Project title:	Baiting and diagnostic techniques for monitoring *Phytophthora* spp. and *Pythium* spp. in irrigation water on ornamental nurseries
Project number:	HNS/PO 188
Project leader:	Erika F. Wedgwood, ADAS
Report:	Final report, March 2014
Previous report:	Annual report, March 2013
Key staff:	Erika Wedgwood (ADAS Project Manager) Sarah Mayne (ADAS Research Manager, Year 1) Robert Drummond (ADAS Research Manager, Year 2) Tim Pettitt (Eden Project, now at NPARU, University of Worcester) water testing, consultant, workshop presentation, & extension study examining positive LFD tests
Location of project:	ADAS Boxworth, Boxworth, Cambridge, CB23 4NN NPARU, University of Worcester, Charles Darwin Building, Henwick Grove, Worcester WR2 6AJ
Industry representatives:	Charles Carr Lowaters Nursery (Garden Beauty), Hampshire SO31 9HH Russell Woodcock Bordon Hill Nurseries Ltd., Stratford-upon-Avon, Warwickshire, CV37 9RU
Date project commenced:	1 April 2012
Date project completed (or expected completion date):	31 March 2014

DISCLAIMER

While the Agriculture and Horticulture Development Board seeks to ensure that the information contained within this document is accurate at the time of printing, no warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Dr Erika F. Wedgwood
Research Scientist
ADAS

Signature ..... Date .....

Report authorised by:

Dr Tim O'Neill
Horticulture Research Manager
ADAS

Signature ..... Date .....

CONTENTS

GROWER SUMMARY

Headline

- An apple bait bag combined with the use of a lateral flow device enables growers to conduct on-site checks for *Phytophthora* and *Pythium* spp. in stored irrigation water.
- A positive bait/LFD test indicates that further in-depth testing is required to fully determine the disease risk.

Background and objectives

Legislation, water quality issues and potential shortages are encouraging growers to collect and reuse irrigation water, either for field, container or hydroponically grown crops. Growers would benefit from the ability to have a rapid check that any non-mains water being used on plants was free of *Phytophthora* and *Pythium* spp. water-moulds.

Three reservoirs from businesses producing hardy nursery stock were experimentally bait-tested as part of project HNS 181, and *Phytophthora* and *Pythium* species able to cause root rots were detected using lateral flow devices (LFDs). The current project aims to develop these techniques via laboratory testing using isolates of pathogens which can be found on ornamental plants, followed by nursery testing. The overall objective is to develop procedures and guidelines for "Do it Yourself" testing by growers for species of *Phytophthora* and *Pythium* in irrigation water.

In the first year of the project, laboratory tests were carried out on plant material that would be readily available to growers in order to select the plant material type and quantity that baited-out *Phytophthora* spp. and *Pythium* spp. zoospores successfully. In the second year, baits were set out at intervals throughout the year in potentially naturally infested nursery reservoirs to see if there were seasonal differences in the presence of oomycetes and if the depth in the reservoir and position around the edge of an open reservoir affected zoospore trapping success. Water was also bait tested after passing through the nursery filtration systems prior to use for irrigation. Standard water samples for laboratory plate culturing were also taken for comparison. The ability of lateral flow devices (LFDs) to indicate bait infestation was examined. Most bait bag construction and some LFD use was carried out by growers at the nurseries being sampled to check that the procedures were suitable for general use. An extension study investigated the possibility that killed oomycete material might attach to apple

baits and give false positive LFD tests.

There were five specific objectives to this project:

1. To identify plant tissue baits which have the greatest sensitivity for zoospore detection.

2. To examine the sensitivity of lateral flow devices (LFDs) for detecting *Phytophthora* spp. and *Pythium* spp. in bait material in water recorded as having a range of colony forming units.

3. To determine the optimum number of bait bags, quantity of bait material and placement positions in reservoirs to maximise detection.

4. To determine whether there are any seasonal or weather related influences on zoospore release to use as guidance to maximise detection.

5. To provide step-by-step instructions for nursery staff on bait use and to provide a demonstration of the techniques at two grower events.

Extension study objective:

1. To determine whether or not cellular debris from killed oomycete cells can attach to plant tissue baits and be detected using LFD immunodiagnostic test kits to give 'false positive' tests for live pathogen presence.

Summary

Experimental procedures

In the first year a series of experiments was carried out in the laboratory to develop bait bags that could be used to catch *Phytophthora* spp. and *Pythium* spp. zoospores in irrigation water. Towards the end of the first year, monitoring experiments in nursery reservoirs were commenced to record seasonal and distribution patterns of zoospores using both isolation and baiting techniques. Water samples taken from three reservoirs in August 2012 resulted in the selection of one with a significant concentration of oomycetes for ongoing monitoring. Another nursery with a different design of reservoir was also included in monitoring from early 2013.

In the second year, apple bait bags were made by two growers and placed in reservoirs which collected run-off water from ornamental plants. One reservoir was open and used a reed bed to de-contaminate the inflow, with a particulate filter where water was drawn off. The second reservoir was covered and used a slow sand filter to remove pathogens from the water before use. Bait bags were also placed in samples of water post-filter. Water samples were taken at the same time as the baits were deployed in order to produce records of colony forming units of oomycetes (which include *Pythium* and *Phytophthora* species) on culture plates. Ceanothus leaf baits were also put in the sampled water in the laboratory to become infested by oomycete

pathogens. Monitoring was carried out in January, February, April, May, July, August, September and November 2013.

The baits were left in the water at the nurseries for 48 hours. The bags were then returned to the laboratory for testing with commercially available lateral flow devices (LFDs) sold to allow growers to test plant material for *Pythium* spp. or *Phytophthora* spp.. The baits were left to incubate until 6 days after they had been deployed in the reservoir to allow any *Pythium* spp. and *Phytophthora* spp. to multiply and so increase the probability that they would be detected. On a few dates the growers also tested baits with LFDs directly on bag retrieval from their reservoirs or post-filter.

Both shallow (30 mm) and deep (250 mm) floating baits were used throughout the sampling to determine if their catches might vary because of any difference in the vertical distribution of the oomycetes in the reservoir water over the year. At the open reservoir, baits were placed by the inflow and overflow. An additional three positions were baited twice around its perimeter in May to form a continuous period of baiting over 4 days to determine whether or not differences in *Pythium* spp. or *Phytophthora* spp. presence arose.

Objective 1. Plant bait material with greatest sensitivity for zoospore detection

Water was inoculated in the laboratory with either of two species of water-mould (oomycete) found on ornamental plant nurseries, *Phytophthora cryptogea* and a zoospore-producing species of *Pythium*. Plant bait materials of pieces of either freshly picked leaves of Rhododendron, Ceanothus and Nordmann Fir and apple and carrot flesh were suspended in separate inoculated containers in replicate bait bags made from horticultural fleece. Bait infestation was recorded by isolation onto selective agar. Not all bags were recorded as infested, the greatest number (9 out of 10) was by *Pythium* sp. of carrot, with the next best (5 out of 10) being for apple. *Phytophthora* sp. was recorded from 7 out of 10 apple bait bags, but only one carrot bag. Apple (cv. Golden Delicious) was therefore selected as the bait that would attract both pathogens, and subsequently used by growers at two nurseries to bait their irrigation water.

For the reservoir monitoring, it was speculated that the chance of a small number of zoospores being detected by the LFD would be increased by leaving the bags for four days after retrieval from the water in order to encourage the growth of *Pythium* and *Phytophthora* spp. mycelium through the apple. Positive LFD results were produced more often after warm, dark, incubation when compared with a duplicate bait bag tested straight after two days immersion.

Initial reservoir baiting in 2013 with bags containing either apple flesh or Ceanothus leaves showed that both tissues picked up *Pythium* spp. from naturally infested water, with isolation of *Pythium* spp. from a greater proportion of apple bait pieces. *Phytophthora* spp. was only isolated from an apple bait. Subsequent bait deployments using apple through the year gave positive LFDs to both *Pythium* spp. and *Phytophthora* spp. across the full range of colony forming units recorded from water sampled from a several locations.

Objective 2. Sensitivity of LFDs used on baits from water with differing colony counts

The LFDs for both *Pythium* spp. and *Phytophthora* spp. gave positive readings when used with apple baits retrieved from nursery reservoirs that were shown to be infested by isolation of colonies from water samples. The water sample colony counts (cfus) included all oomycetes, including saprophytes, with the presence of *Pythium* and/or *Phytophthora* spp. colonies noted. This gave an indication of the level of contamination rather than a quantitative assessment of *Pythium* and *Phytophthora* spp.. Detection in baits by LFDs was shown after being placed in water which containing between 20 and 3360 oomycete cfu/L and also with 20 *Phytophthora* or 26 *Pythium* cfu/L. Positive LFD results were however, also sometimes obtained from baits placed in water that had passed through a slow sand filter and for which no colonies had been detected directly from the water sample i.e. false positives. A few LFDs were negative and this was matched by the colony counts i.e. there were no false negatives. The reservoir bait LFD tests were frequently positive for both *Pythium* spp. and *Phytophthora* spp. and this was nearly always matched by these Oomycetes being detected in the water.

The LFDs produced different colour strengths of the test line in the indicator window. An index of line strength was used when recording the positive LFD readings from the baits tested a week after placement and these were compared with the Oomycete colony counts obtained from water collected when the baits were deployed. No correlation was found between the results to indicate that a stronger line on the LFD might be able to be used by growers to indicate a higher colony count in the irrigation water sampled. However, it was not known what proportion of the cfus were either *Pythium* spp. or *Phytophthora* spp. and a correlation cannot be ruled out as the LFD line strength is dependent on the amount of the correct test subject that binds to the antibodies that are labelled with coloured latex indicator material. A small concentration of the target, or debris hindering the antibodies attaching, can both cause a faint line (Malcom Briggs, Forsite Diagnostics, pers. comm.). A small number of LFDs were carried out immediately rather than after incubation of the baits and these gave the paler test lines, suggesting that the higher amount of test subject present later was more clearly detected.

When apple baits were tested in treated water from a commercial-scale slow sand filter that had been successfully operational for over 15 years, they tested positive for both *Pythium* spp. and *Phytophthora* spp. using LFDs. Concurrent laboratory plating and baiting tests on water samples from the filter detected no colonies of either genera, whilst other oomycete and non-oomycete 'indicator' species all indicated that the sand filter was working effectively. Nevertheless, a follow-up DNA test of one of the LFDs with positive *Phytophthora* spp. confirmed the presence of *Phytophthora* spp. DNA, indicating that the apple baits were detecting material from this genus. This presented two possibilities: either the baiting/LFD procedure is more sensitive than established plating techniques, or it is giving false positive tests (possibly by detecting dead pathogen material resulting from the action of the slow sand filter). 'False positive' tests could lead to unnecessary emergency maintenance of water treatment systems and costly clean-ups of water storage tanks (costs that could amount to thousands of pounds), whilst alternatively, confidence in an increased level of testing sensitivity with an 'on-site' procedure would greatly improve disease management practice. Thus, an additional short study was completed examining the possibility that killed Oomycete pathogen material might attach to apple baits and give false positive LFD tests. This study tested two *Phytophthora* spp. LFD detection kits (Pocket Diagnostic® kits, Forsite Diagnostics and Alert LF™ kits, Adgen Phytodiagnostics) against living zoospores and zoospores of two *Phytophthora* spp. (*Phytophthora cryptogea* isolate E556 and *Phytophthora* sp. Isolate C295) killed by three different treatment types (pasteurisation, ultra-violet light and chlorine dioxide). Both LFD kit types gave good detection of zoospores in water when used with apple baits and unfortunately both gave positive tests with apple baits exposed to dead zoospores. This indicates that LFD kits used with apple baits can give false positive test results by detecting dead pathogen debris when used to assess water that has been given a disinfestation treatment.

Objective 4. Seasonal and weather influences on zoospore release

Nursery monitoring in late January and late February/early March 2013 showed that both *Pythium* and *Phytophthora* zoospores are active in collected bed effluent water at this time, although at lower levels than later in the year. Monitoring continued into November in both open and lidded tank reservoirs and in the outflow of their particulate and slow sand filters, respectively. The open reservoir had higher colony forming unit (cfu) counts in water samples taken in May and July 2013 than in the other six sample months spread through the year (**Figure 1**). The reservoir at the second nursery also had most cfu in May. The higher counts might be related to the unusually high rainfall this month, which might have flushed *Pythium*

and *Phytophthora* out of pots and beds and given speedy transport into the reservoirs. The use of the water at any time of the year would lead to plant infestation regardless of the propagule concentration, therefore continual treatment and monitoring of its effectiveness would be needed at all times.

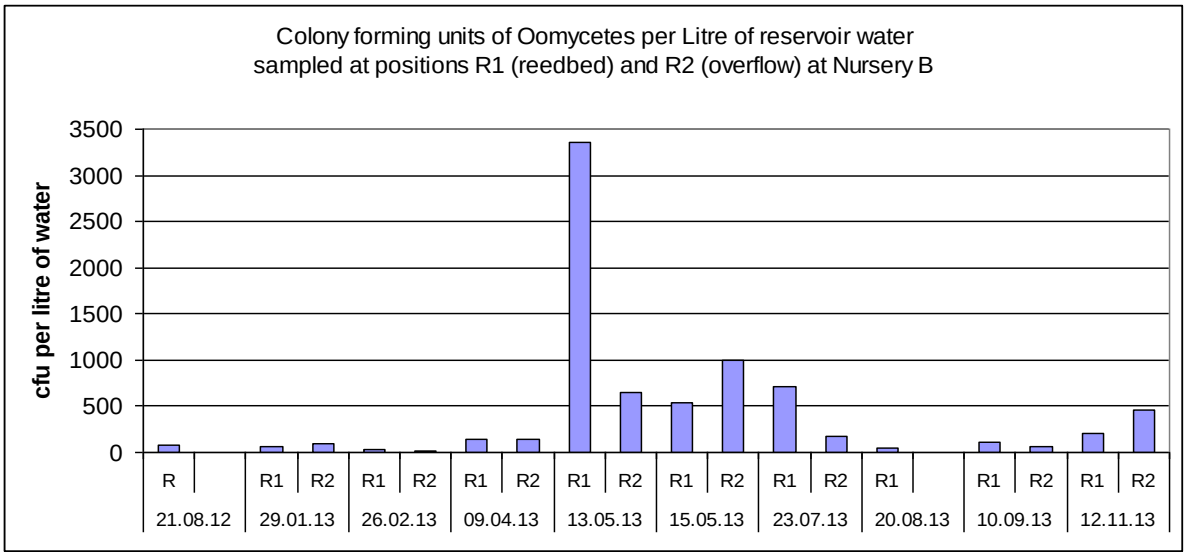


Figure 1: Colony forming units of oomycetes in water samples taken at intervals during 2013 at positions R1 (inflow) and R2 (overflow) in an open reservoir at the time of bait bag deployment showing higher levels in May and July

Objective 5. Instructions of bait and LFD use

An illustrated step-by-step guide to bait construction and deployment for *Pythium* and *Phytophthora* species, and use of the LFD test on bait tissues was devised for the nurseries taking part in the trial and will be disseminated to the wider industry. Bait use was described and/or demonstrated to growers during a number of meetings in 2013 as well as growers in the current work carrying out their own bait bag construction, use and some testing.

Financial Benefits

An on-site test has been developed which allows growers to test their own irrigation water utilising readily available materials (apple and horticultural fleece) and commercially available and relatively inexpensive diagnostic kits. Towards the end of the project, the Pocket Diagnostic LFD kits used in the work became unavailable. However, a kit for *Phytophthora*, (but not *Pythium*), was re-introduced by Forsite Diagnostics in 2014 after validation tests. Another manufacturer, Neogen, produces Adgen kits for both pathogens which use the same antigen source as the other brand. LFDs cost in the region of £16 + VAT for the two kits to detect *Pythium* spp. and *Phytophthora* spp.. Grower use of kits for a single sample location

could save around £50 + VAT plus postage on the cost of the alternative procedure of water sampling for laboratory testing (although a laboratory fee of £100 can cover the cost of five water samples) and the delay while results are returned. The latter can, however, be used to test for other pathogens and extra time is required to put together the bait bags. LFD baiting tests are useful as (a) as a supplement to laboratory procedures and (b) a quick and convenient way to get an idea of potential risks of specific pathogens, especially *Phytophthora.* Not all *Pythium* species are pathogenic.

The use of the baiting test will allow growers to reduce contamination of growing areas e.g. by treating the pathogen infested water or using an alternative water source and so reduce losses to *Phytophthora* and *Pythium* root rots. Root rot pathogens can otherwise spread and cause whole crop loss (particularly in non-woody plants) or loss of vigour.

The use of baits for detection of infested water will contribute to Integrated Crop Management measures that can be utilised to fulfil the requirements of the EU Sustainable Use Directive whereby monitoring is expected to determine the need for, and justify, any chemical control measures.

Action Points

- To minimise the risk of infestation of crops, growers should monitor reservoirs, or the water being drawn off from them, for *Pythium* and *Phytophthora* as these species can be found all year round in collection reservoirs.
- Growers should consider using apple baits, combined with the use of lateral flow devices (LFDs) for *Phytophthora* and *Pythium* species, to monitor the biological safety of their irrigation water with respect to these water-mould root pathogens.
- Negative bait/LFD tests give a good indication of biological safety with respect to *Phytophthora* and *Pythium* species. However, positive tests need to be interpreted with caution, especially when testing efficacy of water disinfestation treatments, as apple baits can detect dead pathogen material. With our current state of knowledge it is right to say that a positive bait/LFD test indicates that further in-depth testing is required to fully determine the disease risk.

SCIENCE SECTION

Introduction

Laboratory testing of nursery water

There is a risk that irrigation water collected from roofs and growing areas is contaminated with species of plant pathogenic *Phytophthora* and *Pythium*. These water moulds are likely to be found in nursery run-off water throughout the year, probably as swimming spores (zoospores). Researchers in Germany, the USA and UK have identified at least ten species of *Phytophthora* in nursery run-off including *P. cactorum*, *P. cryptogea* and the complex of types of *P. citricola* in nursery run-off. Plant clinic samples show that *Phytophthora* and *Pythium* frequently cause rotted roots in ornamental plants without causing obvious foliar wilt or dieback. Zoospores can thus be released without growers being aware that there are infected plants on the nursery. Some growers send bottles of water from their irrigation tanks for laboratory checking for plant pathogens. However, the sampling technique, sample numbers, and sampling intervals are at the discretion of the grower. Frequent tests are needed to allow a reasonably quick response to any failure in water treatment. *Phytophthora* zoospore concentrations as high as 100 spores per litre of water can be found in nursery bed effluent. A badly contaminated reservoir might have 30 spores per litre of water. *Pythium* spore counts can be 200 to 300 per litre in reservoirs. Detection limits of *Pythium* and *Phytophthora* spores in samples by filtering and culturing is down to three spores in a litre of water (Tim Pettitt, *pers. comm.*).

Some growers no longer send in water samples after having received results showing no contamination. However, zoospore release may be more likely under particular conditions and information is lacking on how sampling date affects the likelihood of contamination being detected. Water sampling using sample bottles is a “lucky dip”, particularly when zoospores are at a low concentration as the probability of catching zoospores is reduced. In an alternative sampling procedure, leaf bait bags purchased from test laboratories are floated just below the water surface for a few days. These leaves become infected by the zoospores and develop lesions, but the volume of water “sampled” (i.e. distance to the bait from which zoospores swim) and hence whether more than one bait per reservoir is needed is not known. Laboratory results are returned as colony forming units/litre (cfu/L) of sampled water following membrane filtration-dilution plating and/or percentage of bait leaves infected as shown by isolation onto selective agar. A quantitative immunodiagnostic test (zoospore trapping) has been developed in which viable spores are filtered out and germinated and this can be more sensitive for

Pythium, but is not routinely used (Pettitt *et al*., 2002). It is considered that 1 cfu/L of a plant pathogenic *Pythium* or *Phytophthora* species in irrigation water is of concern.

Use of baits and *in situ* diagnostic tests

Plant tissue (fruit, leaves or seedlings) can be used to assay for *Phytophthora* spp. and *Pythium* spp. in water, with researchers selecting particular material based on availability and past success in detecting the pathogen species present in their locality (Erwin & Ribeiro, 1996). Information is lacking on bait selection for use in detecting specific different *Phytophthora* and *Pythium* root rotting species. Preliminary investigations under HNS 181 (Wedgwood 2010) showed that growers can prepare their own leaf baits out of materials on the nursery (pebbles, polystyrene chips and leaves wrapped in horticultural fleece). More research is needed to give confidence in the use of baits, and to understand more about how placement and other factors affect infestation.

Infection of bait tissue by either *Phytophthora* and/or *Pythium* can be confirmed using a commercially available Lateral Flow Device (LFD) which utilises an antibody reaction. LFD use costs less than a water sample laboratory test and enables growers to know immediately whether or not *Phytophthora* and/or *Pythium* are present and water is safe to use. It is also possible for growers to send the LFD test strip for a PDplus test which samples the DNA and can identify a number of *Phytophthora* species, including *P. ramorum*, *P. kernoviae* and *P. cactorum*, to species level. After verification in HNS 181, *P. cinnamomi* and *P. citricola* will also be included in the PDplus procedure. UK Plant Health Inspectors use Rhododendron and Pieris leaves as baits in rainfall traps for the detection of airborne *P. ramorum* and *P. kernoviae*. In HNS 181, both Nordmann Fir needles and Ceanothus leaves were found to be suitable as baits for *P. cinnamomi* and *P. citricola*.

Research carried out to compare different diagnostic techniques for *Phytophthora* and *Pythium* species in water samples (Pettitt et al, 2002) showed the higher the zoospore concentration in water the greater proportion of rhododendron leaf discs baits became infested, with 6000 zoospores/L giving 100% infestation. Any number of bait pieces infested should allow recording of water contamination by laboratory isolation of each piece onto agar, however the sensitivity of LFDs to a low proportion of infested bait pieces is not known.

Knowledge gap of zoospore behaviour in relation to baits

It is not known whether the zoospores, cysts or resting spores are mainly near the water surface where baits are usually placed. It is possible that they circulate in the water column with light or temperature (as do related algae). At one nursery in the USA, water contained more *Phytophthora* species when collected 1 m below rather than at the surface, and if true elsewhere this could affect test sampling. It is possible that cysts and resting spores are stimulated to germinate by bait tissue exudates. Damaged leaves are said to be infected more than intact ones by *P. ramorum*, but it is not known if this information can be utilised for root pathogens. In HNS 181 larger lesions of water-moulds developed in leaf baits along the line where leaves had become folded, possibly due to release of exudates resulting in a higher zoospore location and infestation success.

In the laboratory, *Phytophthora* and *Pythium* sporangia are stimulated to release zoospores by cold shock below 6°C. There may be peak periods of zoospore release by plants in winter which growers should be aware of so monitoring can be increased. A greater understanding of zoospore behaviour would allow more confidence in bait testing. It is possible that with information gained on zoospore behaviour and further research, a method (e.g. absorbent mats or mass-bait trapping) could be developed for collecting or killing zoospores. Such control methods could be used when *Phytophthora* or *Pythium* species propagules are detected in water storage tanks.

Future irrigation water costs and potential shortages are encouraging growers to collect and reuse irrigation water, either for field, container or hydroponically grown crops. Growers would benefit from the ability to have a rapid check that the water being used on plants was tested free of *Phytophthora* spp. and *Pythium* spp..

Some nursery stock nursery reservoirs were experimentally bait-tested in project HNS 181, and *Phytophthora* and *Pythium* species able to cause root rots were detected using LFDs. The current project aimed to develop these techniques via laboratory testing using isolates of pathogens which can be found on ornamental plants, followed by nursery testing. The overall objective was to develop procedures and guidelines for “Do it Yourself” testing by growers for *Phytophthora* spp. and *Pythium* spp. in irrigation water.

Information was sought on: the selection of a plant material type and quantity that will bait-out zoospores successfully and be readily available to growers, and to determine if the position of the bait in the reservoir affects zoospore trapping success. Once these were determined, bait

monitoring of naturally infested water was carried out at intervals over the second year of the project to see if there are periods of greater detection. The ability of lateral flow devices (LFDs) to indicate bait infestation was also examined. The assistance of some growers was sought to test out bait construction and testing and the clarity of an accompanying instruction sheet on the techniques, with workshops then run to encourage wider uptake.

During this study, when apple baits were used in treated water from a commercial-scale slow sand filter that had been successfully operational for over 15 years, they tested positive for both *Pythium* and *Phytophthora* spp.. Concurrent laboratory plating and baiting tests on water samples from the filter detected no colonies of either genera, whilst other oomycete and non-oomycete 'indicator' species all indicated that the sand filter was working effectively. Nevertheless, a follow-up DNA test of one of the positive *Phytophthora* spp. confirmed the presence of *Phytophthora* spp. DNA, indicating that the apple baits were detecting material from this genus. 'False positive' tests could lead to unnecessary emergency maintenance of water treatment systems and costly clean-ups of water storage tanks, whilst alternatively, confidence in an increased level of testing sensitivity with an 'on-site' procedure would greatly improve disease management practice. An extension study was completed to investigate whether the baiting/LFD procedure is more sensitive than established plating techniques or whether killed oomycete material might attach to baits and give false positive LFD tests.

There were five specific objectives to this project:

1. To identify plant tissue baits which have the greatest sensitivity for zoospore detection.
2. To examine the sensitivity of lateral flow devices (LFDs) for detecting *Phytophthora* spp. and *Pythium* spp. in bait material in water recorded as having a range of colony forming units.
3. To determine the optimum number of bait bags, quantity of bait material and placement positions in reservoirs to maximise detection.
4. To determine whether there are any seasonal or weather related influences on zoospore release to use as guidance to maximise detection.
5. To provide step-by-step instructions for nursery staff on bait use and to provide a demonstration of the techniques at two grower events.

Extension study objective:

1. To determine whether or not cellular debris from killed oomycete cells can attach to plant tissue baits and be detected using LFD immunodiagnostic test kits to give 'false positive' tests for live pathogen presence.

Materials and Methods

Plant tissue baits

Work in both the laboratory and on nurseries was carried out with bait bags made of a square of horticultural fleece, containing a polystyrene packing piece float and weights. The fleece was clean (cut off and used directly from the roll). Heat-sterilised stones (quartz or flint so that they were not absorptive) were used in the laboratory and the first few reservoir assessments. The stones were then substituted by clay baking beads as these were readily obtainable, cheap, clean and of a standard 1.2 g weight. With 6-7 g, a floating depth of the plant material inside the bag of around 35 mm below the water surface was obtained. The fleece corners were gathered up to form a bag, and the neck secured with polypropylene twine which was also then used to suspend the bag so that it could be readily retrieved from the water. The fleece size was increased from 200 mm x 200 mm to 280 mm x 280 mm after the preliminary tests because this gave a wider gap between the weight and the float for the plant material so that water was able to pass around the latter more freely. In the reservoirs a greater depth of 250 mm below the water surface was compared with the near-to-surface bait. The weights in the bait bag were increased and the bag prevented from sinking by attaching it by the required length of twine to a float of a new square of expanded polystyrene.

In Year 1 (2012) a number of bait materials were tested (**Table 1**). All bait material was examined before use to ensure it had no wounds or spotting that could have introduced contamination into the test. None of the plants which provided leaves had received any fungicides for at least two months. Tables 2 and 3 summarise the focus of each of the ten Year 1 experiments (test type), the type and quantity of the plant material used as bait, how many replicate bags of baits were used in each test, the way the plant material was cut to obtain the specified number of bait pieces in each bait bag and the immersion period of the baits in the zoospore infested water. The laboratory experiments summarised in Tables 2 and 3 are described below, with fuller details provided in the Year 1 report.

Table 1. Details of the plant material selected from to use as baits in Year 1.

Plant material type	Variety	Source
Rhododendron leaf	Cunningham's White	Potted plants in polytunnel
Ceanothus leaf	*C. thyrsiflorus* var. *repens*	Potted plant held outside
Nordmann Fir needle	unknown	Christmas tree plantation
Apple internal flesh	Golden Delicious	Retail store
Carrot internal flesh	unknown	Retail store

All the materials tested had been previously used as baits (Singleton *et al*., 1993; Erwin & Ribeiro, 1996; Jennings, 2007; Wedgwood, 2011), although not necessarily with the pathogen species being tested in the current work.

Apple flesh (cut from inside the skin) is the least likely to already have *Pythium* spp. or *Phytophthora* spp. present. It is possible for carrots to have cavity spot caused by species of *Pythium*, especially *P. violae* and *P. sulcatum,* with a delay before symptoms are seen. Surface contamination via dust and rain is possible for leaves and also *Phytophthora ramorum* can occur on shrubs and may be latent inside the leaves.

Table 2. Plant bait materials, size and number of pieces per bag, and time left in water with either *Pythium*, *Phytophthora* or uninoculated

Experiment	Test type	Bait types used in bait bags	No. replicate bags with (+) or without (-) zoospores		Plant material and preparation	No. baits per bait bag	Time immersed
			+	-			
1	*Phytophthora* spp. Bait type. Bait amount. +/-Wounds.	Nordmann	1	1	intact leaf	5	64 h
		Nordmann	1	1	one leaf torn in two	10	
		Ceanothus	1	1	intact leaf	2	
		Ceanothus	1	1	one leaf torn in two	4	
		Rhododendron	1	1	half a leaf torn in two	2	
		Rhododendron	1	1	two thirds of a leaf	1	
2	*Phytophthora* spp. Bait type. Bait amount.	Apple	1	1	cut squares 5p sized	3	65 h
		Carrot	1	1	cut squares 5p sized	3	
		Rhododendron	1	1	one leaf into halves	2	
		Rhododendron	1	1	two leaves into four	8	
		Rhododendron	1	1	two quartered leaves	8	
		Rhododendron	1	1	two quartered leaves (indoors)	8	
		Ceanothus	1	1	one leaf in half	2	
		Ceanothus	1	1	four leaves in half	8	
3	*Phytophthora* spp. Bait type. Bait size.	Apple	10	0	cut with No.3 borer	8	64 h
		Carrot	10	0	cut with No.3 borer	8	
		Nordmann	10	0	leaf split across midrib	8	
		Ceanothus	10	0	one leaf torn in two	8	
		Rhododendron	10	0	one leaf cut into eight	8	
4	*Phytophthora* spp. Eden lab. check-count.	N/a	N/a	N/a	N/a	N/a	N/a
5	*Pythium* spp. Bait type.	Apple	10	0	cut with No.3 borer	8	70 h
		Carrot	10	0	cut with No.3 borer	8	
		Nordmann	10	0	leaf split across midrib	8	
		Ceanothus	10	0	one leaf torn in two	8	
		Rhododendron	10	0	one leaf cut into eight	8	
6	*Pythium* spp. zoospore survival.	N/a	N/a	N/a	N/a	N/a	3 to 5 h

Table 3. Carrot and apple bait tests with number of pieces and time left in water with either *Pythium*, *Phytophthora* or uninoculated

Experimental	Test type	Bait types used in bait bags	Number of replicate bags prepared with (+) or without (-) zoospores		Plant material and prep-aration	No. baits per bait bag	Time immersed
			(+)	(-)			
7	*Pythium* spp. LFD detection on increasing numbers baits (10 ml bottles)	Carrot	1 rep of 1,3, 5 or 8 infested bait pieces per bag	0 (all bags with infested baits)	cut with No.3 borer, not put in bag	8	48 h
8	*Phytophthora* spp. LFD detection on increasing numbers baits (10 ml bottles)	Apple cv. Golden Delicious	1 rep of 1,3, 5 or 8 infested bait pieces per bag	0 (all bags with infested baits)	cut with No.3 borer, not put in bag	8	48 h
9	*Phytophthora* spp. bait sensitivity to decreasing zoospore conc.	Apple cv. Golden Delicious	10 reps 25, 125 & 250 spores /L	10 reps 0 spores /L	cut with No.3 borer	8	46 h
9b	*Phytophthora* spp. LFD sensitivity to baits from low conc.	Apple cv. Golden Delicious	4 reps 25, 125 & 250 spores /L	4 reps 0 spores /L	cut with No.3 borer	8	46 h
10	*Pythium* spp. bait sensitivity to decreasing zoospore conc.	Apple cv. Golden Delicious	10 reps 25, 125 & 250 spores /L	10 reps 0 spores /L	cut with No.3 borer	8	70 h
10 b	*Pythium* LFD sensitivity to baits from lower conc.	Apple cv. Golden Delicious	4 reps 25, 125 & 250 spores /L	4 reps 0 spores /L	cut with No.3 borer	8	70 h

General methods used in year 1: *Phytophthora* sp. and *Pythium* sp. inoculum production

The oomycete pathogens used in this work were all zoospore producing (**Table 4**). Originally *Pythium irregulare* was to be used, but the culture from the ADAS collection was found to be contaminated and although new isolates were obtained from the Royal Horticultural Society they could not be stimulated to produce zoospores (isolates of this species are known to have variable zoospore production).

Table 4. Oomycete isolates used for the water inoculation in laboratory experiments in 2012

Species	Reference number	Year isolated	Host	Plant tissue
Phytophthora cryptogea	E556 (ex T. Pettitt)	2009	Heliopsis	Collar
Pythium sp.	BX 10/60a (ADAS)	2010	Gerbera	Root

P. cryptogea was confirmed by molecular testing (Polymerase Chain Reaction, (PCR)).
The *Pythium* species was either *P. diclinum, P. lutarium, P. dissotocum* or *P. coloratum* (Fera PCR testing reported that the DNA sequence data used matched 100% to these four different species and these require morphological characters examination for their separation).

All agars and culture solutions were produced according to standard mycological recipes, such as given in Erwin & Ribeiro (1996).

P. cryptogea was grown on potato dextrose agar (PDA) in the dark at 20 °C and squares of mycelium floated in clarified V8 broth to produce mycelial mats. The culture solution was then rinsed from the mats and they were left in sterile soil water overnight to produce zoospores. Synchronised release of zoospores was obtained by refrigeration for an hour followed by another hour when they were held at room temperature.

Pythium sp. was grown on V8 agar in the dark at 20 °C and squares of mycelium (10 mm x 10 mm) were incubated in sterile rain water overnight at 20 °C to produce zoospores.

The suspensions of zoospores in water were counted under the microscope using a haemocytometer, just before they were required for the test. The volume of zoospore infested water required to achieve the target spore count in the test container of water was then calculated. In the principal tests 10 replicates of each concentration were prepared; an extra sample of the target zoospore concentration was sent to a laboratory carrying out water tests for growers, in order to see how the two estimations of zoospore numbers compared.

General Methods used in Year 1: *Phytophthora* sp. and *Pythium* sp. infestation

Bait bags were added to the zoospore suspensions within half an hour. The period of bait immersion varied between tests (**Tables 1** and **2**) and in the main experiments was carried out under diurnal lighting (out of direct sunlight) within an air-conditioned room at 20 °C. The weighted bait bags were pushed down in the water straight after deployment to wet the fleece to ensure they were submerged at the start of the incubation (as with no movement in the containers it could take a period for the water to enter the pores in the fleece).

General Methods used in Year 1: *Phytophthora* sp. and *Pythium* sp. detection

When bait bags were removed from the water they were kept separate from each other. Baits that were to be cultured on agar were opened in a laminar flow cabinet to ensure sterile conditions, and equipment was sterilised between handling each bag. When tests were carried out with the LFDs, care was taken to avoid cross-contamination, but the procedure was not carried out in a laminar flow cabinet.

When isolation was required, the bait pieces were gently blotted and placed on an agar selective to water-moulds (P5ARP growth medium), with one piece per plate. The number of bait pieces per bag with either *Pythium* or *Phytophthora* (depending on which was used in the test) was recorded and notes made of any contaminants (as fast growing species such as *Mucor* can overgrow water-moulds). The morphology of the colonies, and microscope examination (to confirm the presence of non-septate hyphae typical of oomycetes) was carried out with reference to the original cultures and also diagrams of *Phytophthora* (Waterhouse, 1970) and *Pythium* (Van der Plaats-Niterink, 1981) in order to identify isolates. Plates were assessed after five days, with further examination a week later. To be able to examine sporangia during diagnosis it was necessary to cut out sections of mycelium from colonies and float them in sterile rain or soil water for up to three days to stimulate sporangial production. Growth of coralloid mycelium confirmed *P. cryptogea*; plates with identical colonies were classed as *P. cryptogea*.

The LFDs were used according to the instruction sheet prepared for growers (**Appendix 1**), with all the baits from one bag being put in one buffer bottle and shaken for around a minute until colouration appeared in the liquid to show that the tissue had been broken down by the ball-bearings. Drops were taken from the bottle to use on both a *Pythium* and a *Phytophthora* LFD. The results were always read after 10 minutes, with the strength of the blue test line scored using a 1-5 index using a standard reference picture of intensity (5 = very strong) as this might give information about the extent of infestation of the baits which could in turn be

related to the number of zoospores colonising the pieces. All used LFDs were labelled and retained in their foil packets.

Specific Methods used in Year 1: Preliminary laboratory experiments

Experiment 1. *P. cryptogea* - bait type, amount and need for wounding.

Preliminary work started on 17 July 2012 using distilled water without and with *P. cryptogea* zoospores. Two bait bags of each of the selected leaf baits Ceanothus, Rhododendron and Nordmann Fir were made (**Table 1**), one to go in the inoculated water, the other in the uninoculated water. There was no replication. All the leaves were rinsed in freshly distilled water before use. For each bait type, half the bags had leaves which were torn in half to see if wounded leaves were more attractive to zoospores.

The experiment was carried out in natural daylight and ambient temperatures outside (temperature range 10 °C to 19 °C) against the north side of a building. Zoospores were added to give 900 spores per inoculated 750 ml glass jar (1200 zoospores per litre). Baits bags were then immersed 30 mm below the water surface for 3 nights.

Experiment 2. *P. cryptogea* - bait type and amount.

Work was done with *P. cryptogea* jars containing 1000 spores per L (750 spores per inoculated 750 ml glass jar) on 24 July 2012 using Rhododendron and Ceanothus leaf baits with up to 8 pieces per bait bag, as shown in Table 2. Additionally, apple and carrot pieces were also tested. As previously, duplicate bags were prepared for placement in the inoculated and uninoculated water. There was no replication of the different compositions of baits in bags. Work was carried out in the laboratory with water in the jars at 20 °C, except for one rhododendron bait bag jar which was placed outside to allow comparison with Experiment 1.

Specific Methods used in Year 1: Main laboratory experiments

Experiments 3 (*P. cryptogea*) and Experiment 5 (*Pythium* sp.) - high spore concentration bait selection.

These and all future experiments were carried out on shelves in an air-conditioned room at 20°C, as it was thought that temperature fluctuations outside with the small water volume could differ more than those in a reservoir. The water volume used was increased to 1 L and

translucent polypropylene (HDPE) bottles used, with lids resting over the bottle necks during the running of the experiments. Ten replicate bottles each containing one bait bag were used, with treatments randomised within each replicate block (two blocks per shelf on each of five shelves). As no uninoculated water was baited, samples of the plant material used were placed directly onto agar plates to check for any natural infection or contamination with *Pythium* spp. or *Phytophthora* spp.

The preliminary tests with *P. cryptogea* found a lot of secondary fungal growth on the agar plates and so after the leaf pieces were cut to size they were surface sterilised with 75% ethanol before being rinsed twice in sterile distilled water. There was no need to surface sterilise the apple and carrot as, after washing them under the tap, their outer surfaces were removed and inner tissue used for the baits.

After the preliminary tests, the bait pieces were made smaller (to increase the amount of edge tissue which may be favoured by the zoospores) and a greater number of pieces were used to get a slightly larger surface area. Each bait bag thus contained eight pieces of freshly wounded or cut material, aiming to produce a similar surface area of 25 mm x 25 mm (as given in manufacturer's instructions for leaves for the LFD test). This required sections taken from one Rhododendron leaf, four large Ceanothus leaves cut in half and eight Nordmann Fir largest needles from current season growth to provide sufficient material for all 10 replicates (**Table 2**). For the apple and carrot pieces, a sterilised No. 3 corer (6 mm wide) was cut through a 7 mm thick cross-section of internal flesh to produce a plug weighing around 1.4 g.

With each of the eight bait pieces plated-out onto agar per treatment bottle, a total of 400 plates for each of the *Pythium* sp. and *Phytophthora* sp. experiments were examined over a period of two to three months. The number of bait pieces infested per bag was recorded. Agar with white/colourless fungal mycelium was placed in sterile rain water floats to confirm the presence of *Pythium sp.* or *Phytophthora sp.* sporangia because water-moulds seldom sporulate on agar. Growth of either coralloid mycelium or pear-shaped sporangia confirmed *P. cryptogea*. The *Pythium* sp. used in the work produced filamentous sporangia. The infestation of bait pieces producing growth on agar of identical colony morphology which was then able to be identified.

For Experiments 3 (on 7 August 2012) and 5 (on 28 August 2012), to improve the results from the preliminary tests the spore concentration used was increased. In work on bait methods for detection of *Pythium* spp. and *Phytophthora* spp. zoospores in water (Pettit *et al.*, 2002) 6000 spores per litre were required to give good (10 out of 10) Rhododendron bait infestation. This

concentration was achieved for *P. cryptogea* for Experiment 3 (although zoospores of *P. cryptogea* had been produced overnight rather than after chilling just before required), but only 4800 spores per litre was available for *Pythium* sp.. Freshly made distilled water was used in the 1 L bottles. The haemocytometer zoospore counts were checked by laboratory culture tests at the Eden Project commercial testing laboratory.

Experiment 4. *P. cryptogea* - zoospore check count.

The results from the Eden laboratory for Experiment 3 did not match the spore concentration calculated from use of the haemocytometer prior to making the dilutions for the bait bottles at ADAS Boxworth. To investigate this difference in spore counts between the laboratories, a volume of *P. cryptogea* spores was produced at a concentration of 5000 spores/L without baits being added. This water sample was sent to the Eden laboratory on 21 August 2012 (as before, by "next day delivery") to determine the concentration by filtration and isolation. The pH and temperature of various types of water were checked in case there were significant differences between the rain water zoospore production float dishes and the 1 L bottles of distilled water that might cause either zoospore encystment or rupturing.

Experiment 6. *Pythium* sp. - water type and zoospore survival.

This experiment, on 10 December 2012, sought to determine whether zoospores were affected by the water they were suspended in for the laboratory experiments. This could also have relevance on nurseries to the baiting of different sources of irrigation water. Distilled water was used in previous experiments, rather than tap water, because it did not contain the chlorine which might affect zoospore behaviour, such as causing encystment. It was possible that the lack of ions in distilled water might affect the osmotic balance of zoospores, causing them to swell and rupture. Tap water can be made "safe" for fish using a proprietary hydrosulfite salts product e.g. "Prime" intended for aquariums that removes chlorine and chloramine and it was possible that this could be utilised in the experiments to improve zoospore counts. If tap water does affect zoospores then this would also be useful to know, as some nurseries mix reservoir and tap water for irrigation. Rain water had not been used previously because it would have required autoclaving to remove micro-organisms and the breakdown products could possibly affect the zoospores or the baits.

A five-replicate observation experiment was thus carried out to record the behaviour of zoospores, in particular any encystment, bursting or changes in movement frequency in the different water sources shown in Table 5. An inverted microscope was used to examine

zoospores in a 25-chambered (5x5) transparent dish which allowed all the treatments to be adjacent (randomised within each replicate column of five chambers), so removing any differences in environmental conditions. Each chamber held 2 ml of spore suspension which had been drawn off from 500 ml of the specified water type to which 1 ml from the (concentrated) float culture had been added. Observations were started after three hours, counting the number of active and encysted zoospores. Observations were made in replicate order and were complete after two hours.

Table 5. Water types tested during observations of *Pythium* sp. zoospore behaviour (Experiment 6)

Water used in test	Comments
Sterile rain water (autoclaved)	Collected in a water butt from a glasshouse roof
Distilled water	Freshly made
Tap water (stood)	Stood for 7 days to reduce chlorine
Tap water (fresh)	Freshly drawn from the tap
Tap water (treated with “Prime”)	Freshly drawn & treated with 2 x 0.5 ml drops / 4L

The experiment was repeated. The first experiment (on 6 December 2012) used around 130 to 230 *Pythium* sp. zoospores per chamber; the second (on 7 December 2012) used around 300 to 400 *Pythium* sp. zoospores per chamber.

Experiment 7 (*Pythium* sp.) and Experiment 8 (*P. cryptogea*) - LFD sensitivity.

It was not known whether apple and carrot bait materials would be able to be utilised with LFDs as previous experience of their use had been with plant stems and fibrous roots. It was possible that the sugar in the apple could prevent the buffer solution being draw across the indicator paper of the LFD, and that the carrot might produce an orange stain that obscured the test window indication. It was also not known whether the LFDs might show different strengths of positive indication with varying amounts of infested bait pieces. The LFDs were Pocket Diagnostic kits for *Pythium* spp. and for *Phytophthora* spp. manufactured in the UK by Forsite Diagnostics, Sand Hutton, York, YO41 1LZ.

To obtain different bait strengths, a standard zoospore suspension was made up to have 10 spores in 10 ml of distilled water (i.e.1000 per L) and 1 bait plug was put in each small glass bottle with the 10 zoospores (estimated by dilution) of either *Pythium* sp. or *Phytophthora* sp. and left in diurnal lighting at room temperature of around 20 °C. The bait plugs were left for 4 days (10-14 August), by which time it was expected that all the zoospores should have come

into contact with the bait material. Varying numbers (1, 3, 5 or 8) of infested baits were then mixed with fresh uninfected baits (7, 5, 3 or 0) to create 4 bait strengths (**Table 6**). The buffer bottles were shaken vigorously for a standard 90 seconds before drawing off liquid to use on the relevant LFD. The test line was assessed after 10 minutes and intensity recorded using a 0 to 5 Index (negative to strong positive).

Table 6. Combinations of infested and uninfested bait plugs used to create four strengths of *Pythium* sp. and *P. cryptogea* infected tissue to test by LFD

Experiment	Bait type and pathogen	No. infested bait plugs	No. uninfested bait plugs used	No. bait plugs put into buffer for LFD test
7	Carrot and *Pythium* sp.	1	7	8
		3	5	8
		5	3	8
		8	0	8
8	Apple and *P. cryptogea*	1	7	8
		3	5	8
		5	3	8
		8	0	8

Experiment 9 (*P. cryptogea*) and Experiment 10 (*Pythium* sp.) - bait and LFD sensitivity.

These experiments used sterilised rain water. The baits were left in place for 2 nights (Tuesday pm to Thursday am), rather than 3, as 48 hours had been used in other work (Jennings, 2007) and the shorter time might be preferable for use on nurseries. It was also likely that there would be faster decay of apple baits in water than for leaf pieces.

A range of spore concentrations was used (**Table 7**) to see if this affected the proportion of plugs affected. The concentrations chosen were closer to what might be present in a nursery reservoir. Both pathogens were tested using apple bait bags with each spore concentration (and the uninoculated) replicated ten times. On retrieval, these baits were plated out onto agar as in previous experiments. In addition to these 10 replicates, another 4 replicates were set up with the same range of spore dilutions in order to test the sensitivity of the LFDs to baits infested by this relatively low range of zoospore concentrations. It was anticipated that the LFDs might show a decreasing test band intensity the lower the spore concentration baited.

Table 7. Zoospore concentrations used of *P. cryptogea* and *Pythium* sp. (Experiments 9 and 10)

Zoospore concentration		Baits per bag
0	spores / L	8 apple plugs (Golden Delicious)
25	spores / L	8 apple plugs (Golden Delicious)
125	spores / L	8 apple plugs (Golden Delicious)
250	spores / L	8 apple plugs (Golden Delicious)

Reservoir water sampling and nursery testing of bait bags

In preparation for carrying out baiting experiments in nursery reservoirs, 1 L water samples were taken at two nurseries ("A" and "B") in August 2012 in order to select a reservoir for use in 2013 to examine bait positioning and any seasonality of zoospore catches. Samples were taken from four locations at nursery "A" (reservoir, lagoon, pond, ditch) and from four locations at nursery "B" (Site 1 reservoir, Site 1 bed run-off, Site 2 reservoir, Site 2 bed run-off). Samples were taken from run-off in order to assess if there was *Pythium* spp. and *Phytophthora* spp. present on the nurseries. Bed run-off would be expected to have a higher pathogen concentration than in the reservoirs and so they would be more easily detected.

The Nursery "B" site 1 reservoir was selected for continuing observation as *Pythium* was detected in the water samples taken from this site in August 2012. This was a butyl-lined open reservoir (about 15 m x 70 m) with a reed bed filtration area across the width at one end where water from the beds entered. Water was taken out from the centre of the reservoir via a particulate filter. Excess water drained out by an overflow at the opposite end to the reed bed. In January 2013, bait bags were used to investigate whether there might be differences in the zoospore populations at two different sides of the reservoir and at different depths. Water samples were taken to send to the Eden Laboratory and baits placed on tethered strings at two positions around the edge of the reservoir. An extra 1 L water sample was taken at the outfall end to return to ADAS Boxworth laboratory for baiting. This was baited in the same way as at the Eden Laboratory – with Ceanothus leaves floating freely in the water. Apple bait bags with eight pieces (as used in the final laboratory experiments) were tested. Ceanothus leaf baits bags (four leaves torn in half per bag) were used in addition as these had been used previously in reservoirs before to bait water-moulds (in HNS 181), in order to compare with the apple bait bags in this initial reservoir experiment.

Both bait bag types were floated at two depths. This was done by using 7 g and 10 g of stones inside to give respectively shallow (30 mm) and deeper (250 mm) flotation below the water

surface. Each bag also had expanded polystyrene pieces in to help create a space in the bag. These were tied to the bank at the reed bed (R1) and the outfall (R2) end of the reservoir so they could float within a water surface area of about 0.75 x 0.75 m. The bags at the two depths at each position were tethered apart so they did not touch when floating. An extra bait bag of each type was also floated at the shallow depth at the outfall end to test with an LFD. The bags were set out, with the participation of the nursery staff, on Tuesday 29 January 2013 and retrieved by the staff on the 31 January (after 48 hours). A temperature logger was also floated in the water (**Appendix 4**).

On retrieval, each bait bag was placed in its own bag and posted the same day to ADAS Boxworth, together with the logger. The bags were received at Boxworth on Monday 4 February and isolations and LFD testing were carried out (6 days from immersion). The bait pieces were placed without surface sterilisation on individual plates of P5ARP agar (which favours the growth of oomycetes rather than fungi) as for the laboratory experiments in 2012. Some Ceanothus leaves direct from the source bush were also isolated from to check if they had any water-mould infestation. These leaves were dipped in 75 % ethanol to surface sterilise them, in the same way as those used in the bait bags. For the two extra bait bags, a single LFD buffer bottle was used for all eight pieces of apple and another bottle for the eight leaf bait pieces. The same apple or leaf bait containing buffer bottle with was then used to provide extract drops to test on both a *Pythium* and a *Phytophthora* LFD.

In February 2013, the Nursery “B” site 1 reservoir was baited by the grower at the same locations and depths as in January. The grower produced his own bait bags using a locally purchased Golden Delicious apple and the stones, fleece, floats and string supplied by ADAS. All baits after February 2013 were made with a Golden Delicious apple sent by ADAS to each nursery from the same bag of apples, with a new bag purchased each month. The apples were washed and sent together with clean bait bag materials, plus labelled grip-seal bags to return each bait bag. Instructions on how to make the baits (**Appendix 1**) and a demonstration bait bag were supplied by ADAS. Only apple baits were used in February. An additional shallow floating bait bag was used at each location so that these could be tested on-site by the grower using LFDs immediately on retrieval of the baits. Instructions on how to test the baits with the LFDs were provided to the grower (**Appendix 1**), together with LFDs from the same batch as those to be used at Boxworth. Bag duplication to allow testing immediately on retrieval was also done in March, but otherwise all LFD testing was done at ADAS. The remaining shallow and deep bait bags from each location were returned to ADAS Boxworth for LFD testing (which replaced the culturing method used in January in order to fulfil Objective 2). In addition, an apple bait bag was placed in water collected from the outflow from the

particulate filter and stood in the nursery office to obtain a steady temperature. The bait bag was retrieved from the water and posted to ADAS at the same time as the reservoir bait bags. When bags were taken out of the water they were blotted to remove excess moisture, left tied up, and packaged in individual plastic bags. The baits in the Nursery "B" reservoir and post-filter were placed on 26 February 2013 and 1 L water samples were taken from these three locations at the same time and posted to the Eden laboratory to be received and filtered for isolation-plating, and a sub-sample baited with Ceanothus leaves, the next day. The baits used for the grower LFD tests were retrieved after 48 hours on Thursday 28 February. The used LFDs were returned to ADAS to confirm the grower's reading. The remaining baits were left in place until Monday 4 March (6 days immersion owing to a mis-interpretation of the instructions) and then posted to ADAS together with a temperature logger from the reservoir. The baits were tested using LFDs at ADAS on 6 March. A 0 to 5 test line intensity index was recorded for each LFD.

In February 2013, apple bait bag materials were sent to a further nursery, "C", with a lidded reservoir tank collecting water from the nursery site. The grower made the bags with freshly cut apple pieces (utilising instructions as given in **Appendix 1**) before placing them for 48 hours on 26 February 2013 in water which had come in to a slow sand filter from glasshouse roofs (Objective 5). The bags were weighted to float at the same shallow and deep depths, (30 mm) and (250 mm), as at Nursery "B". From May 2013, Nursery "C" commenced baiting water from after the slow sand filter as well as baiting and sampling the covered tank. The filtered water was baited at 30 mm depth in the water collection container.

Baiting, water sampling and LFD testing of apple baits was carried out throughout 2013 at both nursery "B" and "C", with deployment planned at intervals (April, May, July, August, September and November) to obtain a seasonal record of infestation (Objective 4), and to compare zoospore bait trapping results (as given by LFD use) with those of colony forming units and *Pythium* and *Phytophthora* identification from water samples (Objective 2). A shallow-floating temperature logger was put into the water and retrieved at the same time as the baits at both nurseries and returned to ADAS with each set of bait bags.

Nursery "B" placed bait bags and sampled water at the inflow (R1) after passing through a reed bed and overflow pipe (R2) ends of the reservoir (**Figure 2**). The reed bed filtered water that entered the reservoir after collection from the standing beds of container plants. During May, additional bait positions were temporarily introduced at Nursery "B" (positions R3, R4 and R5 in **Figure 2**) and a second sets of baits put in straight after retrieval of the first set in order to determine if positive or negative detection differed around the reservoir and if so

whether this was consistent if baiting was repeated within a short period of time (for Objective 3). Water samples were taken by the grower using a bucket on a rope which was pulled through the water and then up and out before the bucket sunk, thus sampling about the surface 300 mm. At the same time as placing baits in the reservoir the grower took two 1 L samples of reservoir water that had passed through a particulate-filter ready to be circulated for irrigation. One water sample was sent for laboratory testing and the duplicate was held indoors at the nursery and a bait bag added for 48 hours.

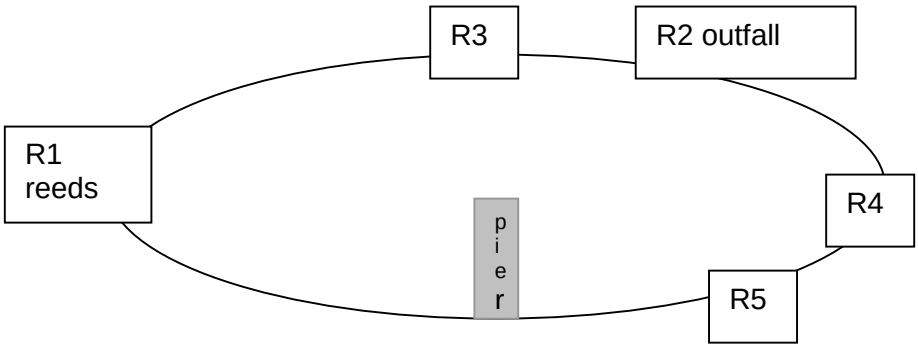


Figure 2: Baiting and water sampling locations around the edges of the open reservoir at Nursery "B". R1 and R2 positions were used throughout 2013. R3 to R5 were only used in May. Water piped out of the reservoir at the pier via a particulate filter was also sampled for laboratory testing and baited

Bait bag placement in each reservoir was usually on Tuesday mornings, with bag retrieval on Thursday mornings for posting to ADAS. Testing of the baits with LFDs was done by ADAS at a six day interval after the bags had been placed in the reservoir i.e. bait bags arrived on Friday mornings at ADAS and were incubated at room temperature until Monday morning the following week). The bait bags were left to incubate in the plastic bag each had been collected into and kept within the padded postal bag on a shelf at room temperature. The four day delay before LFD testing was used to allow oomycete mycelial growth in the baits and increase the chance of the LFDs detecting low *Pythium* or *Phytophthora* levels from what would originally be a single cell for each zoospore attracted. Post-retrieval incubation was used as standard throughout the monitoring in 2013, but when micro-organisms are more active, above about 6°C, then zoospore bait colonisation is more likely to be detected following just the 48 hour of

bait deployment in a reservoir. That detection without incubation is possible was confirmed at site “B” from baits set on 9 April 2013. That incubation improved detection was indicated by the setting of duplicate baits on 26 February 2013, as after retrieval only the incubated baits tested positive.

It was noted after deploying the February baits that the deep baits were not floating as deeply as intended (only about 60 mm) and this was improved for subsequent tests by suspending the more heavily weighted bait bag on a 250 mm length of polypropylene string below a floating piece of expanded polystyrene (Nursery “B”) or an empty water bottle (Nursery “C”). The polystyrene float pieces were still retained inside the bait bag to help keep the fleece from resting on the apple pieces and affecting water flow over the baits. Stones were replaced by ceramic baking beads as these can be bought from shops and do not require boiling before first use like natural quartz nursery-collected stones.

In August 2013, water and bait bag samples were taken from the reservoir and particulate filter of Site 2 at Nursery “B” at Site 2 as well as the usual samples from the Site 1 reservoir. In addition, at Nursery “B” and “C” duplicate bags were placed at each position. On this date only, the bait bags were sent to the Eden Laboratories for isolations to be made from the apple in the duplicates of the shallow depth bait bags that would tested by LFDs. A 3 L water, sample in addition to the 1 L, was also taken from after the slow sand filter at Nursery “C” in order to determine whether any zoospores could be detected given a larger sample.

Extension study investigating ‘false positive’ results - zoospore suspensions

Suspensions of zoospores of two *Phytophthora* isolates (E556 *Phytophthora cryptogea* from *Heliopsis* collar rot and C295 *Phytophthora* sp. from *Leucothoe* crown rot) were prepared by placing 7-day-old mycelial mats grown in clarified V8 broth into ‘starvation’ conditions for 24h in sterile pond water to induce sporangium formation, and releasing zoospores from these by cold-shock treatment at 4 ^{o}C for 1 h followed by 1 h at room temperature. The concentration of each suspension was determined by haemocytometer counts and the appropriate volume of suspension was added to 2 litres of sterile pond water to give a ‘stock suspension’ of concentration 250 spores/ml (2.5 x 10^5 spores/L). These stock suspensions were then taken through dilution series in sterile pond water to provide ranges of spore concentrations down to 1.25 spores/L for carrying out detection sensitivity tests.

In addition to zoospore suspensions, dilution series of pulverised mycelium were used in sensitivity comparisons as well as to give some indication of the amounts of mycelium being

detected in infected bait tissues. Mycelial mats were grown in clarified V8 broth as above, washed in distilled water, drained, gently-blotted on paper tissue to remove excess water and approximately 0.2-0.3 g weighed out before pulverisation by grinding in 5 ml of LFD extraction buffer in a mortar and pestle with the addition of a pinch of acid-washed silver sand. This suspension was tested directly and 1 ml was diluted in 5 ml of extraction buffer and this suspension was taken through a five step 1 in 5 dilution series giving mycelium concentrations down to 0.004 g/L. Each dilution was tested with an LFD kit.

Extension study investigating 'false positive' results - LFD kits used and sensitivity comparisons

Three batches of LFD kits were assessed. These were:

- Pocket Diagnostic® kits (Forsite Diagnostics, www.forsitediagnostics.com) from the same production run as used in the main part of the PO/HNS 188 study (Batch BS01) and referred to here as 'old'. These stopped being available in the early part of 2014, but a small number of these kits were used in this study to provide a cross-reference to the main study.
- Pocket Diagnostic® kits (Forsite Diagnostics, www.forsitediagnostics.com) from the production run that started to be made available in Summer 2014 (Batch BT04, 15 July 2014, PHYT15405) and referred to here as 'new'.
- Alert LF™ kits (Adgen Phytodiagnostics, www.plant.neogeneurope.com), as used for routine health checks focussed on *Phytophthora ramorum* exclusion at Eden Project (LOT 14F1002; expiry date July 2015)

All LFDs were deployed as described in the main HNS/PO 188 report and the intensity of test stripes were measured using an ESEQuant Lateral Flow Reader (Qiagen, www.quiagen.com). The kits listed above were compared for their sensitivity in detecting *Phytophthora* both directly on dilution series of suspensions of pulverised mycelium and on 'Golden Delicious' apple bait pieces following incubation for 48 h with dilutions of zoospore suspensions following the procedures described in the main HNS/PO 188 report. The two kit types have slightly different extraction procedures. The Pocket Diagnostic® kits deploy a bottle containing 5 steel ball bearings into which samples are placed and vigorously shaken to break up the tissues, whereas Alert LF™ kits employ a robust bag in which the sample and extraction buffer are sealed and the tissues are broken up by applying pressure to the outside using an object like the side of a marker pen. The volumes of extraction buffer are different; 5 ml in the Pocket Diagnostic® and approximately 10 ml in the Alert LF™ kits. To make comparisons between the kits, the volume of extraction buffer used in the Alert LF™ kits was reduced to 5 ml.

In all tests of sensitivity using zoospore suspensions, spore viability and cfu/L were determined by plating 0.5 ml samples of suspension onto selective agar or at lower zoospore concentrations, sub-samples of 100 ml were filtered through 3 µm cellulose nitrate membrane filters, re-suspended in 2 ml 0.1 % agar and plated onto selective agar (Pettitt *et al.,* 2002). In addition, subsamples (20 bait pieces) of all bait tests were plated onto selective agar to determine percentage bait infection.

Extension study investigating 'false positive' results - water disinfestation treatments

Three water treatments were assessed in this study:

1. Heat
2. Additions of chlorine dioxide
3. Ultra Violet (UV) radiation.

Dilution series of zoospore suspensions were prepared and assessed by the plating procedures described above before being subjected to one of the treatments listed, after which they were assessed again using the same plating procedures. For the heat treatment zoospore suspensions were placed in a controlled-temperature water bath and heated to 90^{o}C as indicated by a thermometer in an identical bottle containing just pond water, then allowed to cool to room temperature before being tested with Golden Delicious apple pieces. For UV radiation treatment zoospore suspensions were placed in clear polythene containers and placed under a G15W T8 lamp (Sylvania, www.havells-sylvania.com) for 5 h before baiting. A concentrated chlorine dioxide solution was obtained from a catalytic chlorine dioxide system (Cloxide™, Clearwater Technology Ltd, www.clearwater.eu.com) and put through a dilution series prior to estimation of its concentration using chlorine dioxide HR tests strips (Churchill Environmental Services, Cornwall, PL26 8LX, UK). The appropriate dilution was then used to prepare a chlorine dioxide dilution series (0-25 ppm) in a stock zoospore suspension of *Phytophthora cryptogea* to prepare an efficacy curve from which to determine an appropriate concentration to subsequently apply to zoospore dilution series (from this 10 ppm was selected to give a guaranteed 100 % kill).

Results

Laboratory experiments in 2012 to develop baiting technique

Table 8 summarises the results of the ten laboratory experiments carried out in 2012. The detailed results of each experiment are explained further below.

Table 8: Summary of laboratory tests evaluating the effect of bait type, water type and zoospore concentration on the detection of *Pythium* sp. and *P. cryptogea* in water – 2012. Detection was assessed as the proportion of baits with growth from baits on selective agar and by LFD tests

Experiment	Date	Pathogen	Test Type	Spores/L	Size of container	Baits	Total % infected of the number of bags or individual baits per experiment
1	17.07.12	*P. cryptogea* E556	Baiting and culture	1200	750 ml	Mixed	Zero
2	24.07.12	*P. cryptogea* E556	Baiting and culture	1000	750 ml	Mixed	Zero
3	07.08.12	*P. cryptogea* E556	Baiting and culture	6000	1 L	Mixed	24% bags. 5.5% baits.
4	21.08.12	*P. cryptogea* E556	Baiting and culture	5000*	1 L	Not Baited	Not Baited
5	28.08.12	*Pythium* BX10/60A	Baiting and culture	4800 **	1 L	Mixed	56% bags. 13.3% baits.
6	6.12.12 & 7.12.12	*Pythium* BX10/60A	Zoospore observ-ation	up to 400, 000	2 ml	N/a	N/a
7	10.12.12	*Pythium* BX10/60A	Baiting and LFD	1000	10 ml	Carrot	very faint +ve for 1, 3 & 5 infested plugs per bottle
8	11.12.12	*P. cryptogea* E556	Baiting and LFD	1000	10 ml	Apple	Zero
9	12.12.12	*P. cryptogea* E556	Baiting and culture	0 - 250	1 L	Apple	2% bags. 0.2% baits.
9b	12.12.12	*P. cryptogea* E556	Baiting and LFD	0 - 250	1 L	Apple	One faint +ve
10	18.12.12	*Pythium* BX10/60A	Baiting and culture	0 - 250	1 L	Apple	28% bags 5.8% baits
10 b	18.12.12	*Pythium* BX10/60A	Baiting and LFD	0 - 250	1 L	Apple	Two faint +ve

*Experiment 4; Eden laboratory culture tests indicated 67 – 187 cfu/L

******Experiment 5; Eden laboratory culture tests indicated 47 spores/L

Experiment 1. *P. cryptogea* - bait type, amount and need for wounding

No *Phytophthora* was recovered from any of the bait material after four days on agar. Rhododendron leaf pieces were too large and curved to sit down on the agar so smaller pieces were to be used in future.

Experiment 2. *P. cryptogea* - bait type and amount

This repeat of Experiment 1, but using more (a total of eight) bait pieces did not produce any positive isolations of *P. cryptogea.* There were a lot of secondary non-target fungi (probably *Fusarium* spp. and one *Pythium* sp.) on the Ceanothus baits on the agar plates. As a result of the failure to detect *P. cryptogea* in these preliminary experiments, a higher zoospore concentration was used in subsequent work.

Experiment 3. *P. cryptogea* - high spore concentration bait selection

A mixture of fungi was recovered from the baits after plating onto selective agar. *Fusarium* was predominant on the apple, carrot and Rhododendron plates, generally starting as white mycelium similar to that recorded for *Phytophthora* and *Pythium* spp.. The presence of contaminant fungi would not matter with LFD testing as this selects for either *Phytophthora* spp. or *Pythium* spp. (depending on the device used). In total, the number of baits confirmed to be infested by Phytophthora (with morphology checking of the isolations including taking samples of mycelium to floats) was relatively few (**Figure 3**).

Apple pieces were most frequently infested by *Phytophthora* (16 out of 80 pieces), but only 7 out of 10 bags were positive, and within these there were commonly only 3 or fewer out of 8 pieces infested (maximum 4 pieces positive per bag). None of the Nordmann Fir needles became infested. In total across all bait types, out of 50 bags used there were 12 with *Phytophthora* i.e. 24% became infested (**Table 9**).

Although 6000 zoospores were added to each bottle, the laboratory water filtration and plate test (Eden laboratory) found zero colony forming units (spores or mycelial fragments). This result suggests no viable spores were present in the sample when it was tested by the water filtration and plate test. Nursery water samples also arrive by post and using the same methods, and *Phytophthora* and *Pythium* species are commonly detected so these

procedures do not account for the lack of spores. It was possible that the air-gap left in the top of the bottle caused a pressure change/physical impact which caused the spores to burst. Normally any knocking or other chemical or physical shock can cause the zoospores to encyst, however encysted spores are filtered and cultured from water in the same way as un-encysted zoospores.

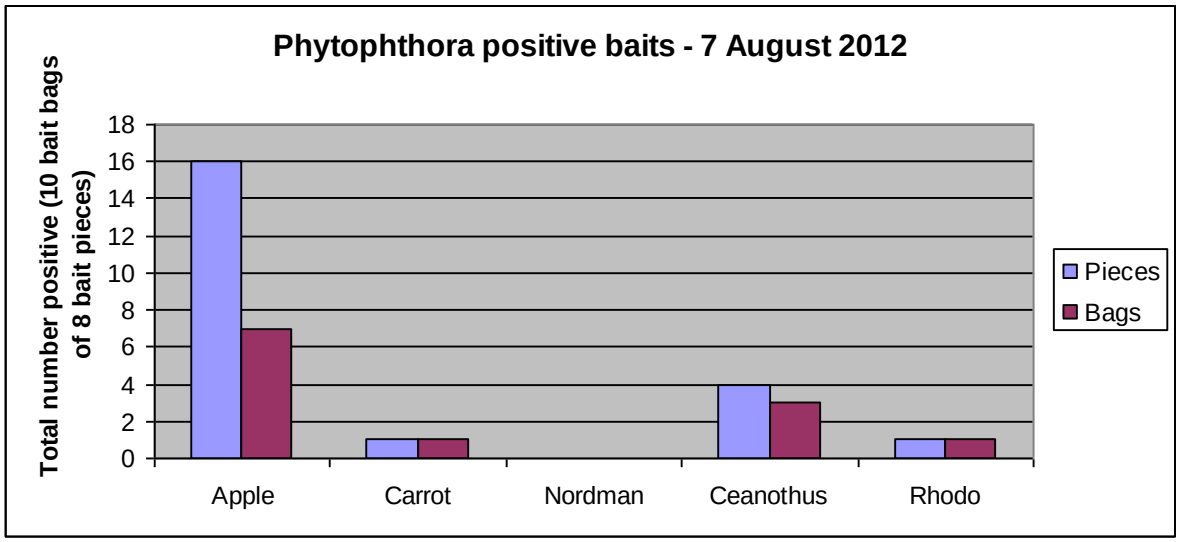


Figure 3: Detection of *P. cryptogea* by tissue baits showing the total number of bait pieces and the total number of bait bags from which *Phytophthora* was isolated (Experiment 3)

Experiment 4. *P. cryptogea* - zoospore check-count

It was noted the zoospores were starting to encyst on the haemocytometer slide before making up the spore suspension to send to the Eden laboratory. Two methods were used by this laboratory to count the spores with results of only 67 or 187 colony forming units per litre, for a suspension originally calculated to contain 5000 spores/L. When 10 Ceanothus leaf baits were added to the zoospore suspension, there was 100% infestation recorded by both direct examination and by agar plating of the leaves. No tissue baiting of the original suspension was done by ADAS in this experiment.

The Eden laboratory also confirmed the culture being used was *Phytophthora cryptogea.* A *Pythium* LFD on the agar culture in the ADAS laboratory had given a strong positive for *Pythium*. However, mycologists Tim Pettitt (Eden Project laboratory) and Charles Lane (Fera) have found in the past that if agar cultures are tested in this way the reaction of the LFD test kit can be wrong (Tim Pettitt, pers. comm.). This means that LFD tests cannot be used to confirm in the laboratory the identity of mycelium growing onto agar from baits.

Measurements on water used in the laboratory experiments showed the sterile rain water was pH 7.4 and the distilled water pH 5.9. The bottle water temperature was 18 °C and the spore suspension in the float dishes 22 °C. Neither of the waters used in the experiment gave an EC reading. These water pH and temperatures were not considered to have an adverse effect sufficient to kill zoospores (Tim Pettitt, pers. comm.).

Experiment 5. *Pythium* sp. - high spore concentration bait selection

For *Pythium* sp., many of the agar isolation plates grew white colonies which were initially thought to be *Pythium* when examined without the aid of a microscope. Out of 80 plates this growth was seen on 78 apple, 72 carrot, 66 Ceanothus, 46 Rhododendron and 27 Nordmann plates. However, when the bait isolation plates were flooded with sterile rain water and each of the agar plates examined under an inverted microscope for zoospore-producing swollen hyphae and the absence of septa in young hyphae, then fewer were confirmed as *Pythium* (**Table 10 and Figure 4**).

Carrot pieces were most commonly infested by *Pythium* (26 out of 80 pieces), with 9 out of 10 bags positive, and within these 1 to 5 out of 8 pieces were positive per bag). In total, out of 50 bags used there were 28 from which *Pythium* grew i.e. 56% were infested (**Figure 4**).

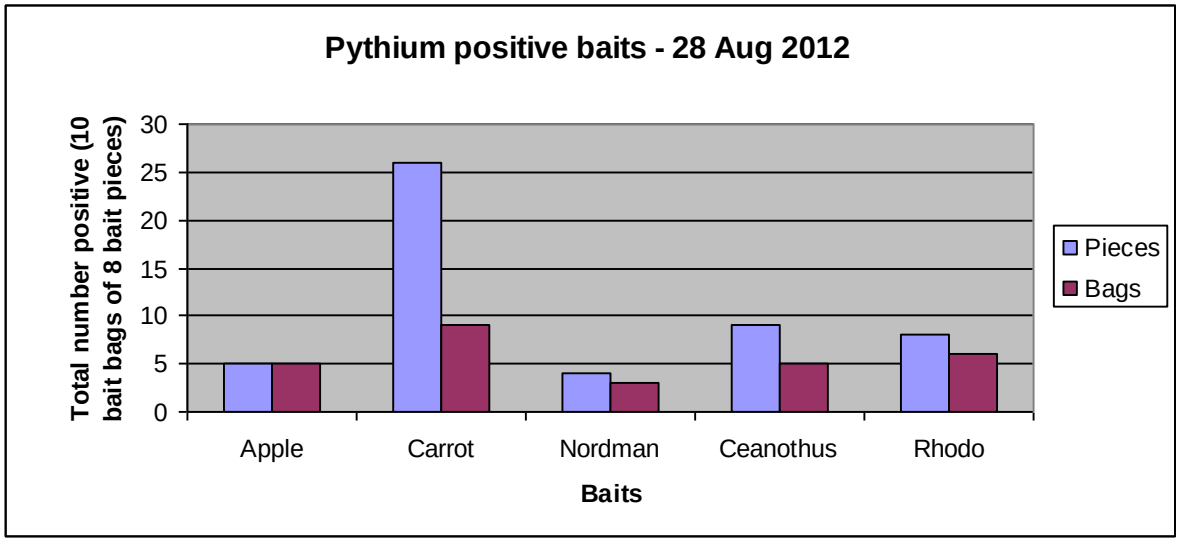


Figure 4: Detection of *Pythium* sp. by tissue baits showing the total number of bait pieces and the total number of bait bags from which *Pythium* was isolated (Experiment 5)

There were 4800 spores in each litre bottle based on laboratory calculations on the infestation day, but after postage to the Eden laboratory only 47 colony forming units were counted. The Eden laboratory obtained 100 % Ceanothus bait infestation at this low water infestation level,

compared with 11 % of bait pieces recorded by ADAS using the zoospore suspension before a sample of it was posted to the Eden laboratory. The reason for this discrepancy was unknown, but Experiment 6 examined the water type used as a potential influence.

Experiment 6. *Pythium* sp. - water type and zoospore survival

There were no apparent differences in zoospores present and the proportion encysted between the different water samples (**Table 13**) following two separate observation experiments. There was no evidence of zoospores having burst in the distilled water, or encystment resulting from immersion in tap water. In all the water types the zoospores had encysted within three hours, leaving only around 10 to 20 % still motile in the first run, and fewer (around 2%) in the second run which had a higher total count added to the counting chambers initially. Results for individual 2 ml chambers and means are given in Appendix 2. The numbers of zoospores plus cysts counted after 3-5 hours in various types of water were within the range expected from the original haemocytometer counts of zoospores.

Table 13. *Pythium* sp. zoospore survival and encystment three to five hours after adding to various types of water (Experiment 6) for laboratory tests on 6 and 7 December 2012

	6 December 2012			7 December 2012		
Water type zoospores were placed in	**Mean number zoo-spores**	**Mean number cysts**	**Total count**	**Mean number zoo-spores**	**Mean number cysts**	**Total count**
Sterile rain water	30	106	136	9	342	351
Distilled water	21	174	195	6	354	360
Stood tap water	21	158	179	8	324	332
Fresh tap water	25	174	199	6	342	348
'Primed' fresh tap water	28	155	183	8	312	320

Experiment 7 (*Pythium* sp.) and Experiment 8 (*P. cryptogea*) - LFD sensitivity

These tests used a different ratio of infected: uninfected baits. There were few positive LFDs across the two pathogen tests, and the indications were only faint. It was not known whether the zoospores did not infest the baits, or if the LFDs were not able to detect the infestation. In theory, where the maximum eight infested baits were used then there could have been infestation at 80 points by the zoospores across all the baits (10 spores x 8 baits), which would have been expected to have been sufficient to be detected by the LFD.

Experiment 9 (*P. cryptogea*) and Experiment 10 (*Pythium* sp.) - bait and LFD sensitivity

There was little detection of *Phytophthora* by isolation, with only one apple bait becoming infested (**Table 11**). Only one *Phytophthora* LFD (for 125 spores/L) was positive, (faintly = Index 1). A higher number of baits and bait bags were infested by *Pythium* sp. (**Table 12**), with most in the bottles with 250 spores / L (with 60% of bags infested). Bait infestation in the 125 and 25 spores / L was similar, (with 40% of bags infested). The LFD results were not very good, however, with faint test lines (Index 1) for *Pythium* sp. from baits taken from two of the 125 spores/L bottles.

Table 9: Laboratory baiting Experiment 3 (6000 *Phytophthora* zoospores/L) on 7 August 2012 showing % of bait pieces and bait bags infected

Bait tissue	% positive baits (out of 8 per bait bag) as determined by isolation onto agar										Mean % infected baits per bag	% of reps with an infected bait bag
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10		
Apple	25	37.5	0	0	12.5	50	25	0	37.5	12.5	20	70
Carrot	0	0	0	0	12.5	0	0	0	0	0	1	10
Nordmann fir	0	0	0	0	0	0	0	0	0	0	0	0
Ceanothus	25	0	0	12.5	0	0	0	12.5	0	0	5	30
Rhododendron	0	12.5	0	0	0	0	0	0	0	0	1	10

Table 10: Laboratory baiting Experiment 5 (4800 *Pythium* zoospores/L) on 28 August 2012 showing % of bait pieces and bait bags infected

Bait tissue	% positive baits (out of 8 per bait bag) as determined by isolation onto agar										Mean % infected baits per bag	% of reps with an infected bait bag
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10		
Apple	12.5	12.5	0	12.5	12.5	0	0	12.5	0	0	6	50
Carrot	50	37.5	12.5	0	37.5	25.0	62.5	25.0	25.0	50.0	33	90
Nordmann fir	0	0	0	0	0	0	0	25.0	12.5	25.0	5	30
Ceanothus	0	12.5	37.5	0	0	25.0	25.0	0	12.5	0	11	50
Rhododendron	12.5	12.5	25.0	12.5	0	0	25.0	0	12.5	0	10	60

Table 11: Laboratory Experiment 9 (0 to 250 *Phytophthora* zoospores/L) on 12 December 2012 showing % of bait pieces & bait bags infected

Zoospores per litre (per litre bottle)	Bait	% positive baits (out of 8 per bait bag) determined by isolation										Mean % infected baits per bag	% of reps with an infected bait bag
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10		
0	Apple	0	0	0	0	0	0	0	0	0	0	0	0
25	Apple	0	0	0	0	0	0	0	0	0	0	0	0
125	Apple	0	0	0	0	0	0	0	12.5	0	0	1	10
250	Apple	0	0	0	0	0	0	0	0	0	0	0	0

Table 12: Laboratory Experiment 10 (0 to 250 Pythium zoospores/L) on 18 December 2012 showing proportion of bait pieces & bags infected

Zoospores per litre (per litre bottle)	Bait	% positive baits (out of 8 per bait bag) determined by isolation										Mean % infected baits per bag	% of reps with an infected bait bag
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10		
0	Apple	0	0	0	0	0	0	0	0	0	0	0	0
25	Apple	0	0	0	0	0	12.5	0	12.5	25.0	37.5	9	40
125	Apple	0	12.5	50.0	0	12.5	0	0	12.5	0	0	9	40
250	Apple	37.5	37.5	0	25.0	50.0	0	0	0	25.0	12.5	19	60

Reservoir water sampling

The results from the Eden Laboratory of colony counts and any identifications of the organisms present in the individual water samples taken across the sample period are given in **Appendix 3**.

Preliminary sampling in 2012 to find a suitable location for monitoring in 2013

In August 2012, Nursery "A" had a clean reservoir and lagoon with nil *Pythium* or *Phytophthora* detected, although *Pythium* spp. were present in the 1 L water samples from a pond and ditch on the nursery as shown by both baiting with Ceanothus leaves and by agar plate tests. At Nursery "B", isolates of *Pythium* were obtained from water in one of the two reservoirs and in the runoff from both sets of beds collected in the reservoirs (**Appendix 3 and Table 14**). Nursery "B" Site 1 reservoir was used for subsequent bait testing in 2013.

Table 14. 1 Litre water sample bait infestations and colony counts showing live oomycetes in various for water collection sites in August 2012 (Eden Laboratories).

Water sample source	Ceanothus leaf pieces (8/bag) with oomycetes after baiting water samples in the laboratory	Colony forming units (cfu/L) filtered from sample
Nursery "A" reservoir	0 %	0
Nursery "A" lagoon	0 %	0
Nursery "A" pond	100 %*	200
Nursery "A" ditch	100 %*	450
Nursery "B" site 1 reservoir	80 %*	80
Nursery "B" site 1 runoff	70 %*	113
Nursery "B" site 2 reservoir	0 %	0
Nursery "B" site 2 runoff	90 %	900

* a mixture of fast and slow growing *Pythium* species and non-pathogenic *Saprolegnia* sp.

Interpretation of laboratory test results in preliminary and reservoir baiting experiments

In the laboratory tests of water the sample is micro-filtered and plated-out on selective-agar plates that allow the growth of all oomycetes, not only *Pythium* spp. and *Phytophthora* spp., with the saprophyte *Saprolegnia* sp. commonly being detected. It is possible for non-oomycete *Mortierella* spp. to be picked up in these tests, but this wasn't seen in the current project. The

colonies are counted as growth just starts (each colony being generated from a propagules such as a zoospore) when oomycete families are indistinguishable. Colonies can be transferred to new plates to grow and so aid identification by colony morphology by a skilled diagnostician. Oomycete colonies appear superficially similar, producing colourless non-septate mycelium whose growth pattern and amount of aerial growth differs depending on the type of agar they are grown on. For more positive identification the colony needs to be transferred to water in order to produce sporangia and other structures that can be measured under high power magnification and their shapes compared with published descriptions. Thousands of colonies were present in some samples and the resources to separate out *Pythium* and *Phytophthora* species were not available. It is unclear whether it is advisable to use LFDs to determine if a colony on agar is either a *Pythium* sp. or *Phytophthora* sp..

Baiting with Ceanothus leaves can be expected to produce zero infestation even when colonies are counted by the plate method as the technique is said to be far less sensitive. The baiting is useful, however in picking up potential pathogens. Commercially, water testing is usually done to confirm that water treatment has been effective and the test looks for a zero result for any living fungus or oomycete (Tim Pettitt, pers. comm.)

In addition to the tests for *Pythium* spp. and *Phytophthora* spp. counts were made at the Eden Laboratories of fungal propagules per litre and the number of colonies belonging to the families of *Trichoderma* and *Fusarium.* These are shown on the tables of results returned from the laboratory (**Appendix 3**). The high levels seen are expected from a reservoir, especially one open to the environment (as at Nursery “B”), and will include many saprophytes. Some fungi might be expected to be drawn off from water passing through a slow sand filter (as at Nursery “C”) as beneficial micro-organisms are grown in the filter to retain and digest pathogens. The particulate filter at Nursery “B” is used to remove suspended particles that could otherwise block irrigation nozzles, and the frequent similarity of the results between water before and after the filter shows that this type of filter cannot be used for pathogen removal. An unexplained exception to this occurred in September 2013 when Nursery “B” filtered water had no *Trichoderma, Fusarium*, *Pythium* or *Phytophthora* species recorded on the test plates when the reservoir had significant counts.

A molecular test called PDplus can be carried out by Forsite Diagnostics. This was originally intended to identify a small range of *Phytophthora* species of interest to Plant Health Inspectors such as *Phytophthora ramorum* and *Phytophthora cactorum*). It was used in the present work to double-check that matter from one or more *Phytophthora* species was actually present in a positive-indicating LFD. Three LFDs were PDplus tested from the July 2013

placed baits of R1 Shallow, post-filter at Nursery “B” and post-slow sand filter at Nursery “C” and the PDplus proved positive for *Phytophthora* spp. (not the species *P. cactorum*) for all. Forsite stated that there was a higher concentration of DNA (quantity not stated) in the LFDs from Nursery “B” than those from Nursery “C”. The water at these positions had contained 293 cfu/L *Phytophthora* spp. in R1 and 107 cfu/L *Phytophthora* spp. post-filter in the Nursery “B” samples, but zero oomycete cfu had been found in the water sample sent from the slow sand filter at the time the bait bags were deployed. This was investigated further in the extension study.

Reservoir baiting in 2013 at Nursery “B” open reservoir

The first bait deployment in 2013 compared the use of two of the materials used in the laboratory experiments in order to see what they would bait from naturally infested water (Objective 1). On 29 January 2013 when the baits were set out at the reed bed (R1) and outflow (R2) locations it was unusually mild for the time of year with an air temperature of 13°C and a water temperature of 6 °C. Oomycetes were isolated at ADAS from both the Ceanothus leaf and the apple flesh baits directly after retrieval (**Figure 5**), showing the presence of zoospores in the water this early in the year. *Pythium* and *Phytophthora* were identified on the basis of colony morphology on P5ARP agar and zoospore-producing structures in the water floats. *Phytophthora* was only recovered from one of the eight bait bags and in this bag only on one bait piece out of the eight was infested which could mean that the infestation was at a low level in the water (**Figure 5**).

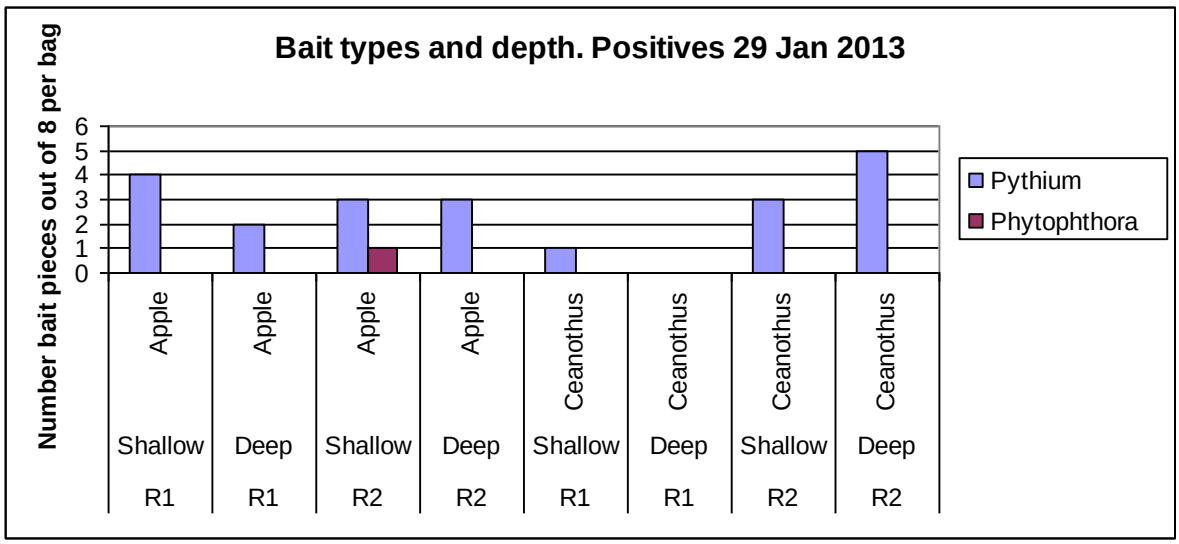


Figure 5: Isolation of species of *Pythium* spp. and *Phytophthora* spp. from apple and Ceanothus baits immersed for 48 hours in the reservoir at Nursery “B” in January 2013 and the number of bait pieces infested per bag of eight per location (R1 = by reed bed, R2 = by overflow) and flotation depth.

Positive LFDs for both *Pythium* and *Phytophthora* spp. were obtained from both the reed bed and outfall position baits placed for 48 h in the reservoir in January and recorded when received the next day at ADAS, with infection of the shallow baits of both the Ceanothus leaves and the apple pieces (**Table 15**). Both bait isolation and the LFDs showed *Pythium* and *Phytophthora* zoospores had been present at the outfall. It is possible that the higher LFD index from the apple than the Ceanothus relates to a greater quantity of the pathogen present which could be either because more zoospores were trapped, or that mycelium growth during post-retrieval incubation was greater in the apple bait than in the leaf tissue.

Table 15. LFD tests carried out six days after baits were added in the Site 1 reservoir on 29 January 2013 at Nursery "B". Assessed as Index 1 = positive but faint test, to Index 5 = Test line as intense blue as the Control line

Deployment positions (and code) for baits	Bait type	*Pythium* LFD Index	*Phytophthora* LFD Index
R2 outfall, Shallower bait	Apple	4	4
R2 outfall, Shallower bait	Ceanothus	4	2

No *Pythium* spp. or *Phytophthora* spp. were isolated from the Ceanothus bait leaves tested without immersion in the reservoir. This confirmed that the water-moulds detected in Ceanothus leaves were from the reservoir, not from previous infection. Of the five Ceanothus leaves placed in the January water sample at the ADAS laboratory, three leaves baited out *Pythium* spp. and one leaf had *Phytophthora* spp. (as determined by agar culture and water float).

The second bait deployment of 2013, in February, was utilised to see how testing directly on retrieval by LFD would compare with the results when the test was carried out after a week from deployment (time in the post plus incubation at room temperature in the dark). After 48 hours immersion, at temperatures principally below 5°C (**Appendix 4**), on 28 February, apple baits from the shallow depths at the reed bed and outfall locations were LFD tested by the grower (and the LFD readings later confirmed at ADAS). Negative results were shown for *Pythium* and *Phytophthora*. A longer than 48 hour bait placement period in the reservoir (Tuesday 26 February to Monday 4 March 2013) occurred in error with the remaining baits tested in the laboratory on Wednesday 6 March 2013 on receipt at midday. These showed positive LFD tests for both *Pythium* and *Phytophthora* at both locations and both depths (**Table 16**). It was not known if negative result after immediate testing resulted from a) the shorter (48

hour) bait placement period in the reservoir, or b) the quicker use of the LFD on retrieval because the positive baits had both seven days in the water and a delay in testing during posting to ADAS.

In February, the shallow and deep baits were seen to have both been floating within the top 50 mm of water, and their LFD indices were similar. Both baits from near the reed bed had a stronger *Phytophthora* test line (higher index) on the LFD than outfall location baits. This might indicate more *Phytophthora* spp. material present and able to bind to the antigen bound to the blue latex particles in the kit to cause more of them to form into the test line. The Index was only used as part of this project and for routine diagnostic usage any visible test line counts as a positive detection.

Results of the various diagnostic techniques in 2013 for the sample positions R1 and R2 and post-particulate filter at Nursery “B” are given in Table 16. Cells have been shaded in either dark (green) or light (blue) colours to show when the LFD results matched or differed, respectively, from the results of colony identifications of *Pythium* and *Phytophthora* spp. from the water samples. Colony identifications from the Eden Laboratory are given for the individual sample dates in **Appendix 3** and record the presence of these species (not their abundance) in the water samples. The results from both the shallow and deep bait can be compared with the single water sample taken at their deployment position as it included water at their flotation heights and to around 250 mm below the surface.

In July, it is likely that the LFD positives for both *Phytophthora* and *Pythium* spp. were correct although they were not individually named in the Oomycete counts for the water. The majority of deviations were the recording of *Phytophthora* spp. in the laboratory LFD tested apple baits when absent in the water sample cultures (i.e. a potentially false positive) but 88 % of *Pythium* spp. and 72 % of *Phytophthora* spp. results matched. To declare a false positive would require water samples to be taken on a continuous basis during the 48 hour period to pick up diurnal vertical movement of zoospores and/or contamination arriving during the baiting period that could be missed by the one-point-in-time water sample. Negative LFDs tested in the laboratory for *Pythium* and *Phytophthora* spp. seven days from bait placement matched the water sample colony records (i.e. there were no false negatives).

Shallow and deep baits at the same location all matched as positives, and baits at the R1 (reedbed end) and R2 (outflow end) locations were also both positive, so giving indication that multiple baits gave duplicate results. When baits from water sampled after the particulate filter at Nursery “B” were positive they had a low LFD index which probably indicated that there was little DNA of the test species present.

Two *Pythium* spp. LFDs used on-site in February (by the grower on baits tested directly on retrieval after 48 hours immersion) showed negative whereas low levels of *Pythium* spp. had been detected in the colony counts. In September, the grower obtained LFD positives for both *Pythium* spp. and *Phytophthora* spp. and matched the higher water sample counts, but the test line strength was not as great as when baits were left before testing. No R1 Deep bait was tested in the laboratory in September, because this was used in error by the grower instead of the R1 shallow bag.

Ceanothus leaf bait records are also shown in Table 16 as these were used in the Eden Laboratory to check if either or both *Pythium* and *Phytophthora* spp. were present in a sub-sample of the water (the two Oomycete families were only distinguished in the August samples, **Appendix 3**). These are not considered definitive indications of the absence of *Pythium* and *Phytophthora* spp. (they do not necessarily match the results from the colony counts) (Tim Pettitt, pers. comm.) and have not been used in Table 16 to compare with the LFD results, only identifications from the colony count plates.

Table 16. LFD results and test line index from apple baits from R1 and R2 positions at Nursery "B" in 2012 and 2013 for shallow and deep baits and after filtration. Oomycete colony counts from plate tests and Ceanothus leaf-bait infestation from water tested in the laboratory. Comparison of LFD results with identifications from test plates.

LFD tests done 5 days after bait retrieval, except those carried out immediately by the grower.
Dark (green) shading = *Pythium* spp. or *Phytophthora* spp. presence or absence matched colony identifications on test plates from water samples.
Light (blue) shading = *Pythium* spp. or *Phytophthora* spp. presence or absence did not match colony identifications on test plates from water samples.
No shading = No specific comments recorded on the identification of oomycetes in the colony cultures from the water sample (e.g. when sub-culture became contaminated)
See Appendix 3 for identification records from Eden Laboratory.

Dates baits put in reservoir at Nursery "B"	Bait bag & water sampling locations & bait depths	LFD tests of baits (laboratory or on-site)		1 L water sample testing at Eden Laboratory	
		LFD 1-5 index *Pythium*	LFD 1-5 index *Phytophthora*	Colony forming units (cfu) oomycetes per Litre of shallow + deep water	Ceanothus leaf baits/bag (8 leaf) with *Pythium* and/or *Phytophthora* spp.
21.08.12	R unspecified position. No bait bag.	not done	not done	80	80 %
29.01.13	R1 Shallow	not done	not done	70	100 %
	R2 Shallow	**4**	**4**	100	80 %

26.02.13	R1 Shallow	1	4	30	0 %
	R1 Shallow (on-site grower test)	negative	negative		
	R1 Deep	1	3		
	R2 Shallow	1	2	20	0 %
	R2 Shallow (on-site grower test)	negative	negative		
	R2 Deep	1	1		
	F after Filter	2	2	110	0 %
09.04.13	R1 Shallow	3	3	150	10 %
	R1 Deep	2	4		
	R2 Shallow	4	2	140	50 %
	R2 Deep	1	4		
13.05.13	R1 Shallow	5	4	3360	0 %
	R1 Deep	2	3		
	R2 Shallow	2	2	650	90 %
	R2 Deep	3	1		
15.05.13	R1 Shallow	3	3	540	20 %
	R1 Deep	4	3		
	R2 Shallow	1	1	1000	10 %
	R2 Deep	1	3		
23.07.13	R1 Shallow	negative	1	720	100 %
	R1 Deep	1	1		
	R2 Shallow	2	2	167	60 %
	R2 Deep	1	1		
	F after filter	1	1	506	50 %
13.08.13	R1 Shallow	1	5	55	100 % (apple)$
	F after filter	negative	2	0	0 % (apple)$
16.09.13	R1 Shallow	5	5	113	100 %

	R1 Shallow	**4**	**5**		
	R1 Deep (on-site grower test)	**2**	**1**		
	R2 Shallow	**5**	**5**	60	100 %
	R2 Deep	**5**	**5**		
	F after filter	**1**	**negative**	0	0 %
	F after filter (on-site grower test)	**negative**	**negative**		
12.11.13	R1 Shallow	**5**	**5**	200	100 %
	R1 Deep	**5**	**5**		
	R2 Shallow	**5**	**5**	453	100 %
	R2 Deep	**4**	**5**		
	F after filter	**1**	**negative**	0	0 %
Ratio same:opposite results for ADAS LFD tested baits & colony identification		**30:4 88% match**	**26:10 72% match**		

LFD Index 1 = very faint blue test line, grading to Index 5 = strong blue test line
Baits were shallow (30 mm) and deep (250 mm) below the surface, except in January and February when the deep bait remained at about 60 mm.
$ Apple bait pieces from the reservoir were cultured.
1 L water sample results count all the different oomycete species. Ceanothus bait tests confirm *Pythium* and/or *Phytophthora* spp. presence in water sample.

If LFD index strength were to be related to colony counts by growers it should be noted that 100 Oomycetes per litre (100 cfu) is a significant level. The counts around and in excess of 1000 Oomycetes/L in May were extraordinarily high, however not all may have been *Pythium* or *Phytophthora* (Tim Pettitt, pers. comm.). This was confirmed in September and November 2013, when colony counts specifically for *Pythium* and *Phytophthora* were done by the Eden Laboratory across the water samples in addition to the routine “Total oomycetes” count (Appendix 3). For example, in September at Nursery “B”, at position R1 of the 113 oomycete colonies counted 13 colonies were *Pythium* and 32 colonies were *Phytophthora* spp.. The LFD tests on the baits confirmed water infestation, with strong indicator strip indices of 5 for both species. When high numbers of colonies were cultured on plates it was not possible to determine the numbers of *Pythium* spp. or *Phytophthora* spp. colonies because the colonies grow into each other before identification by mycelial growth pattern can be completed.

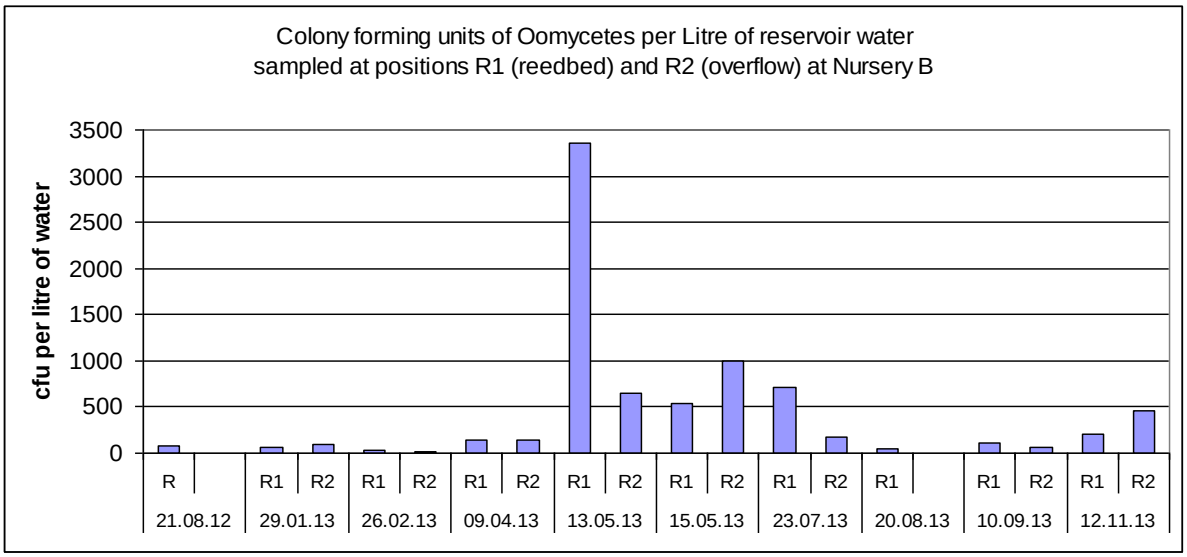


Figure 6: Colony forming units of oomycetes in water samples taken at intervals during 2013 at positions R1 (inflow) and R2 (overflow) in an open reservoir at the time of bait bag deployment. Sample location R in 2012 selected by the grower.

Higher cfus were obtained at Nursery “B” in May and July than earlier in 2013 (January, February and April when water temperature was mainly below 10 °C (**Appendix 4**)). The reservoir was emptied for maintenance after the March sampling and this did not result in any major change in infestation at the April monitoring. The levels fell again in August and September, but there had been an increase at the November sampling. *Pythium* spp. and *Phytophthora* spp. were detected throughout the year in both the water samples and from the apple baits. Ceanothus leaf bait bags in the laboratory are known not to be particularly sensitive (Tim Pettitt pers. comm.) and these did not become infested as frequently as they could have been. Daily sample dates would be need to be certain if microbial abundance was greater all the time during the months with peak samples. However, for the grower any of the levels of infestation are significant if they were to consider using the water without further treatment on their crops.

Table 17. Comparison of LFD indices (test line positive colour strength) from apple baits removed from baiting points around Nursery "B" reservoir edge in May 2013

Dates baits put in reservoir at Nursery "B"	Bait bag locations and depths	Laboratory LFD tests of baits		1 L water sample testing at Eden Laboratory	
		LFD 1-5 index *Pythium* spp.	LFD 1-5 index *Phytophthora* spp.	Colony forming units (cfu) oomycetes per Litre of shallow + deep water*	Ceanothus baits/bag (8 leaf) with *Pythium* and/or *Phytophthora* spp.
13.05.13	R1 Shallow	5	4	3360	0 %
	R2 Shallow	2	2	650	90 %
	R3 Shallow	5	2	780	70 %
	R4 Shallow	1	2	140	0 %
	R5 Shallow	5	3	100	10 %
13.05.13	R1 Deep	2	3	3360	0 %
	R2 Deep	3	1	650	90 %
	R3 Deep	3	2	780	70 %
	R4 Deep	1	1	140	0 %
	R Deep	4	2	100	10 %
15.05.13	R1 Shallow	3	3	540	20 %
	R2 Shallow	1	1	1000	10 %
	R3 Shallow	2	1	1210	0 %
	R4 Shallow	1	1	1110	0 %
	R5 Shallow	1	3	790	0 %
15.05.13	R1 Deep	4	3	540	20 %
	R2 Deep	1	3	1000	10 %
	R3 Deep	1	1	1210	0 %
	R4 Deep	3	4	1110	0 %
	R Deep	1	1	790	0 %

Bait bags suspended 3 cm (shallow) and 25 cm (deep) below the surface
Index 1 = very faint blue test line, grading to Index 5 = strong blue test line
* The sample at each position collected water from both bait bag depths in each 1 L.

In May, when two baiting sessions were carried out with the first set of baits replacing the first directly on their removal for testing at ADAS, stronger LFD Index results were shown for *Pythium* at the first baiting session at the shallow depth (**Figure 7**). This was matched by higher colony counts at the R1 reed bed position (but not the other positions) when the baits

were placed (**Figure 8**). However both *Pythium* and *Phytophthora* were detected by the LFDs at all of the baiting positions, at both depths in both sessions. From the LFD tests of the bait bags, only one bait bag may be necessary for monitoring and it may not matter where water baiting positions are located of what bait flotation depth in the range to 250 mm below the surface. However, while *Pythium* spp. were seen in all of the cultured water samples, *Phytophthora* spp. were not seen at positions R1 and R5 although present at positions R2, R3 and R4 on both the 13 May and 15 May 2013 sampling sessions (**Appendix 3**). During the first half of the week the water temperature (**Appendix 4**) was mainly between 10-15 °C, but on the 16 May daytime water temperatures were above 15 °C (rising to 20 °C).

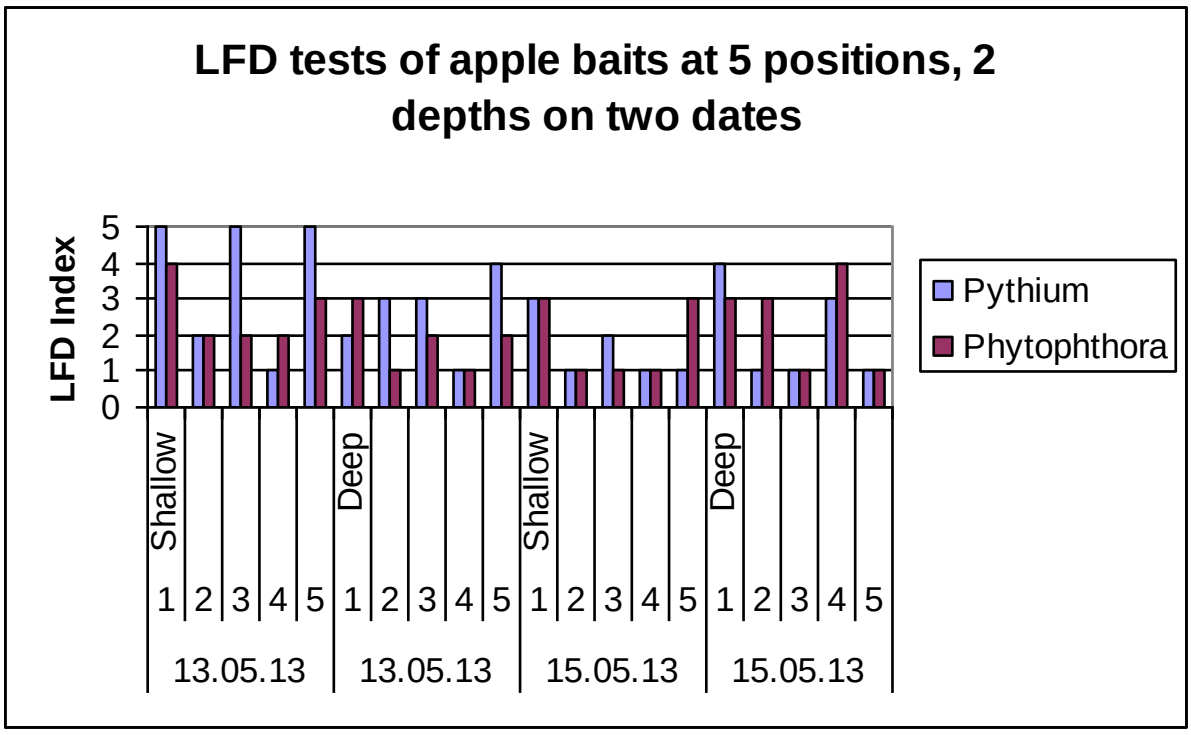


Figure 7: Comparison of LFD 0-5 indices (showing increasing strength of test line, where 5 was the strongest) for *Pythium* and *Phytophthora* spp. in apple baits removed from baiting points around open reservoir edge (positions 1 to 5) at Nursery "B" with baits placed at two depths at each point

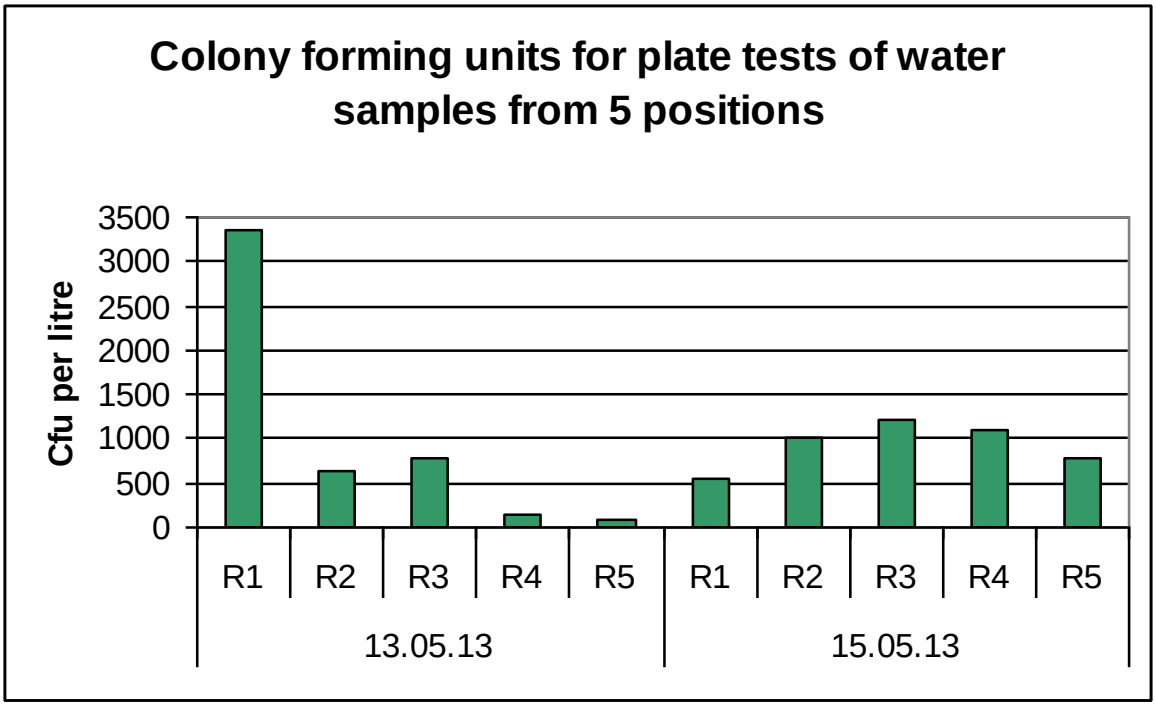


Figure 8: Colony forming units of water moulds on selective agar from 1 L water samples taken from the open reservoir at Nursery "B" for five positions on two dates in May 2013

Reservoir baiting in 2013 at Nursery "C" closed reservoir

At Nursery "C", the first water testing was done on 26 February 2013 and two apple bait bags placed in the water before the slow sand filter tested positive for both pathogens (**Table 18**). It was noted, that the deeper float did not sink much below the depth of the shallow bait bag. The weighting of bags was subsequently adjusted (by tying the weighted deep bag below a floating bottle). All the subsequent LFD records through to the last sample in November 2013 were still the same for the two depths, with positives for both *Pythium* and *Phytophthora* spp. throughout. Except in April, whenever the LFDs were positive for *Pythium* spp. in the reservoir then these species were also detected in the colony counts. Both the February and the April LFDs were positive for Phytophthora when there was no detection in the colony counts, but the positives in September and November were supported by the colony counts. At this nursery and at Nursery "B" the *Phytophthora* spp. LFDs were positive before Oomycete colony counts increased greatly in the unfiltered water in May.

Table 18. LFD results and test line index from apple baits from Nursery "C" reservoir and after filtration in 2013. Oomycete colony counts from plate tests and Ceanothus leaf-bait infestation from water tested in the laboratory. Comparison of LFD results with identifications from test plates.

LFD tests done 5 days after bait retrieval, except those carried out immediately by the grower.
Dark (green) shading = *Pythium* spp. or *Phytophthora* spp. presence or absence matched colony identifications on test plates from water samples.
Light (blue) shading = *Pythium* spp. or *Phytophthora* spp. presence or absence did not match colony identifications on test plates from water samples.
No shading = No specific comments recorded on the identification of Oomycetes in the colony cultures from the water sample (e.g. when sub-culture became contaminated)
See Appendix 3 for identification records from Eden Laboratory.

Dates baits put in reservoir at Nursery "C"	Bait bag locations and depths	Laboratory LFD tests of baits (or on-site)		1 L water sample testing at Eden Laboratory	
		LFD 1-5 index *Pythium* spp.	LFD 1-5 index *Phytophthora* spp.	Colony forming units (cfu) oomycetes per Litre of shallow + deep water	Ceanothus leaf baits/bag (8 leaf) with *Pythium* and/or *Phytophthora* spp.
26.02.13	Shallow	2	3	33	10 %
	Deep	2	4		
09.04.13	Shallow	3	4	0	0 %
	Deep	1	1		
14.05.13	Shallow	5	5	340	20 %
	Deep	1	2		
	Slow-sand Filter	1	1	0	0 %
30.07.13	Shallow	1	2	247	100 %
	Deep	1	2		
	Slow-sand Filter	1	4	0	0 %
13.08.13	Shallow	2	0	253	100 % (apple)$
	Slow-sand Filter	0	1	13	40 %* (apple)$
	Slow-sand Filter 3L water			34	
24.09.13	Shallow	5	3	213	100 %
	Shallow (on-site grower test)	0	0		
	Deep	4	3		

	Slow-sand Filter	**3**	**0**	0	0 %
26.11.13	Shallow	**5**	**5**	146	100 %
	Shallow (on-site grower test)	**not tested**	**0**		
	Deep	**not tested**	**4**		
	Slow-sand Filter	**1**	**0**	0	0 %
Ratio same:opposite results for ADAS LFD tested baits & colony identification		**9:7 56% match**	**9:9 50% match**		

LFD Index 1 = very faint blue test line, grading to Index 5 = strong blue test line
* only Saprolegnia sp. in the apple baits, which when tested with a *Phytophthora* and a *Pythium* LFD did not give a positive.
$ Apple bait pieces from the reservoir were cultured (no Ceanothus laboratory bait test for these water samples).

Compared with Nursery “B”, samples from Nursery “C” had fewer LFDs from baits whose results matched the colony identifications from water samples (i.e. only around half of the LFDs for both *Pythium* and *Phytophthora* matched) (**Tables 16** and **18**). The principal discrepancy was because Nursery “C” had a slow sand filter and no colonies grew on the plates made from the water collected from it. The LFDs would be detecting both alive and dead material, and it is possible that the *Pythium* and *Phytophthora* (or substances that are detected by the antibodies in the LFD) passed through the filter into the sampled water. To test the theory that dead material was being detected by the LFDs a positive *Phytophthora* LFD from 30.07.13 that had tested apple from the slow sand filtered water was sent for PDplus testing to confirm the presence of *Phytophthora* spp. DNA in the device. Neither Ceanothus bait infection by Oomycetes nor Oomycete colonies were reported from the water sample taken at the time the apple bait had been placed in the reservoir. The PDplus result confirmed that DNA of *Phytophthora* spp. was present in the LFD. Confirmation of *Phytophthora* spp. DNA was also obtained for the LFD that tested the shallow depth apple placed on the same date, so supporting the evidence from the Ceanothus leaf infestation in the water sample. Further work was undertaken to investigate the potential detection of dead material.

The sensitivity of the LFDs can be indicated by comparison with the colony counts, although the counts only relate to the time they were taken, not the changes possible during the 48 hours of bait deployment. The lowest number of *Pythium* propagules/L counted in colony counting plates from the water samples was 26 from the Nursery “C” reservoir in November 2013, and 20 *Phytophthora* propagules/L were collected from the reservoir at Nursery “B” in

August 2013 (**Appendix 3**). Both LFDs of the baits placed at the same sample points recorded an Index 5 strong reaction indicating good sensitivity following immersion in water with low colony counts. The August LFD reading was carried out on receipt of the baits through the post at the Eden Laboratories without further incubation. Incubation after bait retrieval is expected to allow the individual propagules to grow on the bait and increase the amount of material for detection. The negative LFD results for *Pythium* and *Phytophthora* species after on-site testing at Nursery "B" in February when there were 30 Oomycete colonies/L when incubation produced positive records (Index 1 and 4, respectively) may indicate low LFD sensitivity. However, as the laboratory Ceanothus water sample baits were also uninfected it is probable that very few of the colonies counted in the water tests were *Pythium* spp. or *Phytophthora* spp. (**Table 16**). Incubation of apple baits before LFD testing improved the detection.

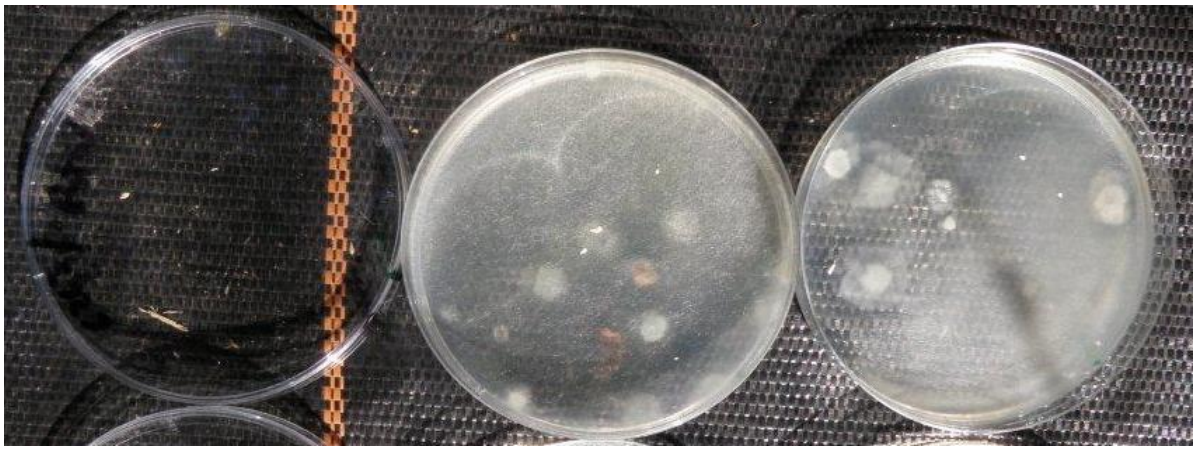


Figure 9: Some of the agar plates for Oomycete colony counts on 7 August after incubation. Water samples taken from after the slow sand filter (clean, left plate) and reservoir water on 30 July 2013 at Nursery "C". The reservoir plates contain several colonies of *Pythium* and saprophytes, and potentially some *Phytophthora* (T. Pettitt)

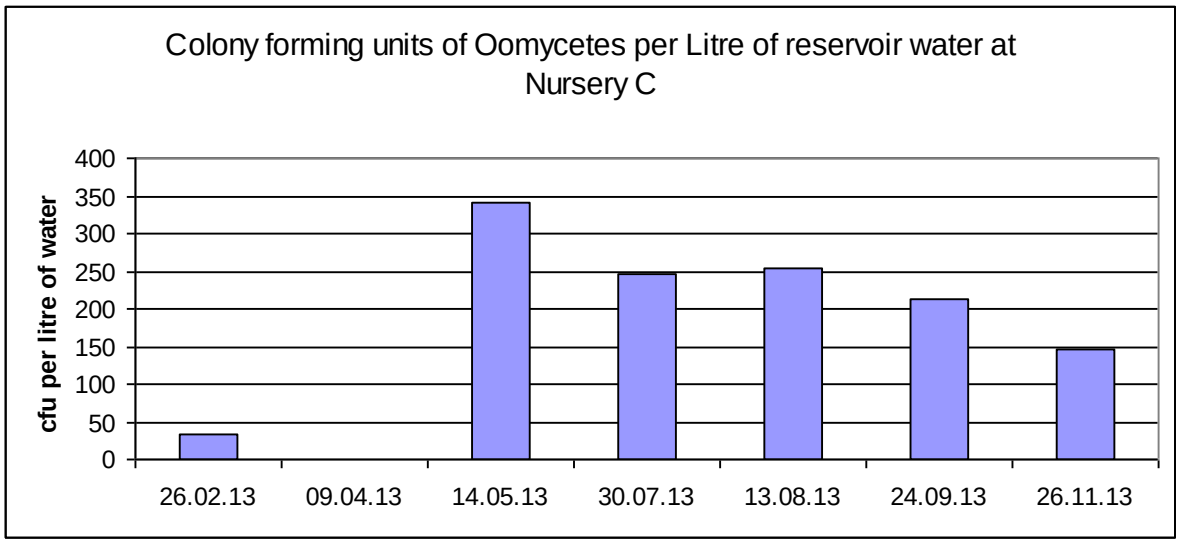


Figure 10: Colony forming units of oomycetes in water samples taken at intervals during 2013 from a closed-top reservoir at the time of bait bag deployment

At Nursery "C" the lower concentrations of Oomycetes in the reservoir were (as for Nursery "B") coming out of the winter period (**Figure 10**). Nursery "C" colony counts were much lower in each month than Nursery "B". A peak was also seen in May in the reservoir at Nursery "C" as also recorded at "B". The numbers then decreased, falling to a still significant 146 cfu by late November. *Pythium* colonies were confirmed present in February, May, August, September and November. *Phytophthora* spp. colonies were confirmed present in May, September and November (**Appendix 3**). Neither *Pythium* nor *Phytophthora* species were checked for specifically in the colony count plates in July.

In the UK in 2013 there was a late winter and an exceptionally cold spring (the coldest since 1962 with March), with unseasonably late snowfalls. There was above average rainfall in May and October. The summer was warm and sunny, but in October there were rain storms (as confirmed by seasonal summaries given at www.metoffice.gov.uk). The cold spring is likely to have held back the multiplication of Oomycetes, and it is possible that the extra rain in May resulted in zoospores and other propagules being washed through out of pots and high water volumes being collected in the reservoirs. It is not known whether or not May would be the month across years when propagules might become most abundant in reservoirs.

August 2013 cross-checks of LFD tests on apple by isolation of apple bait pieces onto agar

In August 2013, the duplicate sets of apple baits received from Nursery "B" Site 1 and 2 and "C" were each either plated out onto agar, with individual isolation from each of the eight pieces, or the extract from all eight pieces LFD tested for *Pythium* spp. and *Phytophthora* spp. at the Eden Laboratory (**Table 19**). The tests were all carried out as soon as the baits arrived

from the grower after overnight delivery. In contrast to Tables 18 and 19, Table 19 compares LFD results with isolations from reservoir-placed baits, not plate test isolations directly from reservoir water samples for cfu counts. Details of the test results are given in Appendix 3.

All the results for both positive and negative detection of *Pythium* spp. in the apple bait plates and the LFDs from the matched (**Appendix 3**).

A complete match of negative presence occurred for all three *Phytophthora* tests on the reservoir water and baits. Detection by the LFD (giving an Index 5) was possible in apple baits removed from the reservoir at Nursery “B” at a position with 20 cfu/L in the water at bait deployment. The plates had *Saprolegnia*-like species on them and a negative LFD test on excised colonies from the plate confirmed the absence of *Phytophthora* spp. and that the LFDs were not detecting other oomycetes.

A positive LFD result was, however, obtained at Nursery “C” following apple baiting of the slow-sand filtered water (although only a faint test line) when no colonies were found in the water sample and no *Phytophthora* spp. colonies grew on agar from the duplicate apple bait bag that had been left in the filtered water (**Table 19**).

Another false positive in filtered water occurred, but after the particulate filter at Nursery “B” (**Table 19**). The remains of the apple left over (without skin) when making the bait bags at Nursery “B” were also sent to the laboratory in a clean polythene bag and divided between isolation and LFD tests showed *Phytophthora* spp., but not *Pythium spp.,* was present. It thus cannot be discounted that some of the apple flesh was the source of the positive detections in deployed baits, although some of the flesh was uninfested because results from two of the eight deployed bait bags were negative for *Phytophthora* spp.. *Phytophthora* spp. infestation of apple before deployment would explain the positive Nursery “B” Site 2 post-filter positive bait test isolation which contrasted with the negative isolation directly from the water sample. It should be noted, however that apples for the bait bags were sent to each of the baiting nurseries from the same plastic-bagged retail pack (with no external rot visible on the fruit) and the skin had been wiped with 75% ethanol to remove any possible surface contamination. Each of the growers cut up the apple for the bags just before use in the bait bags. No *Phytophthora* spp. was detected in the bait bags from Nursery “B”. A further check on apple infestation carried out at the Eden Laboratories in September 2013 showed no oomycetes in plate cultures of 13 unused apple bait pieces returned from Nursery “C”.

The LFD from the August 2013 unused Nursery "B" apple was sent by the Eden Laboratory to the Royal Horticultural Society to see if the DNA could be sequenced to determine the species. However, only a faint band on the DNA fingerprint was obtained when the molecular (PCR) test was done, which was insufficient for sequencing without cloning first to bulk up the amount of DNA. However, the band position was not normal for *Phytophthora* spp.. *Pythium* spp. primers were also tried and the band was not normal for *Pythium* spp. either. This implies that the antibodies in the LFD attached to material they "recognised" and indicated a positive detection of *Phytophthora* spp., but that this material was not bound with either *Pythium* or *Phytophthora* spp. DNA.

Table 19. August 2013. LFD line strength index comparisons with isolations from duplicate apple bags and colony forming units /L for *Pythium* and *Phytophthora* spp.. Nurseries "B" (two reservoirs) and "C" pre and post filter. All tests carried out at Eden Laboratories.

Comparison of LFD results from apple baits with colony identifications from apple bait isolations:
Dark shading indicates = *Pythium* or *Phytophthora* was also identified from either the colony counts from the water, or the culturing of duplicate apple baits.
Light shading = *Pythium* or *Phytophthora* was not recorded the same in the LFD as in either of the other tests
No shading = No specific comments recorded on the identification of oomycetes.
See Appendix 3 for identification records from Eden Laboratory.

	***Pythium* spp.**			***Phytophthora* spp.**			**Total oomycetes**	
Nursery & Sample site	**LFD Index**	**cfu / L water**	**% of baits +ve**	**LFD Index**	**cfu / L water**	**% of baits +ve**	**cfu / L water**	**% of baits +ve**
"B" Site 1 R1 Shallow	**1**	not done	**100**	**5**	20	**75**	55	100
"B" Site 1 after Filter	**0**	not done	**0**	**2**	0	**0**	0	0
"B" Site 2 Shallow	**1**	not done	**75**	**1**	60	**50**	213	100
"B" Site 2 after Filter	**<1**	not done	**75**	**0**	0	**50#**	0	100
"C" Tank Shallow	**1 – 2**	not done*	**75**	**0**	0	**0**	253*	100
"C" after Filter 1L	**0**	not done*	**0**	**1**	0	**0**	13*	40
"C" after Filter 3L					not recorded		34*	

Bait pieces unused at “B”	**0**	not done	**0**	**1 -2**	not done	**100**	not done	100
Ratio same:opposite for LFDs of baits & isolation from baits	**7:0** **100% match**			**5: 2** **71% match**				

* Large numbers of *Saprolegnia* and fast-growing *Pythium* spp.
100% = 8/8 apple bait pieces infested, except 4 unused pieces were only available to test
Duplicate bait results conflict

On-site LFD testing on bait retrieval versus incubation prior to testing

The eight on-site LFD test of baits done by growers immediately on bag retrieval, after 48 h immersion can be compared with baits placed at the same time but tested after incubation. At Nursery “B” the LFD tests when cfus were lower in February 2013 were negative for both *Pythium* and *Phytophthora* species when the laboratory LFD tests of duplicate baits (7 days from immersion) were positive. However although the *Pythium* spp. negatives did not match the water test colony counts (false negative), the *Phytophthora* spp. negatives matched the absence of *Phytophthora* in the colony counts. The on-site positive tests in September at the reedbed end of the reservoir matched those for the laboratory LFDs (although the Index was higher in the latter) and the filtered water was confirmed as negative in the colony counts. At Nursery “C” a false negatives were recorded on-site for *Pythium* spp. and *Phytophthora* spp. in the reservoir as both the laboratory LFDs and the colony identifications picked up this infestation. In November, when the reservoir was again tested by the grower, a false negative was recorded as the laboratory LFDs and colony counts showed *Phytophthora* spp. were present. It is not known whether negatives were recorded erroneously on the nursery because the amount of material in the baits was insufficient to give a positive Test line. The August LFD testing of bait bags (and isolation onto agar of the duplicate set) at the Eden Laboratory was, however, done on receipt from the grower by courier and gave positives for both *Pythium* spp. and *Phytophthora* spp.. without the use of a further incubation period.

Apple baits returned which gave *Pythium* spp. positives tended to have softened and become browner than negative baits and it seems likely that the extended period before testing would allow more rotting and aid detection by the LFD. Freshly retrieved apple often looks little different to when it was put in the bait bag and so when the ball bearings in the buffer did not readily smash the tissue to release the pathogen and discolour the buffer in the bottle a clean crushing rod (as detailed in the instructions to growers) was occasionally used to break tissue open more speedily.

The best incubation interval to be used before LFD (or possibly PCR) testing was not investigated. It was possible that baits placed on a Tuesday and retrieved on a Thursday morning could be tested on Friday morning following incubation at 20°C, as both *Pythium* spp. and *Phytophthora* spp. are fast growing. Less incubation might be required when baits are removed from warmer water or when a higher microbial density in the water is thought possible.

Extension study investigating 'false positive' results - test kit sensitivity comparisons

Two comparisons of sensitivity were carried out using zoospore suspensions of *Phytophthora cryptogea* isolate E556 and baiting with 'Golden Delicious' apple pieces. In the first comparison, the Alert™ LFDs appeared much less sensitive than the Pocket diagnostic® LFDs (Figure 11A). However, this comparison was carried out using the original volumes of extraction buffer and the difference was much reduced when the Alert™ LDFs were restricted to 5ml extraction buffer (Figure 11B), although these were still consistently less sensitive than the 'new' Pocket diagnostic® LFDs. As outlined above, for fairer comparisons, all subsequent Alert ™ LFD tests were carried out using just 5 ml of extraction buffer. A more significant difference was seen between the 'old' and 'new' Pocket diagnostic® tests (Figure 11B) a trend that was also seen in comparisons using mycelial suspensions (Figures 12A & B). The curves illustrated in Figure 11A & B do indicate that measuring baits in this way can be 'quantitative'. All baits plated in both experiments showed 100 % positive for *Phytophthora* infection except those placed in the zero zoospore 'control' suspension (= 0 % infected) and in the 2.3 log zoospore (approx. 200 spores/L) suspension (= 30 % infected in A and 20 % infected in B), indicating that using LFDs can add a meaningful layer of information to using baits for *Phytophthora* detection.

Comparisons of LFD kit sensitivity against dilutions of homogenised mycelium of *Phytophthora cryptogea* (isolate E556) and *Phytophthora* sp. (isolate C295) also showed that the 'new' Pocket diagnostic® kits were consistently more sensitive than Alert™ kits (Figures 12A & B), and that the 'old' Pocket diagnostic® kits were generally the least sensitive. This last result may indicate that the 'old' Pocket diagnostic® kits were possibly partly degraded. Both LFD test kit types were more sensitive to unidentified *Phytophthora* isolate C295 (Figure 12B). However, the intensity of reactions tended to decline at the higher concentrations of mycelial homogenate, possibly as a result of binding/blocking caused by an excess of antigen material.

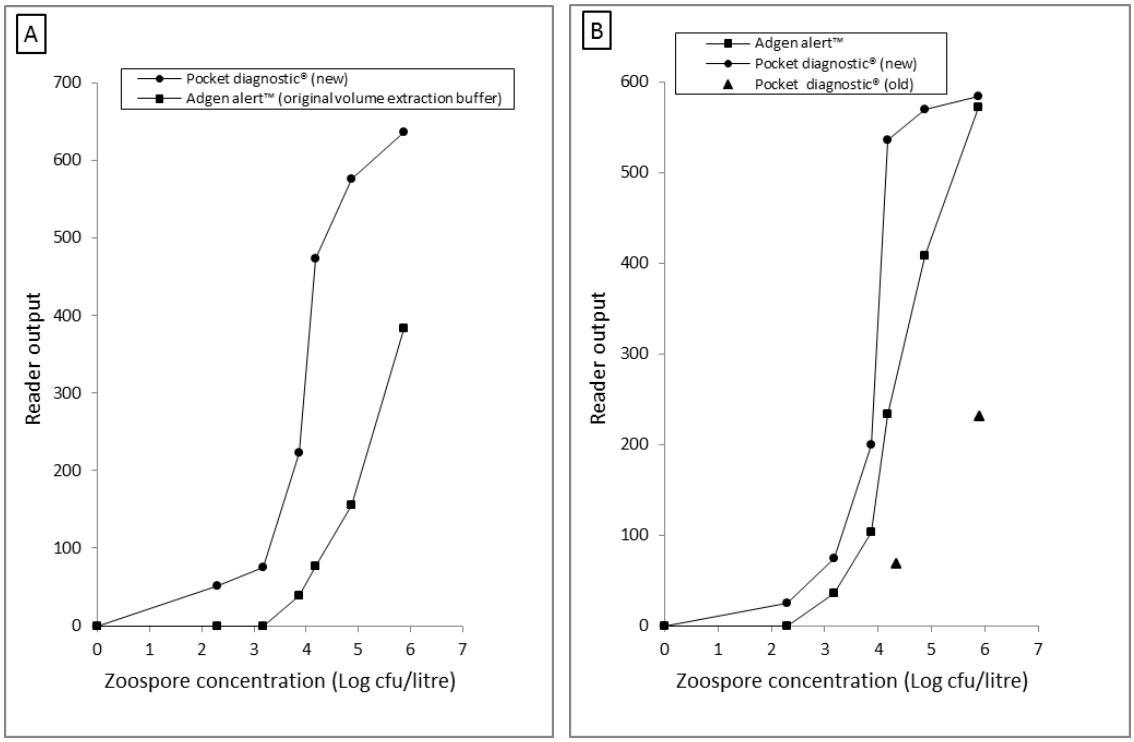


Figure 11: Comparison of sensitivity of Adgen Alert™ and Pocket diagnostic® LFD *Phytophthora* detection kits when used in combination with 'Golden Delicious' apple piece baits to trap and detect a range of concentrations of zoospores of *Phytophthora cryptogea* isolate E556 in sterile pond water. In comparison (A) the kits were deployed with the volumes of extraction buffer supplied, whilst in comparison (B) both kits were deployed with 5 ml of buffer. Also in comparison (B) two older Pocket diagnostic® kits were assessed.

Extension study investigating 'false positive' results - water disinfestation treatments

A range of chlorine dioxide concentrations was tested against zoospores of *Phytophthora cryptogea* (Table 20). Concentrations from 2.5 ppm resulted in 100 % zoospore mortality and from this a concentration of 10 ppm chlorine dioxide was selected for further experiments to guarantee rapid and complete disinfestation prior to baiting. Baits were placed in each chlorine dioxide treatment and tested after 48 h using Alert™ LFDs. These generally showed little variation in detection response across a wide spectrum of spore mortality from 0 to 1.41 x 10^5 spores/L viable (Table 20). The medium strength bands (LF reader outputs of 187.09 to 240.03) seen at 2.5, 12.5 and 25 ppm chlorine dioxide concentrations when there was only dead *Phytophthora* inoculum present, indicate that it is possible to obtain false positive LFD tests in the presence of dead pathogen debris even when using apple segment baits in an attempt to only detect viable and therefore infective propagules.

All three disinfestation techniques used in this study; heat, UV and chlorine dioxide, achieved 100 % kill when applied to dilution series of *Phytophthora cryptogea* zoospores as indicated

by direct plating, membrane filtration-colony plating and plating out bait segments. Dead pathogen debris from all three disinfestation treatments attached to apple segment baits and gave false positive LFD test with both Alert™ and Pocket diagnostic® test kits throughout a wide range of original inoculum concentrations (Figures 13A & B). Positive reactions were substantially less than for equivalent live inoculum at each concentration, but the intensity of reactions did increase with increasing inoculum concentration. Nevertheless, even the very slight positive tests, giving LF reader outputs of 20 to 50 (and still equivalent to 20-200 zoospores/L), were still visible to the naked eye.

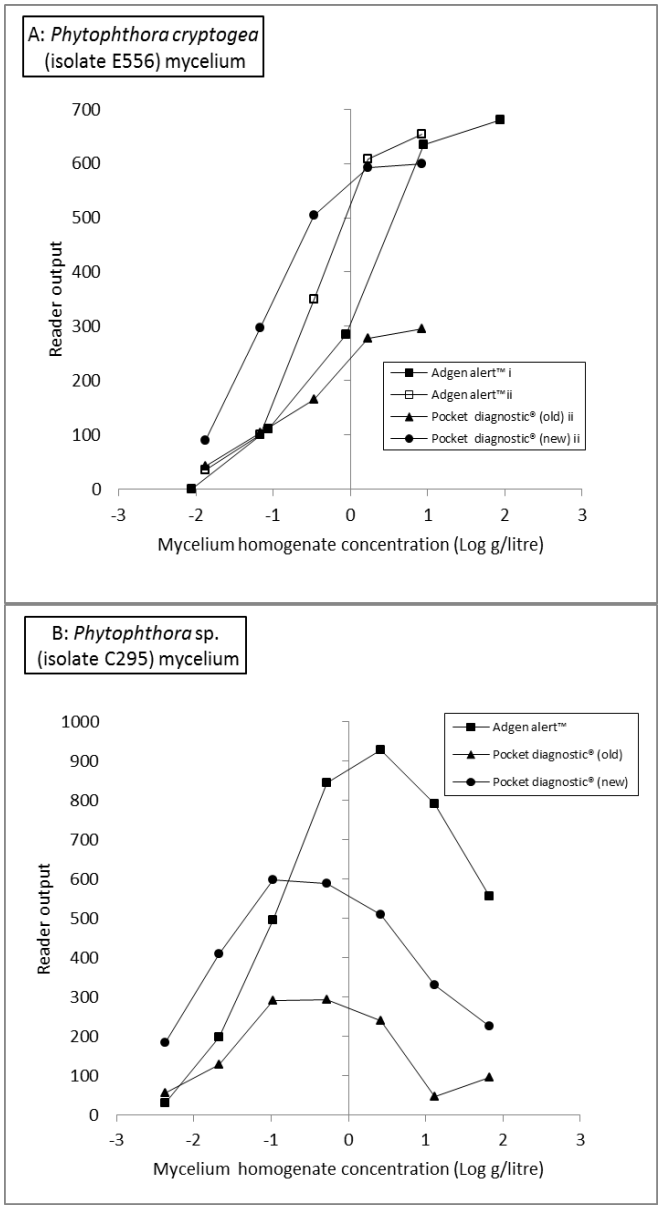


Figure 12: Comparison of sensitivity of Alert™, 'old' and 'new' Pocket diagnostic® LFDs when tested directly on homogenised mycelium of (A) *Phytophthora cryptogea* (isolate E556), and (B) *Phytophthora* sp. (isolate C295).

Table 20: Determination of efficacy of chlorine dioxide treatments against a suspension of *Phytophthora cryptogea* zoospores containing 2.00 x 10^5 spores per litre. Table shows numbers of zoospores estimated using a haemocytometer, numbers of viable spores estimated by plating and therefore the estimated number of non-viable (dead) spores obtained by subtraction. Values in brackets are log zoospore concentrations for cross reference with Figures 11 and 12.

Chlorine dioxide concentration (ppm)	A Haemocytometer-estimated zoospore concentration	B Viable zoospore concentration (cfu/L)	A – B Estimated non-viable zoospore concentration	Lateral Flow Reader Output (Alert™ LFDs)
0	2.00 x 10^5 (5.301)	1.41 x 10^5 (5.149)	5.90 x 10^4 (4.771)	218.16
0.25	2.00 x 10^5 (5.301)	1.59 x 10^5 (5.201)	4.10 x 10^4 (4.613)	143.32
1.25	2.00 x 10^5 (5.301)	7.38 x 10^4 (4.868)	1.26 x 10^5 (5.101)	266.29
2.5	2.00 x 10^5 (5.301)	0	2.00 x 10^5 (5.301)	237.27
12.5	2.00 x 10^5 (5.301)	0	2.00 x 10^5 (5.301)	240.03
25	2.00 x 10^5 (5.301)	0	2.00 x 10^5 (5.301)	187.09

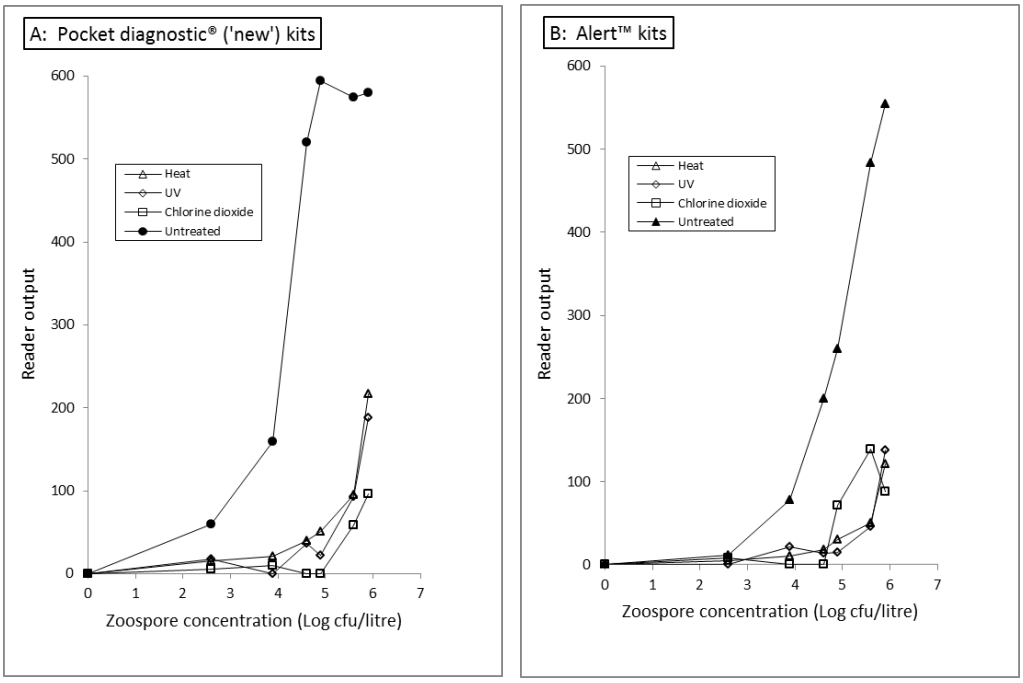


Figure 13: Assessment of the impact of 'total kill' from three different water disinfestation treatments. Heat, UV and Chlorine dioxide, on the results of apple bait/LFD tests on a range of zoospore concentrations in comparison with viable (untreated) zoospores, (A) using Pocket diagnostic® LFD kits, and (B) using Alert™ LFD kits. N.B. The killed zoospore suspensions were originally derived from the same dilution series as the untreated and are thus plotted on the same scale to give an idea of the relative quantities of dead inoculum present despite not any longer 'containing colony forming units' (cfu).

Discussion

Phytophthora and *Pythium* were found in reservoirs throughout the year. Baiting would thus need to be carried out whenever the water was going to be used. This project has shown that the water in an open reservoir can be expected to contain pathogens, and contamination probably at a lower level could still be expected from the environment if being filled from a "clean" source such as a borehole. Monitoring by baiting and/or water sampling should focus on the water drawn off for use. If it is being put through a pathogen treatment system the results will give growers knowledge of whether or not contamination is being removed from the water as there will be no visible indication of operation quality.

In the preliminary laboratory experiments, the discrepancy between the spore concentrations determined from haemocytometer spore counts with calculation for the dilution factor, and the results from standard water tests (filtration followed by culturing to produce colony forming unit counts) was not solved. It was possible that the zoospores encysted on the sides of the HDPE bottles used. Growers generally send samples for testing in drinking water bottles made of PET (T. Pettitt, pers. comm.) rather than HDPE bottles. Encystment will reduce the number of motile zoospores available to infest the bait material. It was noted that in the zoospore observation test the number of spores was close to that sought by dilution, however in the absence of bait material, most zoospores had encysted within 3 hours. It was also hypothesised that as the baits were unable to move in the water in the laboratory bottles that the zoospores were not flushed through the bags. Movement of the bait bags and the water circulation in reservoirs is likely to mean that the bait comes into contact with more zoospores than when placed in a fixed 1 L volume. This could mean that significant bait infestation arises in water samples with relatively low spores per litre sample.

On-site testing of an apple bait bag in February immediately on retrieval gave negative LFD results for *Pythium* and *Phytophthora* spp. which tallied with the Ceanothus leaf bait water testing in the laboratory, however a low Oomycete count and colonies likely to be *Pythium rostratum* were seen in the test plates. By September, the Oomycete colony count was 113, the on-site LFDs tests both positive and matched by *Pythium* and *Phytophthora* spp. presence in the plate test and also infestation of the leaf baits put into the water in the laboratory. Baits removed from the same reservoir in August which had a colony count of *Phytophthora* spp. of 20 cfu per litre at sampling were able to give a strong, positive, test line indication on the LFD following 48 hours immersion and testing within 24 hours. When this bait was deployed temperature in the water and in transit would have been relatively warm. Storing bait bags in the dark at around 20 °C for 24 hours after removal from reservoirs should be tried by growers

before using the LFDs to allow multiplication within the bait pieces, particularly on baits retrieved from cold water. Delaying the LFD test after retrieval by a day would still give a quicker result than laboratory plate tests of water samples.

Bait bags could be made that hold more than one type of plant material, but this project showed that apple gave good results for both *Pythium* and *Phytophthora* spp. in water. If bait bags are used in other locations e.g. soil and for other hosts other material could be investigated as given in reference tables (Erwin & Ribeiro, 1996). The amount of material in the bag is dictated by the LFD test as the manufacturer's instructions for direct sampling of infected plants is to take approximately 0.2g (equivalent to a piece of typical leaf tissue 25 mm square). It might be worth using baits containing both apple and carrot pieces as it seemed in the laboratory tests that carrot was infested more frequently than apple by *Pythium* sp., whereas *Phytophthora* was found more frequently on apple. *Pythium* spp. can be of particular importance during plant propagation. However, if the strength of the LFD test line depends on the number of bait pieces that are positive, then substitution by a bait type less favoured in general could reduce the chance of a positive indication. If a nursery specialises in a particular range of crops then checking that apple baits are the most suitable could be done by performing a "choice test" in the reservoir with an alternative bag containing visibly healthy, washed, host leaves and then testing each bag with the LFDs.

The reed bed was installed at Nursery "B" as a biological filter, a proven technique to clean up run-off containing nutrients and pesticides (Atwood, 2014) and with microbiological activity in the roots that can work in a similar way to a slow sand filter. However, there should ideally be a collection pond which then allows a slow passage of water through the reed bed as it was noted that when there is high rainwater run-off from the nursery, the flow is too great for any benefit from filtration by the reeds. If the reed bed was working efficiently samples from this point collected while water was flowing might have been expected to have lower colony forming units than a more distant sample point where there might have been more opportunity for multiplication of the microbes to have taken place in the standing water.

Live *Pythium* and *Phytophthora* species were shown by the colony counts to be drawn through the particle filter at the water abstraction point at Nursery "B". The nursery has not installed a slow sand filter because the water is only used for irrigation from March to October and a recirculating pump would need to be set to run outside of this period to keep the biofilm organisms oxygenated by water flow.

Slow sand filters are employed on a number of nurseries to remove pathogens from recycled water (Atwood, 2014). The nil colony counts for Oomycetes in water after the slow sand filter at Nursery “C” confirm the effectiveness. Further work is, however, being carried out the Eden Laboratories in 2014 to seek to determine why positive LFD tests for both *Pythium* and *Phytophthora* were obtained from tests of the apple baits which were incubated after retrieval from the filtered water. One explanation of this could be that the baits collected dead *Pythium* and *Phytophthora* spp. material which would still show up as a positive on the LFD (and any PCR testing that could be done). The pathogen material (dead or alive) would need to be able to pass through the slow sand filter.

Water tests for live Oomycetes are only offered by a few specialist plant pathology laboratories e.g. The Eden Project Laboratories, Food and Environment Research Agency (Fera) and Stockbridge Technology Centre (STC) in England. Growers in all sectors are increasingly using recycled water on their crops and need to continually monitor their water for plant pathogens to prevent infestation of their crops and growing areas. With the increasing displacement of traditional mycological skills in testing laboratories by molecular techniques (which cannot distinguish live from dead material) “DIY baiting” may provide an option for regular routine testing – identifying times when water is reasonably safe to use and when more intensive microbiological assessments would be justified.

Dead pathogen debris from all three disinfestation treatments attached to apple segment baits and gave false positive LFD tests throughout a wide range of original inoculum concentrations. Positive reactions were substantially less than for equivalent live inoculum at each concentration, but the intensity of reactions did increase with increasing inoculum concentration.

Conclusions

Although the laboratory bait infestations were not always successful, sufficient information was gained to achieve the objective to identify plant material baits which have the greatest sensitivity for zoospore detection. Nordmann Fir needles were least frequently infested (both by water-moulds and other fungi). Although Rhododendron and Ceanothus leaves were shown to trap both *P. cryptogea* and *Pythium* sp., the possibility of leaves carrying splash-borne *Phytophthora* and *Pythium* species meant that apple (which trapped more *Phytophthora* than *Pythium*) and carrot (which trapped more *Pythium* than *Phytophthora*) were selected for further testing. *Pythium* infestation was, however, seen across all bait types. Internal apple flesh, with eight pieces (7 mm x 7 mm) per bait bag was the final design. These bait bags were

utilised on two nurseries in early 2013 and gave catches of both *Pythium* and *Phytophthora*.

The sensitivity testing of the LFDs to a range of spore concentrations was not achieved in the laboratory, but in nursery reservoirs, positive detection was shown over a range of pathogen propagule concentrations e.g. 20 *Phytophthora* spp. colonies per litre of water gave a strong LFD Index 5 in August as did 26 *Pythium* spp. in November. The LFDs used with the reservoir baits were principally left to incubate and there was not a direct link to the concentration in the reservoir. However, baits tested immediately upon retrieval from the water were more likely to be negative. Although there were some matching trends (particularly with low Index readings before May when the cfu were also low), it is not likely that line strength can be consistently related to the concentration of the tested organisms and the LFD test lines can only be taken to indicate negative or positive. Instructions for nursery staff on bait construction and LFD use have been produced (Appendix 1) and were utilised by growers at Nursery “B” and “C”.

Only one apple bait bag was shown to be needed, floating between 30 mm and 250 mm, and position around the edge did not matter on the larger, open, reservoir. More oomycetes were present in May than earlier in the year, but reservoirs collecting run-off were shown to contain *Pythium* and *Phytophthora* species most of the time. Rather than baiting reservoirs it is probably more important to ensure that an effective treatment procedure is in place and that the water passing through it is monitored. The plant tissue bait/LFD combination has been shown to be a reasonably sensitive water testing procedure. Negative bait/LFD tests can give reasonable confidence of the water’s suitability for irrigation, although this proposition would still benefit from more in-depth study using a wide range of species and concentrations of pathogen inocula. Positive tests need to be treated with caution when testing water that has received disinfestation treatments given the possibility identified in the extension study, of baits collecting and detecting material from dead pathogen propagules. In these circumstances probably the best way to use bait/LFD tests is to identify times when it is advisable to send samples away for in-depth microbiological assessment. Also, guidance is needed on water testing approaches and philosophy, for example an isolated negative test result at the point of water delivery does not necessarily imply that the irrigation system is at low risk of spreading disease since holding tanks or alternative supplies may carry pathogen inoculum. Similarly, a negative test post water disinfestation treatment may not necessarily indicate that the treatment is working as the water being treated may already be free of pathogen propagules. However, if routine tests are carried out at key locations on a nursery, a good idea of the pattern and timing of disease risks from the water should be built up.

Knowledge and Technology Transfer

- Three nurseries have participated in this project, with two growers preparing and setting their own bait bags in their irrigation water. The third nursery took water samples, but oomycete infestation was too low for further investigations at the site. One grower also utilised the baits with LFDs. Instruction sheets have been prepared for the construction of bait bags on the nursery and for the use of LFDs to test baits.

- Information of zoospore behaviour and on water treatment was presented to AHDB Horticulture panel members at the oomycete workshops at Stoneleigh on 1 October 2012 by Erika Wedgwood and Tim Pettitt.

- Erika Wedgwood gave a presentation on the first year of the project to the AHDB Horticulture Herbaceous Perennials Technical Discussion Group on 10 July 2013. This was followed by a demonstration to growers at a nursery of how to make a bait bag with apple, bait deployment in a reservoir and the use of LFD kits for testing. The bait bag tested had been made and deployed by the host grower.

- A presentation on *Phytophthora* and *Pythium* root rots and the monitoring of irrigation water using baiting was given by Erika Wedgwood to growers at a BOPP meeting on 19 September 2013. This meeting resulted in ADAS using the baiting technique with three companies concerned about contamination leading to root rots. Positive detections were made following these enquiries for one propagator of ornamental plants, one producer of bark-mix growing-media and mulches, and a soft-fruit producer re-circulating run-off water for crop irrigation.

- Tim Pettit gave a presentation on water monitoring and treatment for this project at the South West Growers show on 2 October 2013.

- An article on HNS/PO 188 was printed in the AHDB Grower in 2013.

Glossary

Technical terms have been explained within the text.

References

Erwin, D.C. and Ribeiro, O.K. (1996). *Phytophthora* Diseases Worldwide. APS Press.

AHDB Horticulture Factsheet 16/04, (2004). Control of *Phytophthora, Pythium* and *Rhizoctonia* in container-grown hardy ornamentals. Horticultural Development Company.

Jennings, P. (2007) HNS 134: Detection and decontamination of *Phytophthora* spp., including those of statutory significance, from commercial HONS nurseries. Horticultural Development Company.

Lane, C.R., Hobden, E., Walker, L., Barton, V. C., Inman, A.J., Hughes, K.J.D., Swan, H., Colyer A. and Barker I. (2007). Evaluation of a rapid diagnostic field test kit for identification of *Phytophthora* species, including *P. ramorum* and *P. kernoviae* at the point of inspection. *Plant Pathology* **56**, 828-835.

Pettit, T.R., Wakeman, A.J, Wainwright, M.F. and White J.G. (2002). Comparison of serological, culture, and bait methods for detection of *Pythium* and *Phytophthora* zoospores in water. Plant Pathology **51**: 720-727.

Pettitt, T.R. & Pegg, G.F. (1991). The quantitative estimation of *Phytophthora cactorum* in infected strawberry tissue. *Mycological Research*, **95**, 233-238.

Singleton, L.L., Mihail, J. D. and Rush, C.M. (1993). Methods for research on soil-borne Phytopathogenic fungi. APS Press. 265pp.

Van der Plaats-Niterink, A. J. (1981). Monograph of the Genus *Pythium*. Studies in Mycology No. 21. Centraalbureau voor Schimmelcultures, Baarn. (Free online) http://www.cbs.knaw.nl/publications/1021/content_files/content.htm)

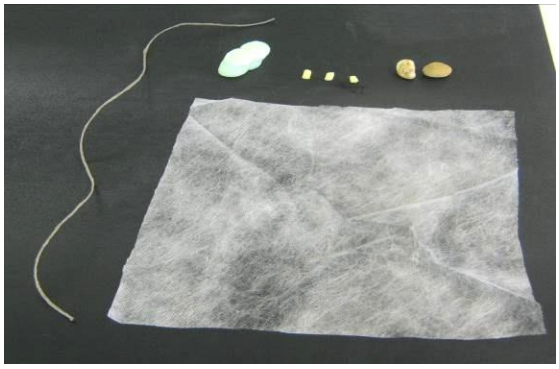
Waterhouse, G. M. (1970). The Genus *Phytophthora* de Bary. Diagnoses (or descriptions) and figures from the original papers. Second Edition. Commonwealth Mycological Institute.

Wakeham, A..J., Pettitt, T.R. & White, J.G. (1997). A novel method for detection of viable zoospores of *Pythium* in irrigation water. *Annals of Applied Biology*, **131**, 427-435.

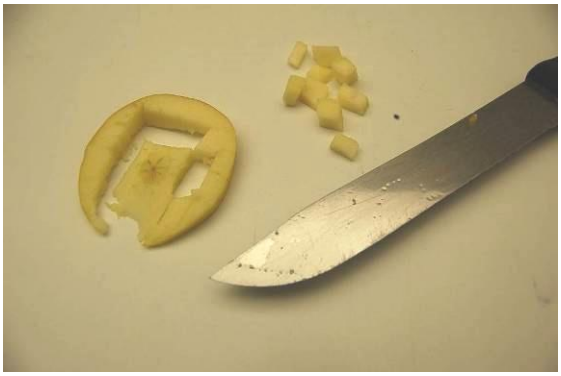
Wedgwood, E.F., (2011) HNS 185: Survey, detection and diagnosis of *Phytophthora* root rot and other causes of die-back in conifers. Horticultural Development Company.

Wedgwood, E.F., (2012) HNS 185: Understanding and managing crop protection through Integrated Crop Management. Horticultural Development Company.

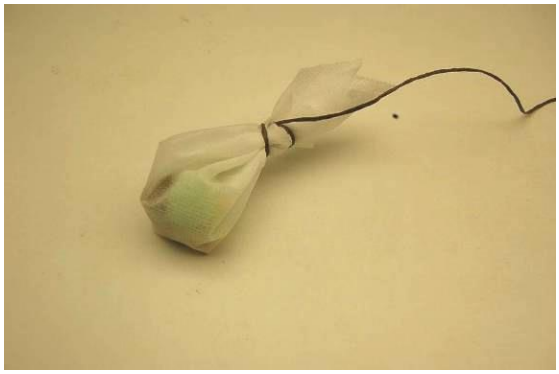
Appendix 1: *Pythium* & *Phytophthora* Water Baiting Instruction Sheet for Growers



1. Components needed for the bait bag; 7-10 g boiled stones, polystyrene, apple pieces, string, fleece (28 x 28 cm).



2. Cut a slice of Golden Delicious apple 7mm thick. Cut out eight squares approx 7 x 7 mm using a clean knife.



3. Place the apple pieces in the centre of the fleece with the stones and polystyrene. Tie up with the string to produce a loose bag.

4. Place the bait bag in the reservoir. Once the fleece is wetted the bag should float below the water. Tether the string at the baiting location for 48 hrs.

5. Untie the collected bag. With washed hands, place the apple pieces in the buffer bottle. Shake buffer bottle vigorously for at least 1 min until the buffer becomes coloured by the apple.

6. Draw up apple solution from the buffer bottle and pipette 2-3 drops into the well on the LFD test device. The C line should show blue. If a blue T line then appears within 10 mins the test is positive.

Appendix 1 contd.: Instructions for using Pocket Diagnostic Lateral Flow Device

Store test kits at room temperature (up to 40°C), not refrigerated or frozen.

Step 1; Plant material selection

- Undo or cut open the bait bag and find all eight apple pieces.
- Unless the pieces look soft then break up the apple pieces a little (handle with washed hands or knife) before adding to the buffer bottle, or add to the bottle (see below) and squash the apple a little with an item e.g. biro rinsed in washing up liquid.

Step 2; Extraction in buffer

- Unscrew the extraction bottle lid and add all the plant material pieces from one bag. Replace the lid tightly. One buffer bottle per bait bag will be used for both the *Pythium* and *Phytophthora* tests.
- Label the bottle with the sample identity if there is more than one sample.
- Shake the bottle firmly for 60 seconds so that the ball bearings break the plant cells apart. Shake until the extraction buffer is no longer colourless.
- The buffer should start to become green or brown as the tissue is broken down. If this does not happen the plant pieces may have been too big, or the shaking not vigorous enough.
- Grasping the bottle during shaking will normally warm it to above 10°C to enable the process to work.

Step 3; using the LFD

- If the test is being performed in conditions below 10°C then warm the packaged lateral flow device (LFD) before opening.
- Remove the test device from its foil packing just before it is needed. DO NOT TOUCH THE VIEWING WINDOW.
- Label the back of the device with the sample identification and date. One *Pythium* and one *Phytophthora* device will be needed per buffer bottle.
- Place on a level surface, or in the hand, with the viewing window upwards. Holding the device is recommended if the temperature is below 10°C.
- Allow the plant material a few seconds to settle in the extraction bottle.
- Remove the lid from the extraction bottle, tilt the bottle and draw some of the liquid into the clean pipette from above the apple bait material.
- Gently squeeze 2 large or 3 smaller drops of the sample liquid into the sample well of the test device (so the liquid is below the rim of the well). Aim to release the liquid without air bubbles as these can break the flow of the liquid across the device.
- After about 30 seconds pale blue dye will appear in the viewing window as liquid flows along the test device.
- If no pale blue dye becomes visible in the viewing window after 30 seconds, another drop of sample can be added to the sample well. Using too much liquid will flood the strip and will cause the test to run incorrectly.
- If the test still runs very slowly tap the device gently to remove any air bubbles.
- If too much debris has been added with the sample liquid the test will run slowly. It may be necessary to use a new device with clearer liquid from the extraction bottle.

Contd.

Instructions for using Pocket Diagnostic Lateral Flow Device contd.

Step 4: Examining the results

- A blue vertical line (the Control line) will appear next to the letter 'C' on the device. This line confirms the test is working properly.
- If the test is positive, a second blue line, the Test line (next to the letter 'T'), will appear. Even a faint line means the result is positive and so the test should be examined in good lighting.
- The lines can appear in *Pythium* and *Phytophthora* kits within 3 – 4 minutes of adding the sample to the device, but may take up to 10 minutes
- Read the result within 10 minutes of adding the sample to the device. Ignore any changes which happen after 10 minutes.
- Where comparison of the strength of the line between samples is being sought for research purposes the LFD should be placed against a similar coloured background and read under the same artificial light.
- After use, the test devices should be returned to the foil packet with the silica gel packet provided. They can be stored for long durations with only slight loss of results if kept dry and out of the light.

Step 5: Interpretation of the results

- A positive result indicates that the plant material sampled contains the fungus under test.
- Under some circumstances, laboratory confirmation of an on-site test result may be necessary.
- A negative result indicates that the target pathogen was not detected in the test sample. As with all diagnostic testing, a negative result does not confirm that the test location is free from the fungus under test.
- A faint or absent line may indicate a low concentration of the pathogen, uneven distribution in the host, or recent infection.

Problems with the readings

- Faint test lines are caused by either low pathogen concentration; uneven distribution; too small a sample; sample not broken up enough; or sample not shaken long enough. If in doubt, repeat with a new device using a fresh sample, or repeat in a few days.
- 'T' line visible, but no 'C' line may be due to a high level of pathogen in the sample, preventing the test from working properly. Dilute sample 1 in 10 and 1 in 100 with fresh buffer and retest with a new device.
- No 'T' line, no 'C' line can occur when too much sample material is added. Retest with a new device.

In LFDs the antibodies in the device are labelled with coloured latex and attach to the pathogen to indicate a positive test. The DNA of the tested pathogen is left on the paper test strip within the device. If the indicator window is flooded the latex can be displaced.

Appendix 2: *Pythium* sp. zoospore survival and encystment after three to five hours* in various types of water (Experiment 6) December 2012

6 December 2012 experiment

Block & Plot	Treatment	Zoospores	Encysted
A1	1	29	120
B2	1	25	60
C3	1	35	100
D4	1	32	150
E5	1	29	100
A2	2	28	200
B3	2	9	130
C4	2	20	180
D5	2	26	190
E1	2	21	170
A3	3	25	210
B1	3	7	110
C5	3	22	150
D2	3	25	200
E4	3	25	120
A4	4	24	160
B5	4	21	190
C1	4	24	195
D3	4	30	150
E2	4	23	150
A5	5	39	100
B4	5	20	200
C2	5	32	100
D1	5	20	175
E3	5	29	200

Treatment	Water type	Mean Zoospore count	Mean Encysted count	Total count
1	sterile rain water	30	106	136
2	distilled water	21	174	195
3	stood tap water	21	158	179
4	fresh tap water	25	174	199
5	'primed' fresh tap water	28	155	183

* counts took two hours progressing from cell A1 to E5 (with treatments 1 to 5 randomised in each block A to E)

Appendix 2 contd.: *Pythium* sp. zoospore survival and encystment after three to five hours* in various types of water (Experiment 6) December 2012

7 December 2012 experiment

Block & Plot	Treatment	Zoospores	Encysted
A1	1	7	450
B2	1	6	350
C3	1	11	300
D4	1	10	310
E5	1	10	300
A2	2	4	400
B3	2	5	300
C4	2	7	320
D5	2	6	450
E1	2	10	300
A3	3	8	300
B1	3	8	400
C5	3	7	310
D2	3	8	300
E4	3	9	310
A4	4	6	300
B5	4	7	300
C1	4	3	400
D3	4	8	310
E2	4	4	400
A5	5	9	320
B4	5	10	320
C2	5	8	300
D1	5	6	300
E3	5	8	320

Treatment	Water type	Mean Zoospore	Mean Encysted	Total count
1	sterile rain water	9	342	351
2	distilled water	6	354	360
3	stood tap water	8	324	332
4	fresh tap water	6	342	348
5	'Primed' fresh tap water	8	312	320

* counts took two hours progressing from cell A1 to E5 (with treatments 1 to 5 randomised in each block A to E)

Appendix 3

Sample 12/C219 collected 9/8/12, processed 10/8/12				
RESULTS OF TESTS ON SAMPLES COLLECTED FROM "NURSERY A" IN WEEK 32, 2012:				
Water sample	Sample 1 Reservoir	Sample 2 Lagoon	Sample 3 Pond	Sample 4 Ditch
Bacteria (cfu l^{-1}) on PDA	-	-	-	-
TOTAL FUNGUS(cfu l^{-1})	1000	2480	6080	4320
Trichoderma spp.	0	1040	880	90
Fusarium spp.	0	0	160	20
Tests for Pythium and Phytophthora				
Bait tests (% infected)	0 (3*)	0 (3*)	100**	100**
Plate tests (cfu l^{-1})	0 (10*)	0 (40*)	200**	450**
Immunodiagnostic tests (viable spores l^{-1})	-	-	-	-
Pythium colony seen	**-**	**-**	**+**	**+**
Phytophthora colony seen	**-**	**-**	**-**	**-**

See next page for key and comments

Sample 12/C219 collected 9/8/12, processed 10/8/12 contd.

* *Mortierella* sp. = not a plant pathogen, often seen in water samples – a group of fungi that grow on *Pythium/Phytophthora* selective media.

** A mixture of fast and slow-growing *Pythium* species (some plant pathogens) and *Saprolegnia* sp. (not a plant pathogen).

Comments by Tim Pettitt at the Eden Laboratory:

- Samples from the reservoir and lagoon look relatively clean.
- The high *Trichoderma* count from the lagoon was of some interest as some *Trichoderma* species can be effective biocontrol agents.
- Samples from the pond and the ditch contained relatively high oomycete counts, with large numbers of *Pythium* propagules present – as stated above, examples of these have been sub-cultured for full identification and possible use in the AHDB Horticulture baiting project if they turn out to be horticultural plant pathogens (and prolific zoospore producers).

Sample 12/C222 collected 21/8/12, processed 22/8/12				
RESULTS OF TESTS ON SAMPLES COLLECTED FROM "NURSERY B" open reservoirs and run-off IN WEEK 34, 2012:				
Water sample	1st site Reservoir.	1st site Runoff	2nd site Reservoir.	2nd site Runoff
Bacteria (cfu l^{-1}) on PDA	1.79×10^4	7.34×10^4	4.78×10^4	1.92×10^5
TOTAL FUNGUS(cfu l^{-1})	3627	4907	1013	6800
Trichoderma spp.	14	173	0	550
Fusarium spp.	133	0	0	50
Tests for Pythium and Phytophthora				
Bait tests (% infected)	80*	70*	0 (20**)	90*
Plate tests (cfu l^{-1})	80*	113*	0	900*
Immunodiagnostic tests (viable spores l^{-1})	-	-	-	-
Pythium colony seen	**+**	**+**	**-**	**+**
Phytophthora colony seen	**-**	**-**	**-**	**-**

See next page for key and comments

Sample 12/C222 collected 21/8/12, processed 22/8/12 contd.

* A mixture of fast and slow-growing *Pythium* species (some possible plant pathogens) and *Saprolegnia* sp. (not a plant pathogen).

** *Saprolegnia* sp. only.

Comments by Tim Pettitt at the Eden Laboratory :

- Isolates of fast-growing *Pythium* sp. were taken from 1st site Reservoir, 1st site Runoff and 2nd site Reservoir Runoff samples for identification to species.
- The 2nd site Reservoir sample looks comparatively clean but the 1st site Reservoir sample has a high concentration of both oomycetes (some possible pathogens) and *Fusarium* spp. (a mixture of members of the '*Roseum*' group {most frequently pathogens of cereals} and *F. oxysporum* a potential pathogen to a wide range of plant species).
- The 2nd site Runoff sample also contained a small quantity of compost and root debris – some of this was plated out onto selective agar and fast-growing *Pythium* spp. were isolated and sub-cultured for identification to species.

Water test results for "Nursery B" open reservoir (1st sampled in August) and closed tank at "Nursery C", Jan/Feb and Apr 2013									
Sample date	29/01/13		27/2/13			27/2/13	12/4/13		10/4/13
Sample no.	C241		C244			C245	C252		C253
Water sample	B open reservoir		B open reservoir			C tank	B open reservoir		C tank
	R1	R2	Filter	R1 reed	R2 outfall	Res	R1 reeds	R2	Pre SSF
TOTAL FUNGUS(cfu l^{-1})	760	910	1520	1780	1680	1973	800	1760	25
Trichoderma spp.	20	60	600	560	720	0	0	0	0
Fusarium spp.	0	40	0	20	480	266	0	0	0
Tests for Pythium and Phytophthora									
Bait tests (% infected)	100	80	0	0	0	10	10	50	0
Plate tests (cfu l^{-1})	70*	100**	110***	30†	20††	33†††	150‡	140‡	0‡‡
Pythium colony seen	+	+	+	+	+	+	+	+	-
Phytophthora colony seen	-	+	-	-	-	-	+	+	-

See next page for details of colonies

Water test results for "Nursery B" open reservoir and closed tank at "Nursery C", Jan/Feb and Apr 2013 contd.

*=*Pythium rostratum*
**= mixture of spp. including *P. rostratum,* a fast growing *Pythium* sp. (looks like *P. sylvaticum*), approx. 20 cfu l^{-1} *Phytophthora* sp., and a *Saprolegnia* sp.
***= mixture of spp. including *P. rostratum,* a fast growing *Pythium* sp. (looks like *P. sylvaticum*), and a *Saprolegnia* sp.
†= *P. rostratum*?? Plus *Saprolegnia* sp.
†† = fast-growing *Pythium* sp. (not identified)
††† = fast-growing *Pythium* sp. (not identified), plus (predominantly) *Saprolegnia* sp.
‡ = Mixture of species including members of Saprolegniacea, *Pythium* spp. and one possible Phytophthora sp.
‡‡ = I think this sample is post SSF as it is very clean!

Sub-culturing data
Baits:
C252/R1 *Pythium* (1); C252/R2 *Phytophthora* (2), *Pythium* (1).
Plates:
C252/R1a *Pythium*; C252/R1b *Pythium*; C252/R2a *Phytophthora*; C252/R2b Lost; C252/R2c *Mortierella*.

Water test results for "Nursery B" open reservoir and "Nursery C" closed tank and after slow sand filter, May 2013												
Sample date	14/5/13		13/5/13					15/5/13				
Sample no.	C256		C257					C258				
Water sample	C tank		Nursery B open reservoir					Nursery B open reservoir				
	SSF	Res (raw)	R1	R2	R3	R4	R5	R1	R2	R3	R4	R5
TOTAL FUNGUS(cfu l^{-1})	93	600	7680	8960	2400	1920	2240	5120	5760	6720	10560	6080
Trichoderma spp.	0	0	80	20	300	0	160	180	80	140	200	40
Fusarium spp.	0	0	0	1120	20	40	0	0	300	60	60	100
Tests for Pythium and Phytophthora												
Bait tests (% infected)	0	20*	0	90*	70*	0	10*	20*	10*†	0	0	0
Plate tests (cfu l^{-1})	0	340*	3360*	650*†	780*†	140*	100*	540*	1000*	1210*	1110*	790*
Pythium colony seen	-	+	+	+	+	+	+	+	+	+	+	+
Phytophthora colony seen	-	+	-	+	+	+	-	-	+	+	+	-

†= Some colonies look strongly like *Phytophthora* sp. *= Sub-cultured for further identification, see next page

Water test results for "Nursery B" open reservoir and "Nursery C" closed tank and after slow sand filter, May 2013 contd.

Comments by Tim Pettitt at the Eden Laboratory
Samples C257 and C258 contained very high numbers of filamentous fungal propagules and gave some of the highest oomycete plate test counts I've seen for some time – I would not recommend using this water for irrigation without treatment first.

Sub-culturing data
Baits:
C256/RAW *Pythium* (2).
C257/R2 *Pythium* all (10); C257/R3 *Pythium* (10), *Phytophthora* (2); C257/R5 *Pythium* (1).
C258/R1 *Pythium* (2); C258/R2 *Phytophthora* ? *cryptogea*? (1), *Pythium* (1 same bait!).

Plates: .
C256/RAWa *Phytophthora*; C256/RAWb *Pythium*; C256/RAWc *Saprolegnia*; C256/RAWd *Pythium* (looks like HS group).
C257/R1a *Pythium*; C257//R1b Lost; C257/R2a *Phytophthora*; C257/R2b *Pythium*; C257/R3a *Phytophthora*; C257/R3b *Phytophthora*; C257/R3c *Pythium*. C257/R4a Lost; C257/R4b *Pythium*; C257/R4c *Pythium*; C257/R4d *Phytophthora*; C257/R5a *Pythium*; C257/R5b *Saprolegnia*.

C258/R1a *Pythium*; C258/R1b *Pythium;* C258/R2a *Pythium*; C258/R2b *Pythium*; C258/R2c *Phytophthora*; C258/R2d Lost; C258/R3a *Phytophthora*; C258/R3b *Phytophthora*; C258/R3c *Pythium*; C258/R3d *Pythium*; C258/R3e *Pythium*; C258/R4a *Pythium*; C258/R4b *Pythium*; C258/R4c *Phytophthora*; C258/R4d *Mortierella*; C258/R4e *Pythium*; C258/R5a Lost; C258/R5b *Pythium*; C258/R5c *Pythium & Saprolegnia!*; C258/R5d *Pythium*; C258/R5e *Saprolegnia*

Water test results for Nursery B and Nursery C, July 2013					
Sample date	30/7/13		25/7/13		
Sample no.	C263		C262		
Water sample	Nursery C		Nursery B		
	SSF	Res (raw)	R1	R2	F
TOTAL FUNGUS(cfu l^{-1})	560	4240	15147	8107	64853
Trichoderma spp.	0	0	107	0	66
Fusarium spp.	0	26	66	53	0
Tests for Pythium and Phytophthora					
Bait tests (% infected)	0	100	100	60	50
Plate tests (cfu l^{-1})	0	247	720 (†293)	167	506 (†107)
Pythium colony seen	_	not reported	not reported	not reported	not reported
Phytophthora colony seen	_	not reported	**+**	not reported	**+**

†= Some colonies look strongly like *Phytophthora* sp. – numbers of cfu = in brackets under the total oomycete cfu count

Water test results for Nursery B and Nursery C, August 2013									
Sample date		21/8/2013					14/8/2013		
Sample no.		C266					C265		
Water sample		Nursery B					Nursery C		
		2nd Reservoir after Filter	2nd Reservoir	1st Reservoir after Filter	1st Reservoir R1	Bait left-overs	Reservoir	SSF	SSF (3 litre sample)
TOTAL FUNGUS(cfu l^{-1})		800	1493	1120	2507	not done	800	960	1547
Trichoderma spp.		0	0	0	533	not done	0	0	0
Fusarium spp.		0	53	0	0	not done	0	0	0
Tests for Pythium and Phytophthora									
Bait tests (counts of infested apple bait pieces from reservoir bags)	Total oomycetes	8/8	8/8	0/8	8/8	4/4	8/8	3/8*	
	Phytophthora†	4/8	4/8	0/8	6/8	4/4	0/8	0/8	
	Pythium	6/8	6/8	0/8	8/8	0/4	6/8	0/8	
Plate tests (cfu l^{-1})	Total oomycetes	0	213**	0	55	not done	253**	13*	34*
	Phytophthora†	0	60	0	20	not done	0	0	
LFD tests (duplicate bait bags)	*Phytophthora*	0	1	2	5	1-2	0	1	
	Pythium	<1	1	0	1	0	1-2	0	

See next page for key and comments

Water test results for Nursery B and Nursery C, August 2013 contd.

* = *Saprolegnia* sp. – did not give +ve LFD tests for either *Phytophthora* or *Pythium*

** = Large numbers of *Saprolegnia* and fast-growing *Pythium* sp.

† = Colonies look strongly like *Phytophthora* sp.

Comments by Tim Pettitt at the Eden Laboratory

- Generally results of plating and LFD tests appear to tie up.
- Bait left-overs from Nursery B contained *Phytophthora* and this may explain the positive for 1st Reservoir filter sample which was clear with plates
- Nursery B 2nd Reservoir filter water also contained *Phytophthora* on the baits but this didn't give a positive LFD test and again plates were clear.
- Also there was a repeat of the positive LFD test on SSF-treated water from Nursery C.
- Nursery C plates showed presence of a *Saprolegnia*-like species – this did not give a positive reaction with *Phytophthora* LFD test.
- No *Phytophthora* was isolated from Nursery C baits.

Water test results for Nursery B and Nursery C, September 2013					
Sample date	18/9/13		12/9/13		
Sample no.	C268		C267		
Water sample	Nursery C		Nursery B		
	SSF	Res (raw)	R1	R2	After Filter
TOTAL FUNGUS(cfu l^{-1})	52	2240	1760	6080	1280
Trichoderma spp.	0	93	40	27	0
Fusarium spp.	0	160	0	67	0
Tests for Pythium and Phytophthora					
Bait tests (% infected)	0	100	100	100	0
Plate tests (cfu l^{-1})					
Phytophthora	0	53?	32?	6?	0
Pythium	0	33	13	47	0
Total oomycetes	0	213	113	60	0

Comments by Tim Pettitt at the Eden Laboratory
Unused apple baits from Nursery C were plated out and none out of 13 tested contained any oomycetes

Water test results for Nursery B and Nursery C, November 2013						
Sample date		26/11/13		15/11/13		
Sample no.		C274		C273		
Water sample		Nursery C		Nursery B		
		SSF	Res (raw)	R1	R2	F
TOTAL FUNGUS(cfu l^{-1})		16426**	11307***	75093**	17493***	26880*
Trichoderma spp.		0	0	0	720	0
Fusarium spp.		0	0	0	427	0
Tests for Pythium and Phytophthora						
Bait tests (% infected)		0	100	100	100	0
Plate tests (cfu l^{-1})	*Phytophthora*	0	120?	47	147	0
	Pythium	0	26	33	40	0
	Total oomycetes	0	146	200	453	0

*=One species, **=Not diverse (< 5 species), ***= Diverse (> 10 species).

Comments by Tim Pettitt at the Eden Laboratory

- All samples contained very high numbers of filamentous fungal propagules (mostly *Penicillium* spp.) – samples R1, F and SSF contained very few species, whilst samples R2 and Raw were very diverse.
- Nursery C reservoir contained a large number of *Phytophthora*-like cfu (unusual for this site) – these have been sub-cultured for identification.

Appendix 4. Water temperature records over 48 hours from floating loggers in reservoirs at Nursery "B" & Nursery "C" during apple bait bag deployments.

January to November 2013.