated in more detail.

(i) Efficacy of chemical treatment on infected pathways (*P. ramorum*)

The contaminated pathway at the southeast garden outbreak site (described above in 2(b)(ii)) was treated with disinfectant during the autumn of 2006. The central area of the path (which over a two year period had consistently been shown to be contaminated with *P. ramorum*) was split into four treatment areas, each approximately 2m x 1m. Three of the four areas were treated with 1L of a 1:20 dilution of Panacide M, to the fourth 1L of water was applied as a control. Three methods of treatment were tested (a) direct application to the path surface, (b) preparation of the surface by raking prior to application or (c) preparation of the surface by forking prior to treatment (raking or forking, were used to ensure penetration of the treatment beyond the gravel surface). Samples were taken just prior to treatment, and then again 24 hours and two months post-treatment. Three replicate samples were taken at each sampling time and all samples were tested using both the rhododendron leaf bait and the quantitative TaqMan® PCR methods.

(ii) Removal of residual host material (*P. ramorum*)

Three areas within a large managed garden in the southeast of England where eradication action (removal of specimen rhododendrons) had been taken during 2002/2003 (monitored in Defra Project PH0195), were revisited in November 2006 and soil samples taken from around the location of the original infection. Three replicate samples were taken at each sampling time and all samples were tested using both the rhododendron leaf bait and the quantitative TaqMan® PCR methods.

(iii) Pruning and fungicide application (*P. ramorum*)

A large mature rhododendron (Figure 3) in a managed garden in Cornwall was first identified as having a low level of infection caused by *P. ramorum* in May 2004. Monitoring began in July 2004 and continued through to March 2006 (reported in Defra report PH0195). At the start of monitoring no eradication action had been taken, however during the course of monitoring, pruning of the bush skirt was carried out in summer/autumn 2004 to remove infected new shoots and increase the distance between the foliage and the contaminated soil. The plant was sprayed with fungicide (Fubol Gold (metalaxyl M/mancozeb) on a regular basis during the pruning action to try to contain the disease and prevent further infection. In addition, all leaf litter was removed from under the plant as monitoring had shown this to be a source of inoculum. These actions were carried out as an alternative strategy to

removal of the entire plant. This plant was re-visited during 2006/2007 and monthly soil samples analysed for ongoing presence of inoculum using rhododendron leaf bait tests.

Figure 3. Large rhododendron at a managed garden in Cornwall

4. Categorisation of inoculum levels at sites where action has been taken by comparison with inoculum levels of *P. ramorum* and *P. kernoviae* at a location heavily infected by both pathogens

(a) Spore dispersal in a woodland previously infected by *P. kernoviae* (infected rhododendrons eradicated)

Rainfall traps to monitor spore dispersal were set up in the two woodland sites described in Objective 2 which had been cleared of all rhododendron during eradication action in November 2004 following a severe outbreak of *P. kernoviae*. Samples were collected on a monthly basis from both low-level rain traps (LLRT) and high-level rain traps (HLRT) placed within the woods between 2006 and 2008. Low level traps were placed on the ground to trap splash-borne spores whereas the high level traps, at 1m above the ground, were designed to trap spores dispersing in wind-driven rain or dripping from infected plants above. Both trap types contained rhododendron leaves in a small amount of sterile water. Each month, the traps were sampled by removal of the rhododendron leaves for isolation of either *P. ramorum* or *P. kernoviae* using a traditional bait test. In addition to the traps located within the woodland, two high level traps were placed in open ground between one of the eradicated areas and a third wood (still heavily infected with *P. kernoviae*) to monitor any longer distance dispersal (the distance between the two areas was approx 300m, with the traps placed at 100m intervals between the two (Trap A nearest the infected area))

Methods for quantification of spores present in rainwater samples were newly developed within this project and validated for use in October 2006 (described in Objective 1). Samples of rainwater from the HLRTs were additionally tested using these new methods from October 2006. The rain water sample was filtered and tested using the quantitative TaqMan PCR. A positive test from the rhododendron leaf confirmed viability of the inoculum measured by the PCR test.

(b) Spore dispersal at a garden site infected by *P. ramorum* and *P. kernoviae* (infected plants *in situ*)

A large managed garden in Cornwall, affected by both *P. ramorum* and *P. kernoviae,* was selected for intensive study. Within the garden, five diseased plants were identified as locations for monitoring of contamination of soil/leaf litter and dispersal of inoculum; site 1 – Magnolia (Figure 4a), site 2 – Rhododendron (Figure 4b), site 3 – Pieris (Figure 4c), site 4 – Drimys (Figure 4d) and site 5 – rhododendron (Figure 4e). Rain traps (low and high level) were set up at each of the five sites and

monitored monthly from May 2006 to March 2008. Quantification of spore levels within the HLRTs was started October 2006 and continued through to March 2008.

a)  b) 

c)  .d) 

e) 

Figure 4. Monitoring sites at a managed garden in Cornwall, a) Magnolia at site1, b) Rhododendron at site 2, c) Pieris at site 3, d) Drimys at site 4 and e) Rhododendron at site 5.

Four further HLRTs were set up in open ground away from plant species susceptible to either *P. ramorum* or *P. kernoviae* with the aim of monitoring long distance spore dispersal. These sites were monitored from June 2006 to March 2008 (site 6), January and February 2007 (site 7), and January 2007 to March 2008 (sites 8 and 9 (Figure 5)). Periods of monitoring were restricted at these sites due to public access to the garden. A final HLRT (site 10) was placed near to a second infected Drimys (multi stemmed) in August 2007 but monitoring was discontinued in October 2007 following removal of

the infected plant. All traps were tested using traditional baiting and quantitative PCR as described above.

Figure 5. A high level rain trap (site 9) placed in open ground in a managed garden in Cornwall.

Following detection of very high levels of spore dispersal at site 4 (Drimys), four additional HLRTs were placed around the tree in June 2007 to provide data on direction of spore movement (traps 4B, C, D and E were placed to the NW, NE, SE and SW of the tree respectively). These trapwere were placed outside the drip zone of the tree canopy but were within a 30m radius of the tree. All traps were tested using traditional baiting and quantitative PCR as described above.

Additional monitoring work was planned to track inoculum dispersal from rhododendron near a watercourse in Devon. This was not possible as the plants were very rapidly removed as part of the eradication action taken at the site.

5. Monitoring of sporulation potential and spatial and temporal aspects of infection development on rhododendrons infected with *P. ramorum* and *P. kernoviae*

Spatial measurement techniques were developed in project PH0318 to create a visual model of a rhododendron plant infected with *P. kernoviae*. Measurements allow analysis of the height of each infection point, the rate of spread of the infection and the distance relationships between new infections and previous infection points and the infected litter on the ground.

Spread of symptoms on diseased plants within ongoing outbreak locations were measured in order to monitor rates of spread of infection on host plants in relation to host species, seasonal factors and environmental conditions. Monitoring of the spatial distribution and development of infections caused by *P. kernoviae* was continued on a diseased rhododendron bush in an infected woodland in Cornwall (started in November 2005 in Defra project PH0318). The plant was assessed in entirety using laser devices to calculate height, bearing and distance of all points of infection on the plant. A sample of infected leaves/stems was taken at each assessment and tested to confirm the continuing presence of *P. kernoviae* as the cause of the symptoms measured. Leaf washings taken at each assessment timing to measures levels of sporulation did not yield any spores. The data were used to generate sequential spatial maps of the development of the infection. Data were collected on the type and extent of symptoms at each point so that the development of infection could be quantified over time.

Measurements of the spatial distribution and development of infections of *P. kernoviae* on a Pieris and a Rhododendron plant were also initiated within a garden in Cornwall during June 2006. The monitoring of a *P. kernoviae* infection on Pieris was included as there were no suitable plants available for monitoring of a *P. ramorum* infection on rhododendron.

Results and discussion

1. New methodologies for monitoring *P. ramorum* and *P. kernoviae*

Calibration curves for *P. ramorum* in soil and water, and *P. kernoviae* in water are shown in Figures 6-8 respectively (see Defra report PH0318 for calibration curve for *P. kernoviae* in soil). Results indicate a strong relationship between number of spores present and the Ct value using PCR but there is more inherent variability in quantification of inoculum in soil compared to water. This is due to the inherent variability in soil samples and the difficulty in obtaining a homogeneous sample. Methods for measurement of inoculum in rainwater proved highly reliable and produced consistent results. In all instances, the methods used could detect DNA levels which were equivalent of 10 or less sporangia per unit of sample (4g of soil or 250ml water). Following the successful validation of these methodologies, the techniques were introduced into the experimental work for routine monitoring of inoculum levels in soil and water. Rhododendron bait testing was continued for further validation to test the viability of the inoculum being detected.

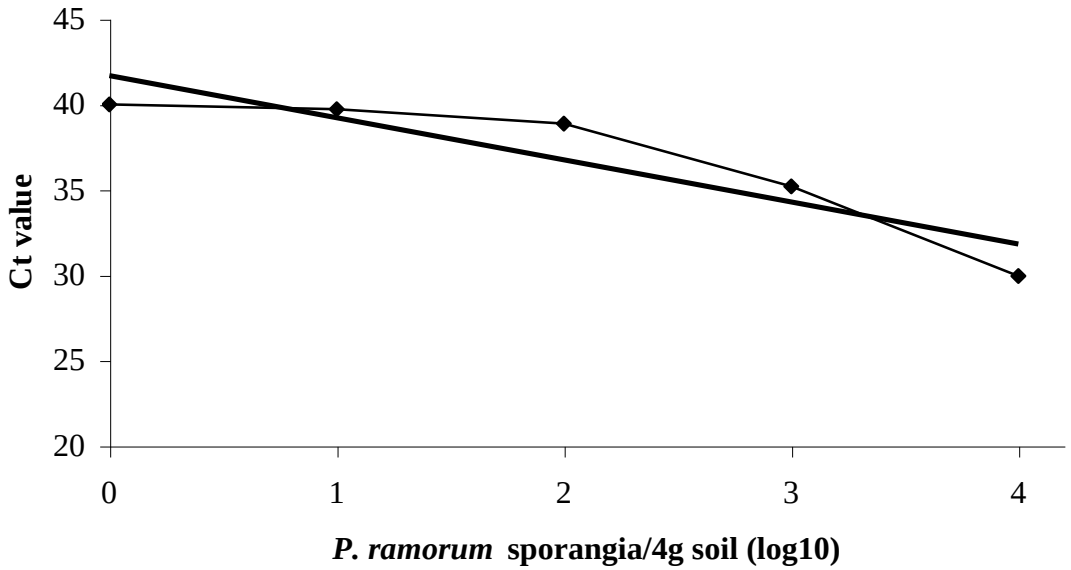


Figure 6. Calibration curve for *Phytophthora ramorum* levels in soil

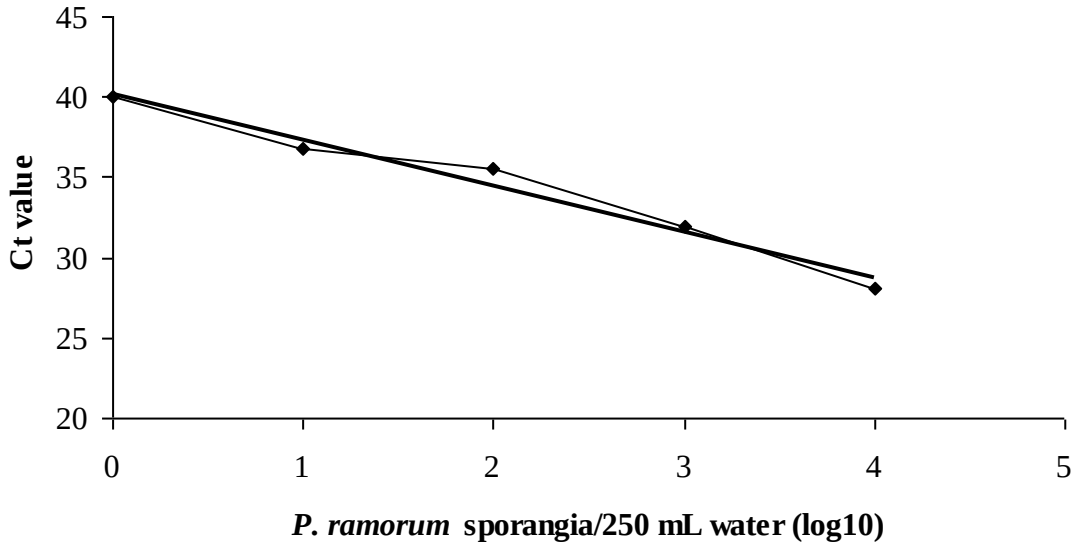


Figure 7. Calibration curve for *Phytophthora ramorum* spores in water

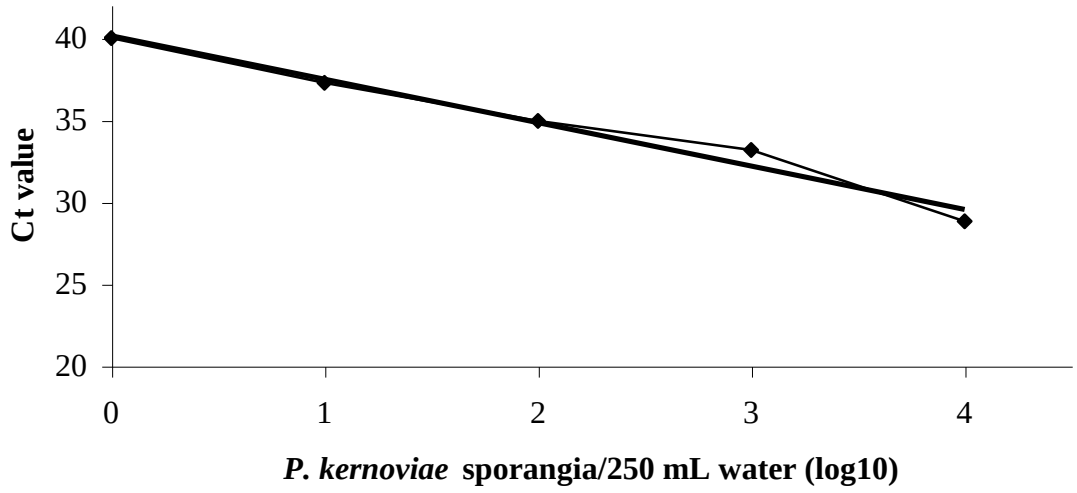


Figure 8. Calibration curve for *Phytophthora kernoviae* spores in water

2. Investigation of sources and levels of residual inoculum at sites where eradication action has been taken for *P. ramorum* and/or *P. kernoviae*

(a) Monitoring of residual inoculum on nurseries

P. ramorum was found in samples collected from three of the four nurseries visited. Fourteen percent of the samples taken were positive by one or both methods (Table 1). However, measurements of the levels of DNA present show that levels of contamination were very low. Two samples were positive using both detection methods, four samples were negative by baiting but positive by PCR and two were the reverse. These differences are probably due to variability in the soil/gravel sub-samples, very low levels of contamination and possibly cases where the PCR has detected non-viable DNA. In all instances, the positive samples were only found in areas where the infected plants had originally been located, indicating that widespread contamination of the nurseries had not taken place. Results indicate that the original statutory action taken had been effective in either eradicating the outbreak or reducing the level of contamination to a level which was epidemiologically insignificant in terms of further disease spread.

Table 1. Detection of *P. ramorum* following eradication action at nursery sites in Cornwall, North Yorkshire, Lancashire and South Yorkshire

Nursery and sample location	Sample type	Leaf bait result	DNA level (sporangia equivalent (/g soil)
Nursery 1 – Cornwall			
Road at bottom of tree unit	Gravel	Negative	0
Path outside potting tunnel	Gravel	Negative	0
1H Drainage water	Water	Negative	0
Roadway along boundary of tree unit	Gravel	Negative	0
Infected bed (stock area)	Gravel	Negative	0
Silt Trap 1	Water	Negative	0
Silt Trap 1	Silt	Negative	0
Silt Trap 3	Silt	Negative	0
Silt Trap 4	Water	Negative	0
Silt Trap 4	Silt	Negative	0
Silt Trap 7	Water	Negative	0

Silt Trap 7	Silt	Negative	0
Area 8	Gravel	***P. ramorum***	0
Area 9	Gravel	Negative	0
Area 10	Gravel	Negative	0
Area 11	Soil	Negative	0
Silt Trap 1	Water bait	Negative	N/A
Silt Trap 3	Water bait	Negative	N/A
Silt Trap 4	Water bait	Negative	N/A
Silt Trap 7	Water bait	Negative	N/A
Drain 2	Water bait	Negative	N/A
Drain 5	Water bait	Negative	N/A
Drain 6	Water bait	Negative	N/A
Nursery 2 - North Yorkshire			
Bay F26 - Irrigation trap 1	Soil	Negative	0
- Irrigation trap 2	Soil	Negative	0
Entrance to field - debris in mypex	Soil	Negative	0
Entrance to field - end of drainage channel	Soil	Negative	0
Large Lagoon - sample near filter	Soil	Negative	0
Bay F25 - Irrigation channel - sample 1	Soil	Negative	0
- Irrigation channel - sample 2	Soil	Negative	0
- Irrigation channel - sample 3	Soil	Negative	0
- Irrigation channel - sample 4	Soil	Negative	0
Bay D9 - Lagoon Inlet	Silt	Negative	0
- Sample 2	Soil	Negative	0
- Sample 3	Soil	Negative	0
- Sample 4	Soil	Negative	0
- Sample 5	Soil	Negative	0
- Sample 6	Soil	***P. ramorum***	**5**
- Sample 7	Soil	Negative	0
- Sample 8	Soil	Negative	0
Bay D10 - Last Sample on D9 run	Soil	***P. ramorum***	**4**
- Sample 1 (irrigation post 1)	Soil	Negative	0
F19 Drain	Leaf litter	Negative	0
Nursery 3 – Lancashire			
1 East - Bay 3	Soil/gravel	Negative	3
- Bay 4	Soil/gravel	Negative	**9**
- Bay 5	Soil/gravel	Negative	0
1 West - Bay 8	Soil/gravel	Negative	0
- Bay 9	Soil/gravel	Negative	0
8 West - Bay 15	Soil/gravel	Negative	2
- Bay 16	Soil/gravel	***P. ramorum***	0
- Bay 17	Soil/gravel	Negative	0
Nursery 4 – South Yorkshire			
Sample 1	Soil	Negative	0
Sample 2	Soil	Negative	9
Sample 3	Soil	Negative	0
Sample 4	Soil	Negative	0
Sample 5	Soil	Negative	0
Sample 6	Soil	Negative	0

(b) Detection of residual inoculum at an eradicated outbreak of *P. ramorum* in a managed garden in south east England

(i) Monitoring of water courses

Results from monitoring work during summer 2006 (Table 2) showed that *P. ramorum* was isolated from 54% of the baits deployed, with the majority of the positive baits being located in three main areas. Recovery of *P. ramorum* from baits was unusually high compared to previous summers; one possible explanation being that the sampling was carried out following thunderstorms which broke a prolonged spell of dry weather, thus high levels of inoculum could have been released all at once. The high levels of recovery from baits deployed in the summer contrasted with the results from the autumn baiting, where *P. ramorum* was isolated from only 15% of the baits deployed. The positive baits were located in similar areas of the stream to those showing high levels during the summer. Levels of detection in the autumn were relatively low compared to previous seasons but sampling followed a prolonged period of heavy rainfall so the spores may already have been dispersed prior to sampling. *P. ramorum* was not detected in any of the water samples collected for quantitative analysis at the summer assessment (data not shown). However, *P. ramorum* was detected, at very low levels, in three of the water samples collected during the autumn (Table 2) and were all in locations where a positive bait was also recorded within the immediate area. As so few of the samples contained any inoculum, and these contained a maximum of 4 sporangia per 250 mL water, it was not possible to carry out observations of the spore types present in the water.

Table 2. Detection of *Phytophthora ramorum* from tributaries feeding into north end of the *Phytophthora ramorum* outbreak site in south east England (See Figure 1 for map of sample points).

Bait number (Roman numerals indicate 10 m baits)	Summer 2006	Autumn 2006	
	Water bait *	Water bait*	*P. ramorum* DNA level (sporangia equivalent/250 mL)
1	-	-	0
2	-	-	0
3	-	-	0
4	-	-	0
5	-	-	0
6	-	-	0
7	-	-	0
8	+	-	0
9	+	-	0
10	+	-	0
11	+	-	0
12	+	-	0
13	-	+	0
14	-	-	0
15	-	-	0
16	+	+	0
17	+	+	0
18	-		0
19	-	-	0
20	-	-	0
21	-	-	0
22	-	-	0
23 (I)	+	-	0
II	+	-	0
III	+	+	0
24 (IV)	-	-	0
V	-	-	0
VI	-	-	0

VII	**+**	-	0
VIII	**+**	-	0
IX	**+**	-	0
X	**+**	-	0
25 (XI)	**+**	-	0
XII	**+**	-	0
XIII	**+**	-	0
XIV	**+**	-	0
XV	**+**	-	0
XVI	**+**	-	0
XVII	-	**+**	0
XVIII	-	-	0
XIX	**+**	-	0
XX	-	-	0
27 (XXI)	-	-	0
XXII	-	-	0
XXIII	-	-	0
XXIV	-	**+**	0
XXV	-	-	0
XXVI	**+**	**+**	**1**
XXVII	**+**	**+**	0
28 (XXVIII)	**+**	**+**	0
XXIX	**+**	-	**1**
XXX	**+**	-	**4**
29 (XXXI)	**+**	-	0
XXXII	**+**	-	0
XXXIII	**+**	-	0
XXXIV	**+**	-	0
XXXV	**+**	-	0
XXXVI	**+**	-	0
XXXVII	**+**	-	0
XXXVIII	**+**	-	0
XXXIX	**+**	-	0
XL	**+**	-	0
XLI	**+**	-	0
XLII	**+**	**+**	0
32 (XLIII)	-	-	0
XLIV	-	-	0
XLV	**+**	-	0
33 (XLVI)	-	-	0
XLVII	-	NS	NS
XLVIII	-	NS	NS
XLIX	-	NS	NS
34 (L)	-	NS	NS

* *P. ramorum* present (+)/absent (-); NS – no sample taken

These data were passed to Dr Mike Shaw at Reading University for analysis as part of the modelling project investigating drivers of spread of *P. ramorum* (Defra Project SD0413 'Understanding the incidence and spread of *P. ramorum* using epidemiological modelling'). Statistical analyses showed weak clustering of captures suggesting that inoculum sources within a few metres of the capture site. As a result of a review of these and other data it was suggested that a host may shed spores into a river in a small zone, which can act as the point source. This can then move by advection ie the parcel of contaminated water moves as a unit, without much dilution and mixing with neighbouring parcels. Mixing dilutes the zoospores but increases the volume containing any. Zoospores can move to a bait by zootaxis only over rather small scales; they would need to be close to or at the boundary layer around a leaf to encyst on it, so the volumes of water sampled by a bait will correspond only to a film a few mm thick moving over the leaves at the average water speed. It can be calculated that, as an order of magnitude, a bait with 100 cm^2 surface, 10 cm long, in a stream flow of 0.5 m/s samples 10^{-2}

m^2 x 10^{-3} m x 0.5 m/s/0.1 m =5 x 10^{-5} m^3/s. A positive baiting result over 3-days, ca 2 x 10^5s, therefore corresponds to at least one spore per 10 m^3. In practice the true concentrations are likely to be one or more orders of magnitude higher than this.

Results of this monitoring work show that local rainfall events can significantly influence levels of contamination in watercourses (previous work has also shown strong seasonal influences (Defra project PH0195). The data indicate that sources of contamination are likely to be very close to where the positive bait or sample point was located. Results give no evidence of significant down-stream dispersal with inoculum being continuously detected for distances of a few 10s of metres rather than 100s. The sporadic detection of very low levels of DNA present also indicates that contamination of the watercourse was not universal along the entire length.

Despite detectable levels of contamination in the watercourse, there were no new plant infections recorded on this site between 2003 and the end of the project in 2008. The results therefore indicate that the contamination in the watercourse had not acted as a source for re-infection of the site and that the inoculum present was sufficiently distant from the remaining susceptible hosts or was at too low a level to initiate a new epidemic within the garden during the monitoring period. The source of the ongoing contamination remains unclear but is likely to be via runoff from residual inoculum sources remaining following the removal of infected plants.

(ii) Monitoring of residual inoculum in pathways
Monitoring of the path during the summer of 2006 indicated that despite the very dry weather conditions, *P. ramorum* was still present in the surface soil/gravel (Figure 9). Samples from all 12 locations within the central sampling grid were positive for *P. ramorum* when tested using rhododendron leaf baits. Levels of *P. ramorum* in the soil/gravel substrate of the path were analysed using quantitative TaqMan® PCR analysis, which indicated that levels in the central grid area of the path ranged between 14 and 361 spores/g soil. The areas of path showing the highest levels of contamination were areas where there was more clay present and higher soil moisture. The path was a natural route for run-off from a large area of the graden where there had previously been a number of infected plants. It is possible that these areas were the source of inoculum moving in run-off and collecting on the path. Monitoring of the path ceased once disinfectant treatment was applied to the path (see Objective 3).

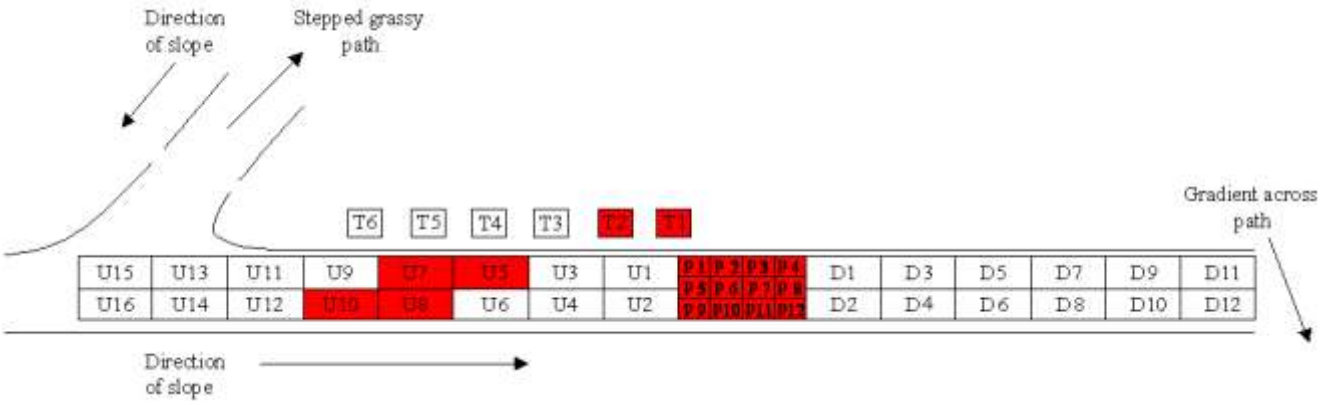


Figure 9. Locations of positive samples on a path contaminated by *P. ramorum*

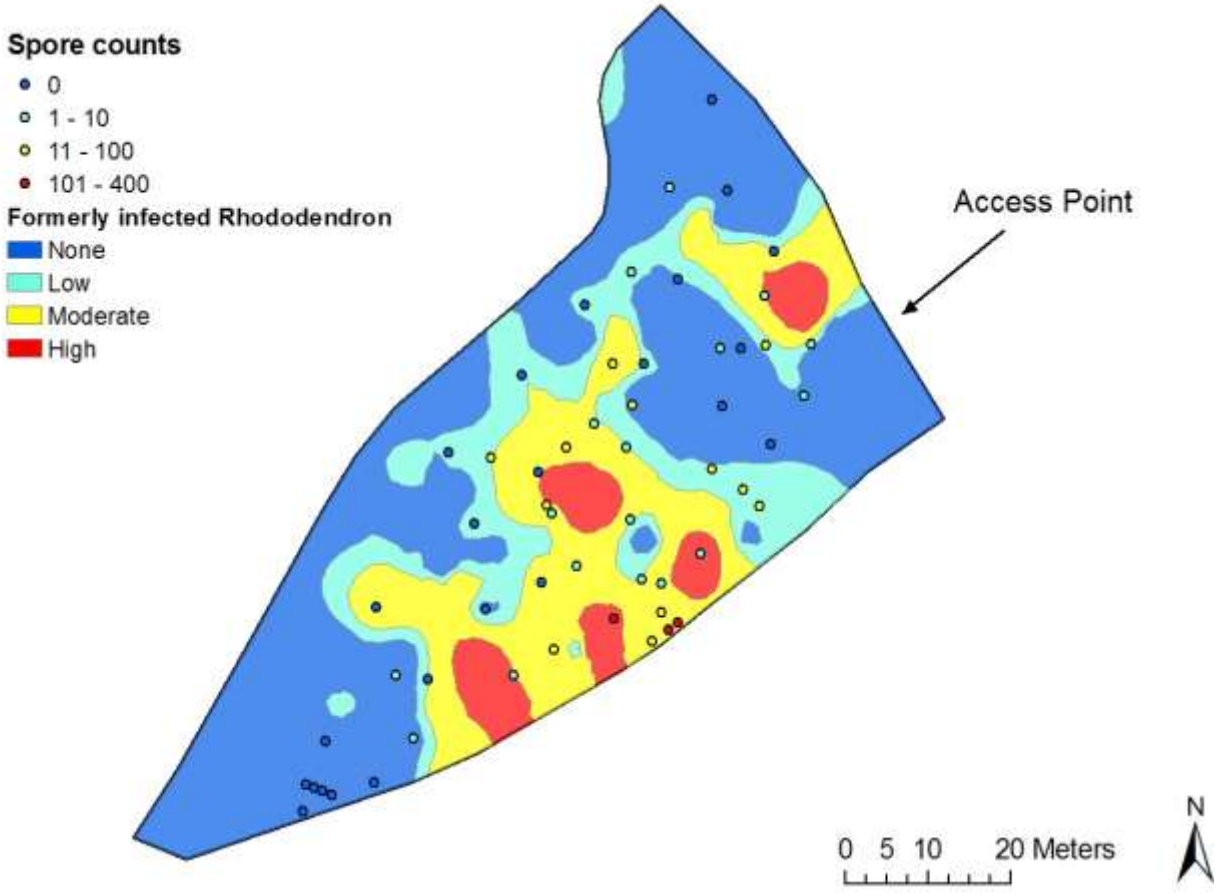
Tests on soil samples taken from an area of runoff below the path were all negative. This area had previously been shown to be consistently contaminated by *P. ramorum* but prior to the sampling the gardens management had cleared an extensive amount of leaf litter from the area and it is likely that this removed the major source of the residual inoculum.

(c) Detection of residual inoculum in soil at an eradicated outbreak of *P. kernoviae* in woodlands in Cornwall

Results from baiting of soil samples collected in October 2006 and June 2007 indicated that only one sample from the October 2006 sampling and none from June 2007 (total 262 samples) contained sufficient *P. kernoviae* inoculum to give a positive result using a rhododendron bait test. However, quantitative TaqMan® PCR analysis of the samples collected in October 2006 showed that DNA of *P. kernoviae* was present in 96 of the 133 samples with the levels of detection ranging between 1 and 97 equivalent sporangia/g soil. Mapping of the sample points onto the pre-eradication disease maps of the woods (produced for project PH0318) showed that samples containing the highest spore levels occurred in areas where disease levels were previously highest or in areas where rhododendron re-growth had previously become re-infected on residual stumps (Figure 10).

Quantitative analysis was continued on a limited number of soil samples collected during June and September 2007 from areas which had been shown to be key areas of ongoing residual contamination. These were mainly samples of soil from around residual stumps were infection of regrowth had occurred. Tests on samples of bark and root taken from the stumps to determine whether the pathogen was present were all negative. Results confirm that residual stumps and the soil surrounding the root zone were primary locations for persisting levels of residual contamination which were sustained further if regrowth occurred which subsequently became infected.

Analysis of results from the soil testing showed that levels of contamination in the soil were higher in the autumn (September and October 2006 and 2007 compared to in the summer (June 2007). There was a drop in overall levels between October 2006 and June 2007 and an increase between June and September 2007 (Table 3). These differences mirror trends previously recorded where levels of contamination in watercourses are generally higher in the autumn/winter compared to the summer (reported in Defra project report PH0195). Trends in disease development on a rhododendron bush monitored in a nearby wood (see later) also show this trend.



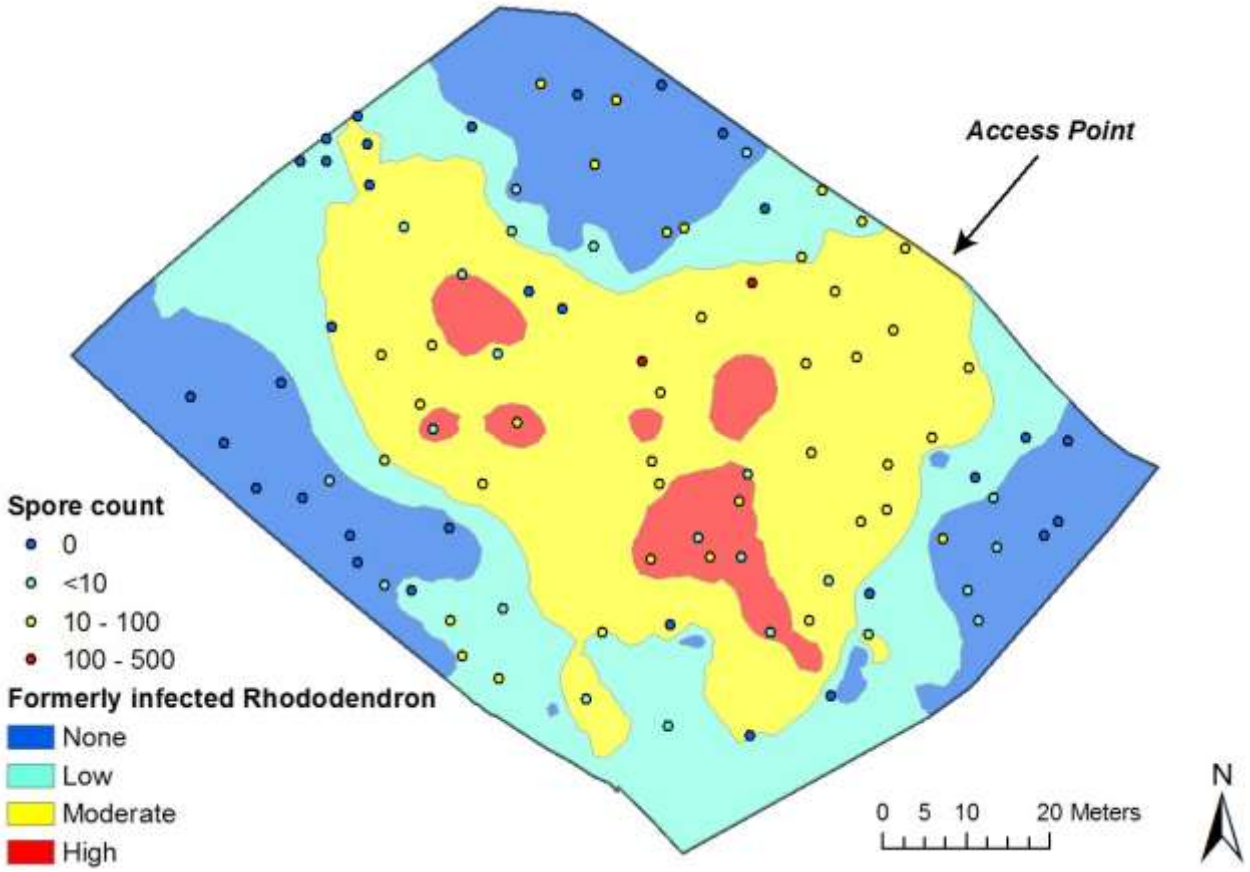


Figure 10. Maps of pre-eradication disease maps for the two south-west wood sites (produced for project PH0318) overlaid with levels of *Phytophthora kernoviae* in the soil from analyses carried out in October 2006 (Spore count = no. of equivalent sporangia/g soil)

Table 3. Changes in levels of inoculum of *Phytophthora kernoviae* in soil at selected sample locations from a wood in the south west of England.

Location	*Phytophthora kernoviae* detection in soil (equivalent sporangia/g soil)		
	October 2006	June 2007	September 2007
PE1	4	0	4
PE2	20	6	23
PE3	3	0	10
PE12	8	1	4
IP1	6	4	4
IP2	30	1	7
IP3	8	2	5
IP4	8	0	5
IP5	2	0	1
IP6	3	2	0
IP7	5	0	0
IP8	3	-	3
IP9	1	-	3
IP16	9	3	3
IP18	19	-	5
I30	20	0	9
I54	8	1	5
Mean	9.1	1.3	5.3

PE = post eradication locations, IP = infected plant locations (stumps), I = intermediate (between tree) sample locations

Samples were also taken from a nearby woodland area where infected rhododendron had been removed along a public pathway but the litter had not been cleared. All samples tested negative for *P. kernoviae* and *P. ramorum* so no comparative analyses could be undertaken.

3. Evaluation of control strategies for management of residual contamination of *P. ramorum* and *P. kernoviae* in nursery and natural environments

A review of previous research work and data generated by other experimental work within this project was undertaken to identify proven strategies for management of residual contamination following eradication action. Conclusions are summarised at the end of this report.

a) Efficacy of chemical treatment on infected pathways

A path which had previously been shown to be heavily contaminated with *P. ramorum* was disinfected using a number of methods and then sampled for ongoing presence of pathogen inoculum. All samples taken prior to treatment (autumn 2006) were positive for *P. ramorum* using both tests (Table 4), with levels detected by PCR being much higher than those in the summer (range 368 - 2,302 spores/g in autumn c.f. 14 - 361 spores/g soil in summer). *P. ramorum* could not be recovered, by baiting, from any of the samples taken 24 hours after treatment with Panacide M but could be isolated from all samples taken from the control area. PCR tests on the same samples taken following treatment showed that pathogen DNA was still detectable (but may not have been viable). Samples taken from the path two months after treatment showed that levels of *P. ramorum* had returned to levels similar to those detected prior to disinfection. This indicates that either the path was re-contaminated by *P. ramorum* or that the disinfectant treatment had not penetrated deep enough to remove all the *P. ramorum* present, thus enabling it to re-contaminate the surface layers of the path as the activity of the disinfectant declined over time. The data may also indicate that Panacide-M was not a particularly effective disinfectant and it is possible that other treatments i.e. fungicides may be more effective. Despite high levels of inoculum present on the path all plants in the vicinity remained healthy and the contamination in the path did not lead to re-infection of the site.

Table 4. Effect of Panacide M treatment on *Phytophthora ramorum* contamination of a gravel path

	Pre-treatment		Post-treatment			
			24 hour		2 month	
Treatment	Sporangia equivalent (/g soil)	*Bait test	Sporangia equivalent (/g soil)	*Bait test	Sporangia equivalent (/g soil)	*Bait test
Control	1,830	+++	297	+++	1,644	-++
Panacide-Rake	368	+++	123	---	479	-++
Panacide-Fork	525	+++	676	---	545	--+
Panacide	2,302	+++	453	---	960	-++

* number of baits (out of 3) positive or negative for *P. ramorum*.

(b) Removal of residual host material

Of the 19 samples taken from the three areas where infections had previously occurred (sites 1 to 3), none were positive using the bait test and only one sample was positive using the more sensitive PCR test (Table 5). Eradication across this site had been very thorough and often involved removal of the residual stump and root material of the infected plant. Results indicate that eradication action in these areas has been very successful, particularly where the host root material was removed as part of the eradication action taken.

Table 5. Detection of *Phytophthora ramorum* (using baiting and PCR quantification) from monitoring sites (Defra project PH0195) within the garden outbreak site in the SE.

Sample location*	Soil bait[#]	Soil quantification (*P. ramorum* sporangia equivalent/g soil
Site 1 – 1	-	0
- 2	-	0
- 3	-	0
- 4	-	0
- 5	-	0
- 6	-	0
- 7	-	199
Site 2 – 1	-	0
- 2	-	0
- 3	-	0
- 4	-	0
- 5	-	0
- 6	-	0
Site 3 – 1	-	0
- 2	-	0
- 3	-	0
- 4	-	0
- 5	-	0
- 6	-	0

* sample numbers do not relate to those in Defra report PH0195; [#] *P. ramorum* present (+)/absent (-)

c) Litter removal, pruning and fungicide application

Monitoring at a second large garden site (first started in project PH0195) indicated that residual inoculum of *P. ramorum* remained at a high level in leaf debris under a Cornish Red rhododendron which had been pruned and sprayed to remove the original infection (Figure 11). Due to the size of the plant and restricted access, the leaf debris had not been cleared on a regular basis and the prevalence of inoculum in the soil as well as the litter indicated a significant level of contamination had built up. The fluctuation in inoculum levels over time may also indicate that the pathogen was cycling within the debris. These levels of contamination were much higher than measured in other situations where action had been taken to remove litter and indicates a clear heightened risk of ongoing contamination if litter is allowed to remain in situ. The data also confirm trends observed from previous monitoring, with levels of contamination in soil and litter declining over the summer months and peaking in the early spring. However, results for summer 2007 were unusually high and probably reflect the very wet summer which may have allowed continued cycling of the pathogen during this period.

Regular assessment of the plant indicated ongoing occurrence of new infections, although at a low frequency (the size of the plant prevented a thorough assessment). Results indicate that the strategy of pruning and treatment of the original infection was not effective in controlling inoculum levels, possibly due to the excessive size of this plant and the possibility of ongoing cryptic infections being present.

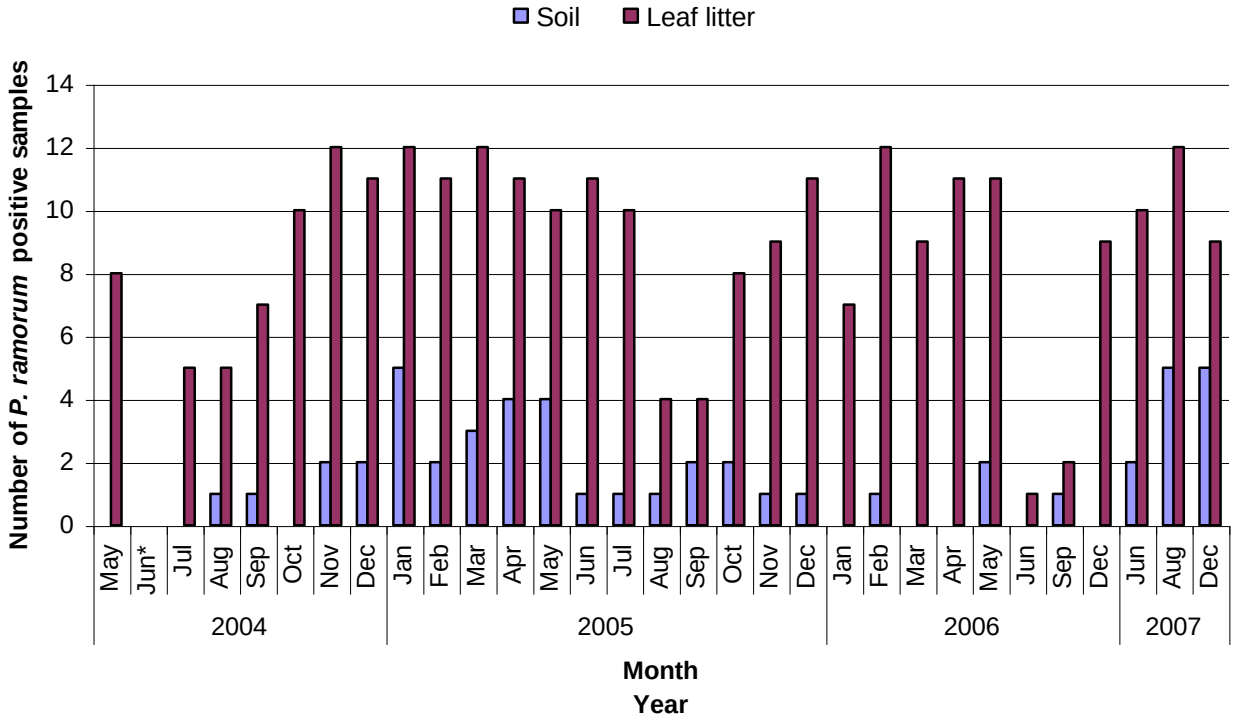


Figure 11. Detection of *P. ramorum* in soil under a Cornish Red rhododendron which had been pruned and sprayed to remove the original infection. * no samples taken

4. Categorisation of inoculum levels at sites where action has been taken by comparison with inoculum levels of *P. ramorum* and *P. kernoviae* at a location heavily infected by both pathogens

(a) Spore dispersal in woodland previously infected with *P. kernoviae* (infected rhododendrons eradicated)

No *P. kernoviae* inoculum was detected in the LLRTs placed in the two woodlands during summer 2006 (June-August, Figure 12). However, there was an overall increase in the number of traps detecting inoculum dispersal through the autumn/winter and into early spring 2007. This seasonal pattern of detection was consistent with those found in previous monitoring projects. However the historical seasonal pattern was not followed in summer 2007, with isolations falling off in the spring and increasing in the summer. This can be explained (as above) by the unusually dry conditions experienced in the spring followed by a particularly wet July. The consistent detection of splash-borne inoculum between 2006 and 2008 shows that the action taken in winter 2004 did not eradicate P. kernoviae within the woodland and the evidence suggests that the pathogen was persisting and continuing to cycle in the environment. However, the monitoring under Objective 2 showed that this inoculum was primarily associated with the remaining rhododendron stumps and regrowth and again highlights the need for thorough removal of infected host material.

P. ramorum was detected at this site for the first time in LLRTs in December 2006, and was detected consistently thereafter. This is noteworthy as there were no susceptible host plants within the woodlands themselves as these had been removed during the action to eradicate *P. kernoviae*. However, *P. ramorum* was shown to appear at the site in December 2006 and then was detected in the majority of samples thereafter suggesting that the pathogen was able to survive and sporulate in the environment via an unknown source.

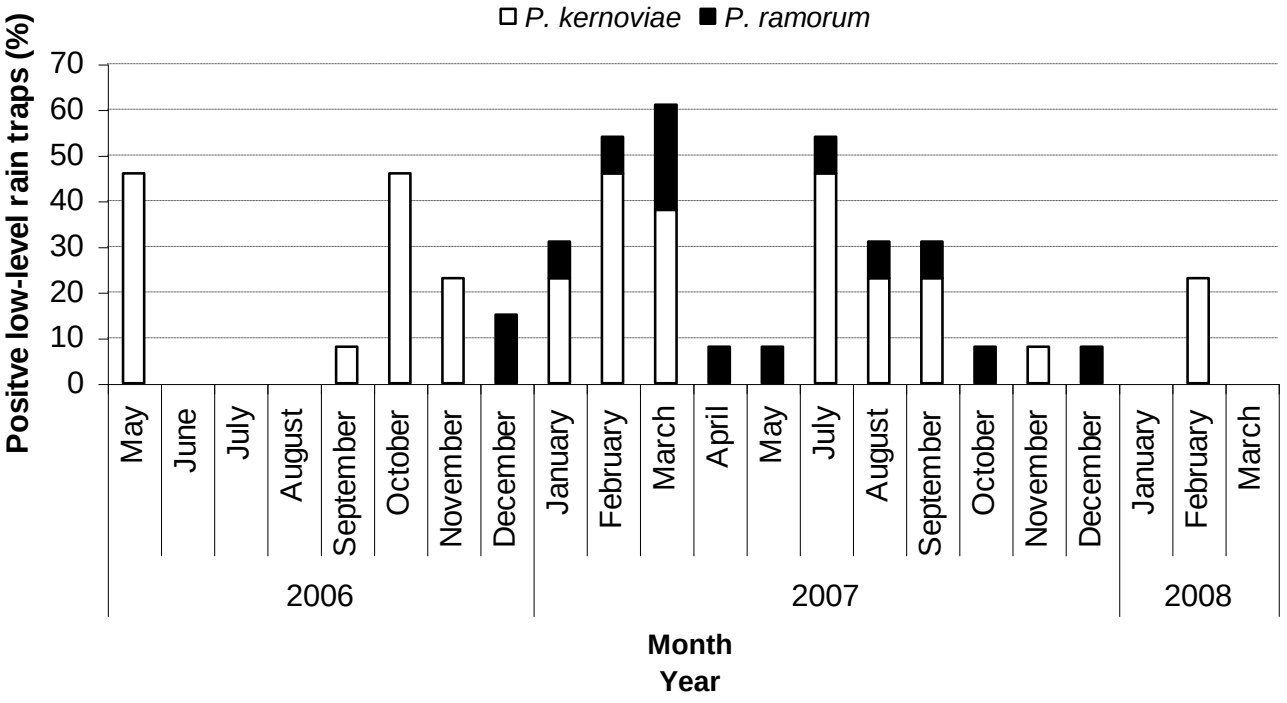


Figure 12. Combined recovery of *P. ramorum* and *P. kernoviae* from low level rain traps within the two woodland sites in the south west of England

No inoculum of either *Phytophthora* species was detected in rainwater collected in any of the HLRTs sampled monthly between June 2006 and March 2008 when tested using rhododendron leaf baits. However using filtering and quantitative TaqMan® analysis, *P. kernoviae* was detected in traps collected from both woods in December 2006 and one of the woods in January 2007 (Table 6). No spores of *P. ramorum* were detected. Results provide no evidence for inoculum of *P. ramorum* being introduced aerially from an external source.

Table 6. Detection of *P. kernoviae* from high level rain traps within two eradicated woodland sites in south west England (December 2006-January 2007)

Location	December 2006 Mean sporangia equivalent /250mL	January 2007 Mean sporangia equivalent /250mL
Wood A (6 traps)	39.2	1.0
Wood B (4 traps)	49.3	0.8

During December 2006 and January 2007, significant numbers of spores were detected in the two high level rain traps located between the two cleared woodlands and a wood still containing rhododendrons infected by *P. kernoviae* (Figure 13). Up to 400 spores of *P. kernoviae* /250mL rainwater were detected by quantitative PCR in the trap nearest to the infected area (within 100m). Low numbers of spores were detected in January, February, April, May and August 2007 and tests in all other months were negative. Bait tests indicated that the inoculum detected was viable. No *P. ramorum* was detected in these traps at any time. These results indicate a major dispersal event within the area monitored in Dec/Jan and that inoculum was dispersed up to 200m in distance.

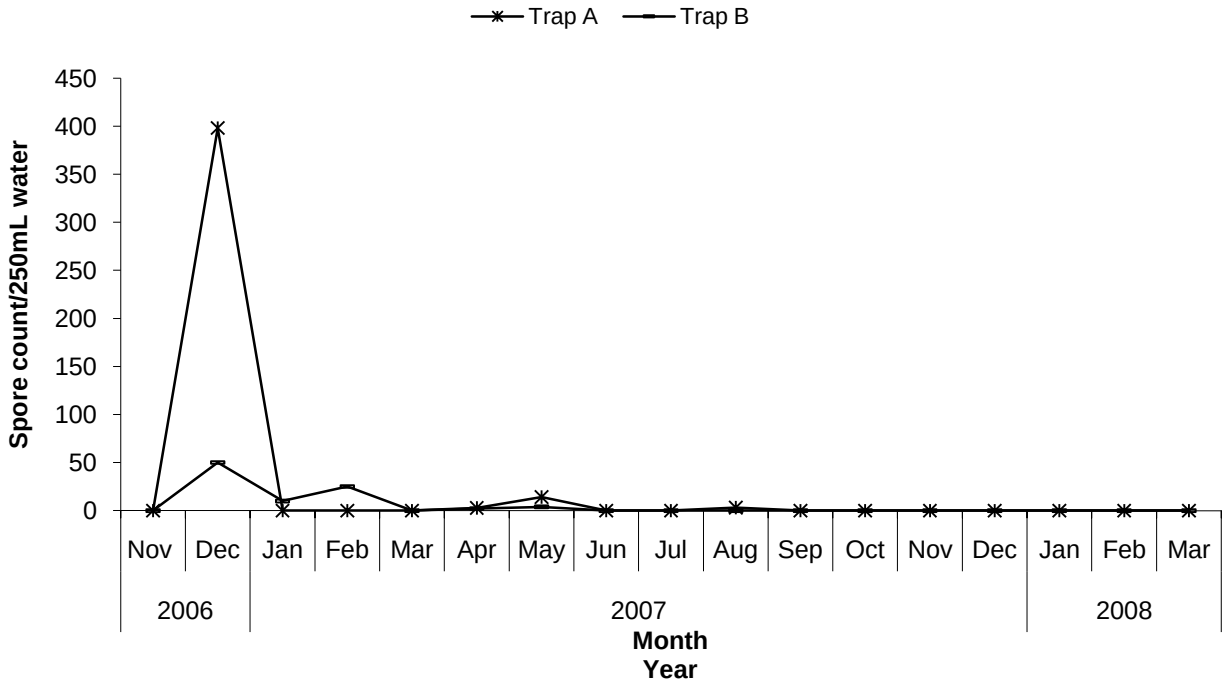


Figure 13. Detection of *Phytophthora kernoviae* spores in high level rain traps placed in open ground between two woodland sites.

(b) Spore dispersal at a garden site infected with *P. ramorum* and *P. kernoviae* (infected plants *in situ*)

Results from leaf baiting of samples from the low and high-level rain traps (Figure 14) initially showed the same seasonal trend seen in previous monitoring work, with low levels of spore dispersal in the summer (2006), which then increased over the autumn and into the winter. However, between spring 2007 and spring 2008 there was little seasonal variation in the frequency of positive traps. This was probably related to the wet summer of 2007 which allowed spore cycling to continue throughout the year. *P. ramorum* was recovered at greater frequency than *P. kernoviae* from LLRTs during the summer of 2006, however the reverse was seen in 2007.

P. kernoviae was detected in the HLRTs from the start of monitoring in May 2006. Neither *P. ramorum* nor *P. kernoviae* were detected in the HLRTs during the summer of 2006, however *P. kernoviae* was consistently detected in HLRTs after late September 2006. In contrast, *P. ramorum* was not detected in HLRTs until December 2006 and then primarily in the winter and spring. Results indicate that inoculum of *P. kernoviae* was dispersing more frequently than that of *P. ramorum*.

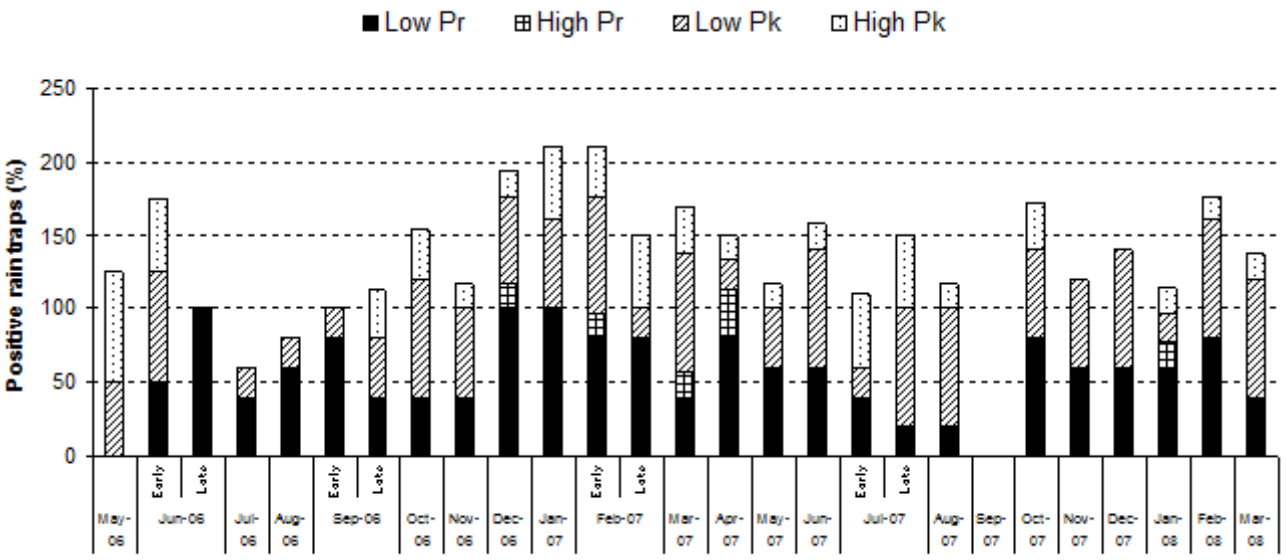


Figure 14. Recovery of *P. ramorum* and *P. kernoviae* from low and high level rain traps using rhododendron leaf baits

Tests on HLRTs using TaqMan PCR proved to be very sensitive and detected inoculum of the two pathogens far more frequently than the leaf bait tests shown in Figure 12. Quantitative data from PCR analysis of traps at five sites indicated that levels of *P. kernoviae* on the site were higher than those of *P. ramorum* (Figures 15 & 16) and indicated a more active and increasing epidemic of *P. kernoviae* compared to *P. ramorum* with levels and frequency of spore dispersal being considerably higher for *P. kernoviae*. This is despite the fact that the two pathogens were first detected on the site at a similar time ((28/01/2005 and 06/05/2005 respectively). There was a cessation of dispersal of *P. ramorum* in June/July 2007, a trend which was not observed for *P. kernoviae*. This may indicate higher moisture requirements for *P. ramorum*. The peak of dispersal of *P. kernoviae* was detected in the autumn (2007) whereas peak dispersal of *P. ramorum* occurred in the early spring and late autumn/winter. These results indicate that levels of inoculum of both pathogens progressively increased on the site starting in late 2006 (eradication action was not being taken on this site at the time) and continued as the epidemic developed.

Quantitative PCR tests on rain water from traps located at distance from susceptible host plants (site 6) detected spore dispersal of both *P. ramorum* and *P. kernoviae* between December 2006 and March 2007, although levels were very low (Figures 15 and 16). This provided the first evidence that longer distance dispersal was occurring on the site (>50 m). These results correlate with data from spore traps located in the eradicated woodland (also in Cornwall) which show similar peaks in longer distance dispersal at this time. *P. kernoviae* was also detected in low spore numbers in the HLRT at sites 8 and 9 during April and May 2007. Records of long distance dispersal at this site coincide with detection of longer distance dispersal at the woodland site (reported above). Analysis of these data in combination with the meteorological data recorded at the sites indicated that the long distance dispersal of spores was correlated with periods of wet and windy weather.

Peaks in dispersal not only occurred at different time for the two pathogens but also for the same pathogen on different hosts. Highest levels of spore dispersal of *P. kernoviae* were detected from Drimys in August/September (up to 40,000 spores/250mL water) and then magnolia in July (up to 1000 spores/250 mL water). Spore dispersal from rhododendron peaked in October (up to 700 spores / 250 mL water).

Highest levels of spore dispersal of *P. ramorum* were much lower than for *P. kernoviae*. There were a number of peaks in sporulation from rhododendron in the spring and autumn (up to 30 spores /250mL water) but the highest level measured was from magnolia in August 2007 (up to 40 spores /250 mL/water).

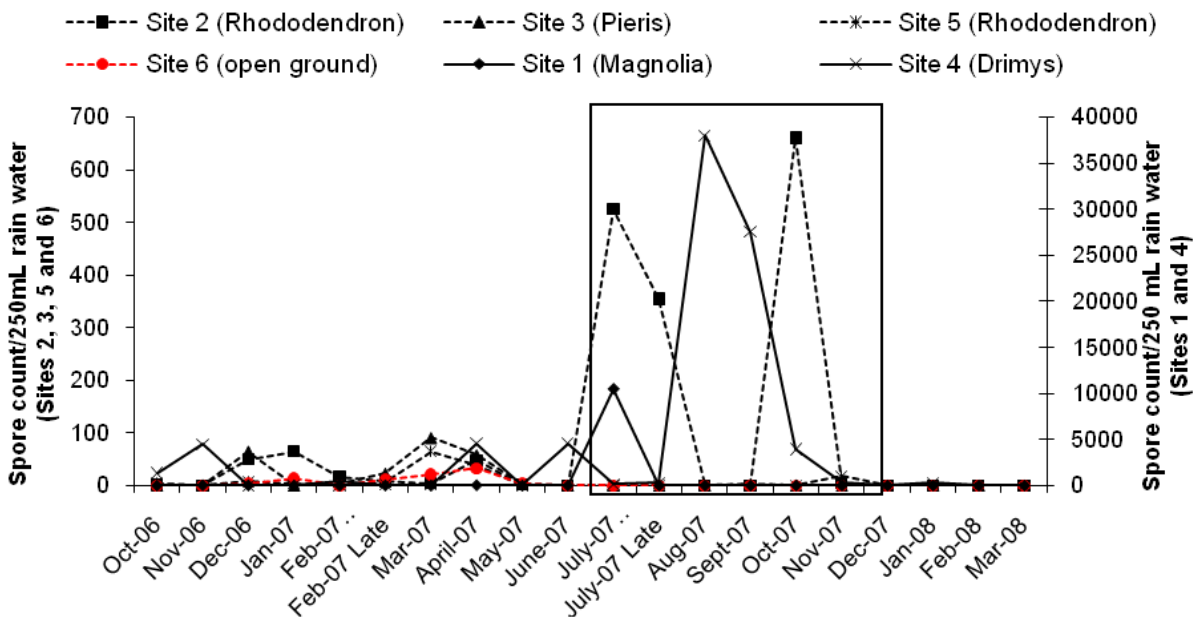


Figure 15. Estimated levels of *Phytophthora kernoviae* spores detected in rain traps at six locations at a large managed garden in Cornwall (October 06 – Mar 08)

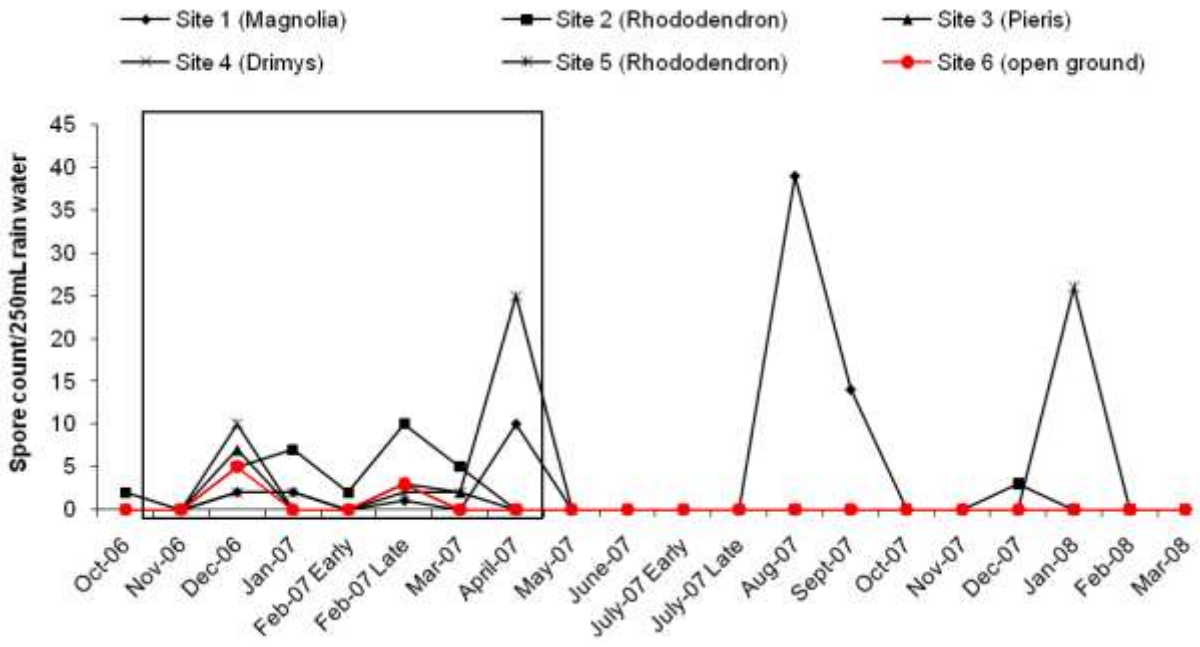


Figure 16. Estimated levels of *Phytophthora ramorum* spores detected in rain traps at six locations at a large managed garden in Cornwall (October 06 – Mar 08)

Data from spore monitoring of *P. kernoviae* at Site 4 (next to a large Drimys plant) indicated a major hot spot of activity with levels of spores detected a hundred fold higher than at other locations (Figure 15). The plant did not exhibit significant disease symptoms but a second Drimys plant nearby was found to be heavily infected. Subsequent laboratory tests showed that this host species could support significant inoculum production by *P. kernoviae* (Table 7) and due to the risks highlighted by the monitoring this plant was removed due to the risks it posed to the rest of the garden. Results also show that Drimys is not a major sporulation host for *P. ramorum*.

Table 7. Comparison of sporulation on Drimys and rhododendron in detached leaf assays*

Pathogen	Host	No. of sporangia/cm^2
P. kernoviae	Drimys	166
	Rhododendron	4
P. ramorum	Drimys	10
	Rhododdendron	10

For methodology for host testing see Defra report PH0194

Results from the additional HLRTs placed around the remaining Drimys at site 4, showed that *P. kernoviae* was detected most frequently in trap 4A, the trap placed directly beneath the infected tree (Figure 17), with the peak in detection between August and October 2007. Detection of *P. kernoviae* peaked in trap 4B (placed NW of the Drimys) in August and December 2007, whereas those placed to the NW and SE peaked in October. This suggests directional spore dispersal around the site which was dependant on wind direction.

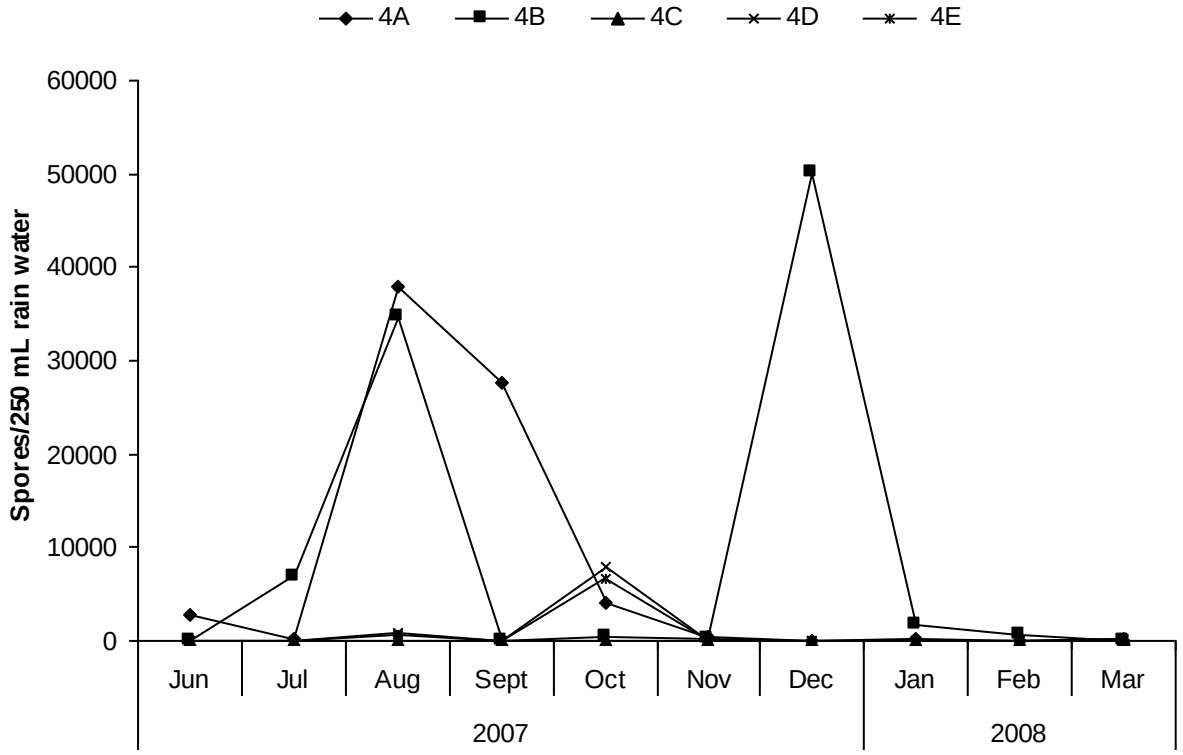


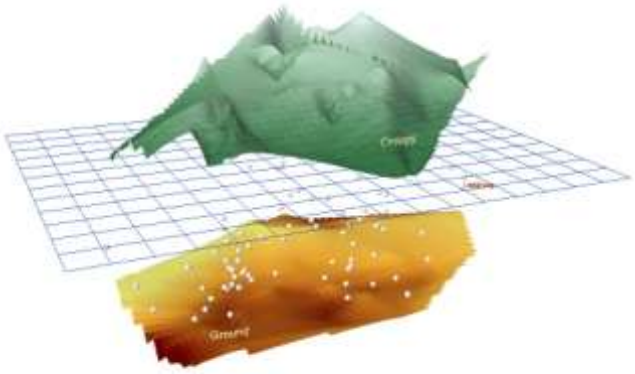
Figure 17. Estimated levels of *Phytophthora kernoviae* spores detected in rain traps at five locations around an infected Drimys in a large managed garden in Cornwall (October 06 – Mar 08). Trap 4A was placed directly under the tree and traps 4B, C, D and E to the NW, NE, SE and SW of the tree respectively.

Overall, data indicate that eradication can have a significant impact on spore dispersal levels. Throughout the monitoring period, levels of dispersal in the eradicated woodland area were less than 100 spores/250 mL of rainwater. This compares with a peak of over 400 spores / 250 mL of water in traps placed nearer to an infected area and up to 50,000 spores /250 mL in traps placed near to an infected host.

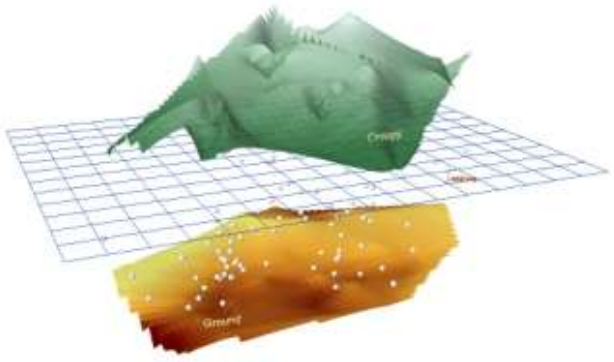
Rotorod aerial spore traps were set up in the woodland and garden monitoring areas but did not catch any spores. Further work needs to be done to determine if this result indicates that spores are not frequently dispersed in the absence of rainfall.

5. Monitoring of the sporulation potential and spatial and temporal aspects of infection development on rhododendrons infected with *P. ramorum* and *P. kernoviae*

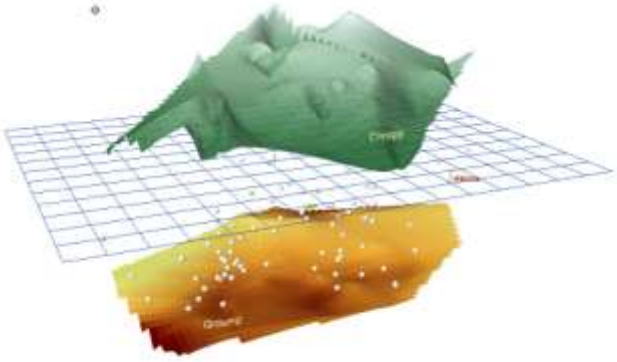
Monitoring of the spatial distribution and development of infections caused by *P. kernoviae* (started in Defra project PH0318) has continued on a diseased rhododendron bush in infected woodland in Cornwall. Mapping of the spatial measurements is shown in Figure 18. The diagrams illustrate the development of disease over time and show the relationships between infection points on the plant. Monitoring of the leaf litter at the base of the plant did not detect any residual inoculum and may indicate new infections on the plant were as a result of spore dispersal from other infected plants nearby. Measurements carried out on the bush during the summers (June) of 2006 and 2007 showed there had been little spread of the disease since the previous monitoring visits. However, significant disease spread had occurred by the autumn in both years (Table 6). Previous studies have indicated that young rhododendron leaves are more susceptible to infection by *P. kernoviae* than more mature ones. In the natural environment, rhododendron bushes produce a flush of new leaves in April and again in September, and as a result it would be expected that disease increases would be most likely to be seen at the visits immediately following these months. In these experiments, small increases in disease were recorded following the April flush (June 2006 and 2007 visits) and major increases recorded following the September flush (October 2006 and November 2007 visits) (Table 6). Comparison of the increases in disease symptoms with seasonal anomaly meteorological data for SW England and S Wales (Met office) does not indicate a consistent pattern between weather conditions and disease development (Table 6); the increase in October 2006 was preceded by warmer drier weather than usual, whereas the increase in November 2007 was preceded by cooler wetter weather than usual.



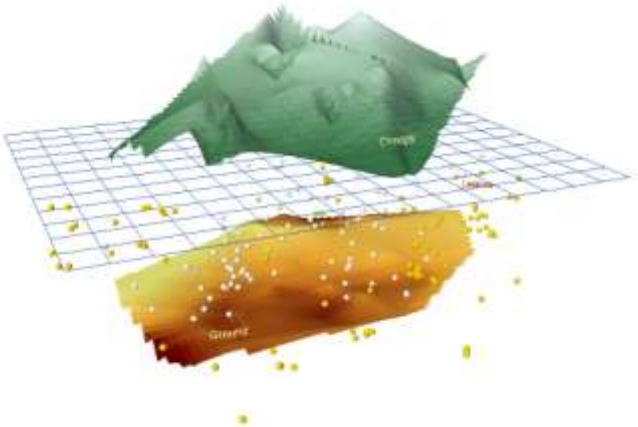
a 30th November 2005



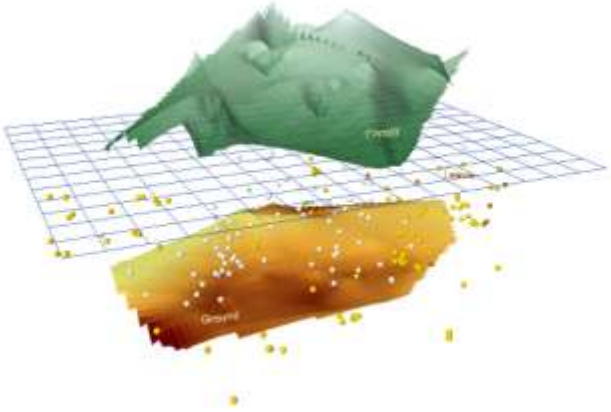
b 28th March 2006



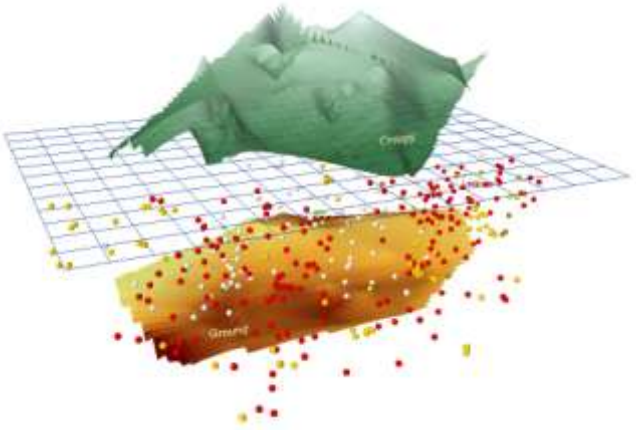
c 20th June 2006



d 17th October 2006



e 5th June 2007



f 20th November 2007

Figure 18. Diagrammatic representation of *Phytophthora kernoviae* disease development on a rhododendron bush located in an infected woodland in Cornwall, a) 30th November 2005 [white spheres], b) 28th March 2006, c) 20th June 2006 [green spheres], d) 17th October 2006 [yellow spheres], e) 5th June 2007 [brown spheres], f) 20th November 2007 [red spheres].

Table 6. Influence of seasonal weather conditions on the development of disease symptoms, caused by *Phytophthora kernoviae*, on a rhododendron found in a Cornish woodland.

Assessment date	Number of new lesions	Weather data (cf mean figures 1971-2000) for SW England and S Wales)*		
		Season	Actual temperature (°C) (anomalous value °C)	Actual rainfall (mm) (anomalous value %)
30th November '05	Baseline	Autumn '05	11.3 (+ 1.1)	411 (+ 10)
		Winter '06	4.4 (- 0.5)	236 (- 40)
28th March '06	0	Spring '06	8.4 (+ 0.2)	310 (- 25)
20th June '06	6	Summer '06	16.5 (+1.6)	156 (- 33)
10th October '06	88	Autumn '06	12.3 (+ 2.1)	419 (+ 13)
		Winter '07	6.7 (+ 1.9)	502 (+ 27)
		Spring '07	10.0 (+ 1.8)	254 (+ 2)
5th June '07	10	Summer '07	14.8 (- 0.1)	388 (+ 68)
20th November '07	183	Autumn '07	10.9 (+ 0.6)	204 (- 45)

* data from www.metoffice.gov.uk

The data may indicate that rhododendron plants are more susceptible to infection following the flush of new leaves in the autumn, rather than in the spring but the role of environmental conditions affecting sporulation, dispersal and infection cannot be fully quantified using the data available. More research would be needed to determine the drivers of these annual and consistent increases in disease during 2006 and 2007.

Similar measurements looking at the spatial distribution of infections on a Pieris (site 3) and a Rhododendron (site 5) plant were initiated within a garden in Cornwall during June 2006. At the time of the first measurement the Pieris plant was infected by *P. kernoviae* (Figure 19), although soil and path samples taken from around the base of the plant contained a mixed *P. kernoviae*/*P. ramorum* population. The Pieris was overhung by a heavily infected rhododendron and the intensity of infection reflects this as the primary inoculum source. By the second visit (October 2006), no disease symptoms were apparent and the plant had produced a significant amount of new growth. Symptoms continued to be absent at subsequent visits so only the initial measurement of the plant was carried out. These data indicate that the Pieris species was able to recover from infection, possibly as a result of the overhanging rhododendron plant dying and the infection source reducing. The data again support a strategy of removal of the infection source for effective management of disease spread.

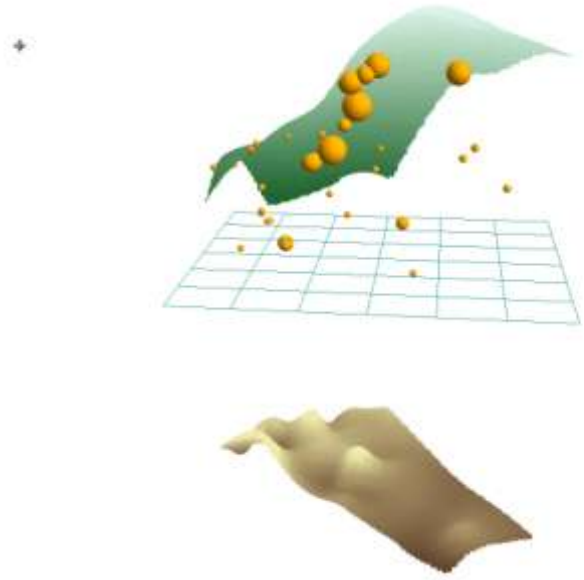
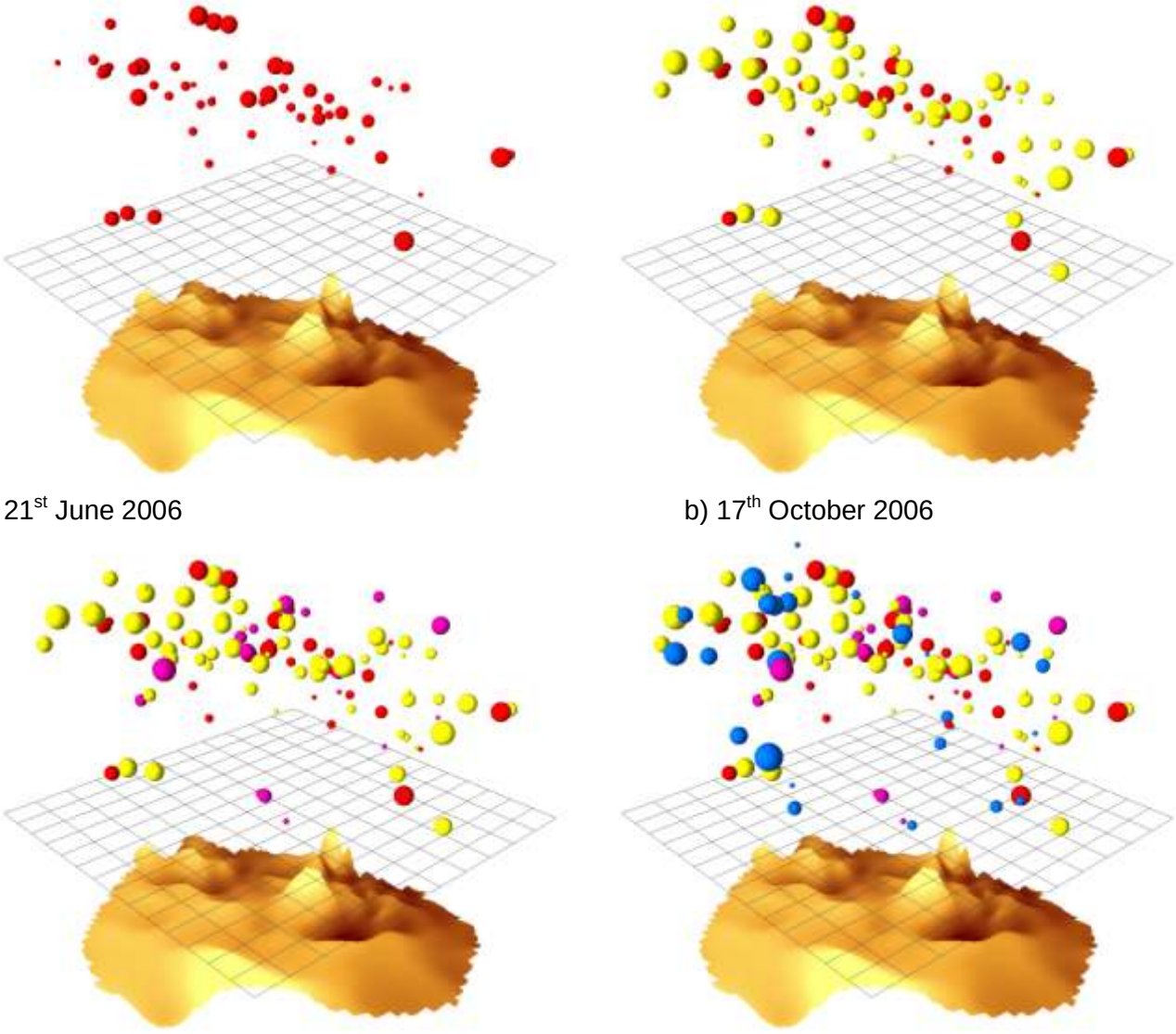


Figure 19. Diagrammatic representation of *Phytophthora kernoviae* disease on a Pieris plant (site 3, PH0318) located within a garden in Cornwall. Assessment date 21st June 2006. Size of lesion indicated by sphere size.

Unlike the disease on the Pieris plant, infections continued to develop on the Rhododendron plant located at site 5 (Figure 20). Results were similar to those from the rhododendron monitored at the woodland site in that the majority of new disease symptoms were found during the autumn assessments. This again indicates a possible higher risk of infection of rhododendron in the autumn. In contrast to the woodland site, the main increase in symptoms was recorded during the autumn of 2006 and not in autumn 2007. However this may be more related to a lack of healthy leaf material remaining on the plant than differences between the sites.



a) 21st June 2006 b) 17th October 2006

c) 5th June 2007 d) 19th September 2007

Figure 20. Diagrammatic representation of *Phytophthora kernoviae* disease development on a rhododendron bush (site 5, PH0 318) located within a garden in Cornwall a) 21st June 2006 [red spheres], b) 17th October 2006 [yellow spheres], c) 5th June 2007 [pink spheres] and d) 19th September 2007 [blue spheres]. Size of lesion indicated by sphere size.

Conclusions and policy implications of the research

New diagnostic methodologies to monitor effectiveness of eradication

- New sensitive methodologies have been developed which can detect very low levels of contamination by *P. ramorum* and *P. kernoviae* in soil and other substrates. Now fully validated, these methods provide inspectors with access to diagnostic tools that will unequivocally determine whether eradication on a nursery or natural outbreak has been achieved or not and provides researchers with the tools to quantify the drivers of inoculum spread.
- Baiting methods were shown to more effective for detection of contamination in large water bodies, as the bait is exposed to potential inoculum for several days whereas direct PCR involves testing of a very small sub-sample taken at one point in time. Conversely, direct PCR was found to be highly sensitive and reliable for testing soil samples and rainwater samples from traps, generating quantitative data on levels of contamination.
- Overall, the use of quantitative methodologies has proven highly successful in monitoring spore dispersal of *P. ramorum* and *P. kernoviae.* The technique has facilitated comparison of frequency and quantity of the two pathogens and allowed the magnitude of epidemics to be measured over time.

Monitoring of residual contamination following eradication

- Sampling on nurseries detected either zero contamination or extremely low levels of residual inoculum present following removal of infected plants. Results suggest that the policy of early removal of inoculum sources (infected plants) on nurseries is very effective in preventing significant contamination of the wider environment hence minimising risks of further outbreaks. However, results also show that contamination can persist for at least a year following an outbreak.
- Monitoring carried out in watercourses showed that inoculum of *P. ramorum* was still detectable 3 years after the original outbreak and over two years after the last plant became infected, although levels still showed an overall downward trend. It was reported previously that inoculum generally decreased significantly in watercourses during the summer months. However, monitoring in 2006 demonstrated that these trends could be significantly affected by rainfall events. Hence levels in the summer were high when monitoring was conducted following a heavy thunderstorm and were lower in the autumn following several weeks of prolonged rainfall. However, no new plant infections occurred in the vicinity of the watercourses and there was no evidence from the monitoring that the contamination present posed a significant risk for re-infection of the site.
- Monitoring of a gravel pathway on one (eradicated) outbreak site consistently detected levels of contamination that were several thousand times higher than in soils sampled from nearby areas where infected plants had previously been located. Results indicate that inoculum could accumulate on paths via run-off and highlight the need to consider pathways as potential sources of spread of inoculum on infected sites. However, no new plant infections occurred in the vicinity of the infected path and there was no evidence from the monitoring that the contamination present posed a significant risk for re-infection of the site.
- Monitoring of soil contamination in woodland in which all rhododendron had been removed showed that limited residual inoculum was present and that the action taken had been very successful in containing the disease. Only one test using the traditional bait test was positive whereas quantitative PCR showed that very low levels of inoculum were present in a few areas either where plant infections had been particularly severe or in areas where subsequent contamination had occurred usually through re-infection of re-growth.
- Similar monitoring in a garden in which all infected plants had been removed following an outbreak of *P. ramorum* also showed that little or no inoculum remained in the soil following removal of the infected plants.

Effectiveness of control strategies

- All monitoring data clearly demonstrate that the strategy of removal of the infected plant and associated leaf litter is highly effective in controlling disease spread and substrate contamination by both *P. ramorum* and *P. kernoviae*, when carried out quickly and effectively.
- Removal of root and stump material as part of the eradication action was shown to increase the effectiveness of eradication still further and in a number of cases resulted in complete eradication of the pathogen from the local environment. Ongoing treatment of regrowth was also observed to be effective in reducing residual inoculums over time.

- Pruning and subsequent fungicide application was shown to be relatively ineffective in controlling disease spread in a large rhododendron plant in Cornwall. Re-infection of the plants occurred and levels of contamination in leaf litter and soil remained high. It is possible that repeated targeted fungicide treatment might have resulted in better control but would be unlikely to be completely effective on such a large plant.
- Tests on the use of Panacide M to decontaminate a pathway heavily infected with *P. ramorum* showed that although the treatment initially appeared to eradicate the inoculum from the path surface, within 2 months levels of inoculum had returned to previous levels. The treatments used may not have penetrated sufficiently to achieve effective control or recontamination could have occurred from external sources during rainfall. Use of other treatments, i.e. fungicides which have been shown to be effective in laboratory tests (Defra report PH0308), may be more effective but would need testing in outbreak situations.

Monitoring of spore dispersal to assess infection risk

- Monitoring in an ongoing outbreak of both *P. ramorum* and *P. kernoviae* in a managed garden showed that localised splash dispersal of both pathogens occurred throughout the year, peaking during the autumn and winter.
- Wind-driven spore dispersal during rainfall was most commonly detected in the early autumn and winter months. Based on monitoring between May 2006 and March 2008 (18 months), the data indicate that dispersal events for *P. kernoviae* occur more frequently than those for *P. ramorum*.
- Long distance dispersal of both pathogens, which coincided with a period of wet, windy weather, was first confirmed in December 2006 at a distance of >50 m from infected plants. These records were obtained near to heavily infected sites and again illustrate the increased risk of infection and spread if sources of inoculum are allowed to remain and contribute to epidemic development and escalating inoculum dispersal.
- Spore trapping was demonstrated to be a highly effective technique for detecting hot spots of inoculum dispersal to assist targeting of management actions.
- Overall, data indicate that eradication can have a significant impact on spore dispersal levels. Throughout the monitoring period, levels of dispersal in the eradicated woodland area were less than 100 spores/250 mL of rainwater. This compares with a peak of over 400 spores / 250 mL of water in traps placed nearer to an infected area and up to 50,000 spores /250 mL in traps placed near to an infected host.

Monitoring plant infection and disease progress

- The most significant increases in disease spread caused by *P. kernoviae* within the host plants monitored occurred following the new flush of leaves in September, rather than following the April flush. Data from monitoring indicates a peak of spore dispersal of *P. kernoviae* which began in late summer/early autumn which would correlate well with the new infections observed in the late autumn. Results may also indicate greater susceptibility of rhododendron to *P. kernoviae* in the autumn but more work is needed to confirm this as the spore monitoring data cover a very limited time scale.
- Comparison of frequency of disease spread with seasonal anomaly meteorological data did not indicate any consistent relationship between weather conditions and disease development. More frequent monitoring and finer detailed weather data may clarify this and assist in further quantifying factors important in driving disease spread.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Turner JA, Jennings P, Humphries G, Parker SR, McDonough S, Stonehouse J, Locklet, D and Slawson D. Natural outbreaks of *Phytophthora ramorum* in the U.K. - current status and monitoring update. Proceedings of the sudden oak death third science symposium. Santa Rosa, CA, p43.