Final Report

Sampling strategies for sensitive, accurate cost-effective detection of grape phylloxera for quantifying area freedom status

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AUTHORS

Vinehealth Australia: Inca Pearce, Suzanne McLoughlin, Brendan Tully

SARDI: Kathy Ophel- Keller, Alan McKay, Danièle Giblot-Ducray, Barbara Hall

Rho Environmetrics: Ray Correll

University of Adelaide: Cassandra Collins

Primary Industries and Regions SA: Geoff Raven

Department of Economic Development, Jobs, Transport and Resources: Greg King

Department of Primary Industries NSW: Chris Anderson

Phylloxera adults, nymphs and eggs (Agriculture Victoria, Rutherglen)

Project Leader contact details

Name: Mrs Inca Pearce
Address: Vinehealth Australia, Level 1, Industry Offices, National Wine Centre, Botanic Road, Adelaide SA 5000
P: 08 8273 0550
E: inca@vinehealth.com.au

PBCRC contact details

Plant Biosecurity Cooperative Research Centre
LPO Box 5012
Bruce ACT 2617

P: +61 (0)2 6201 2882
F: +61 (0)2 6201 5067
E: info@pbcrc.com.au
www.pbcrc.com.au

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1. Executive summary

Of the pests and diseases already in Australia, our greatest biosecurity threat to grapevines is grape phylloxera. This tiny insect destroys vines by feeding on their roots and once infested vines will die within 5-6 years and must be replanted with vines on resistant or tolerant rootstock. There is no treatment for phylloxera. Currently, phylloxera is confined to regions in Victoria and New South Wales, while South Australia, Western Australia, Northern Territory and Tasmania are recognised to be free of phylloxera.

The Australian wine industry contributes $40.2 billion in gross output to the Australian economy each year, and we have some of the oldest vines in the world at 175 years old. With estimates that at least 70% of Australia's winegrape vineyards are planted on own roots, and thus susceptible to phylloxera attack, continued work on improving phylloxera detection methods is vital.

Growers are going through vineyards every week. They are our eyes and ears. Active surveillance for grape phylloxera is what we must prioritise, and that requires practical and effective tools for growers and regulators alike. Traditionally, surveillance for phylloxera is undertaken by a third party, often regulators and not the growers themselves. The current endorsed phylloxera detection method, requires a small portion of roots to be dug up and inspected by trained personnel. This method is time consuming, expensive, and heavily relies on the capabilities of the inspector. Therefore, a simple, rapid, sensitive and accurate method for the detection of phylloxera is needed to enhance grower uptake and to simplify surveillance activities for regulators. This will improve the chance of detecting where phylloxera is and is not, and thus enable protection of vineyards from phylloxera. Additionally, the development of a test that enables rapid collection and analysis of samples will greatly assist not only general surveillance activities, but also management in the event of an incursion.

This project has successfully developed a field sampling protocol for collecting soil cores and validated a diagnostic protocol using qPCR for the detection and quantification of phylloxera ('DNA method'). The estimated detection limit was determined to be 2 phylloxera per 200g dry soil composite sample.

The field sampling protocol developed in this project utilises an inexpensive soil corer to collect soil samples within 10cm of the vine trunk and to a depth of 10cm. Compositing of soil cores was shown as an option to reduce laboratory analysis costs for the DNA method, however, the level of compositing needs to be balanced against potential diminished phylloxera detection capability. This is particularly important in blocks with isolated infestations or low infestation levels.

The way in which soil samples collected in the field must be packaged and transported to SARDI MDC and importantly, timings to ensure minimal DNA degradation during both transport and processing at the laboratory, were confirmed. Specifically, samples must be stored at no more than 20°C during transport and reach the laboratory within 48 hours of collection. On receival at the laboratory, samples must be stored unfrozen at approximately 10-20°C pre-drying and dried within approximately 24-48 hours of receival.

Detection rate of phylloxera was evaluated over seasons. For the three vineyards sampled over three consecutive years, there were no significant difference in the frequency of phylloxera detection based on time of year sampled. However, higher amounts of phylloxera DNA were found from late summer to early winter, peaking in autumn. As a result, the DNA method can be successfully undertaken all year, but preferentially in autumn.

Preliminary investigations demonstrated the potential for the existing SARDI MDC *Vitis* qPCR assay, which provides an estimation of the amount of vine root in a soil sample, to be used as an internal quality control for samples analysed for phylloxera DNA.

The current field sampling density outlined in the NPMP of sampling one vine in every 3rd row in every 5th panel has been shown to be suitable for the DNA method. However, higher sampling density above the standard recommendation may be warranted for any blocks suspected to have a low or isolated phylloxera infestation. In addition, continued evaluation of weak vines as part of any phylloxera surveillance strategy is recommended.

A comparison of the newly developed DNA method to the other primary phylloxera detection methods, emergence traps and visual root inspection, showed differences in detection rates at landscape, block, composite and vine level. Reasons for these differences and the relative strengths and weaknesses of each mehod are discussed. Endorsement of the DNA method alongside the emergence trap and visual root inspection phylloxera detection methods, will provide growers and regulators with an integrated toolkit of field sampling and detection options to utilise as part of national surveillance plans. This will enable greater confidence in area freedom status, delimiting of incursions and upgrading phylloxera management zone status from a PIZ or PRZ to a PEZ.

It is therefore recommended that the DNA method be endorsed by the Plant Health Committee for inclusion in the NPMP as a primary phylloxera detection method.

This project has delivered a significant advancement in the ability of industry and regulators to detect phylloxera, and therefore manage the impact of this devastating insect on vines. Full deployment of the DNA method will be achieved following endorsement by the national Plant Health Committee.

2. Introduction

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch) is a destructive pest of grapevine (*Vitis* spp.) worldwide. The insect can feed on the roots and/or leaves of most *Vitis* species, but on highly susceptible *Vitis vinifera*, this can have a severe impact on vine health and production (Benheim et al., 2012). Phylloxera is native to North America, but its economic importance became evident in the 1860s, when its introduction to the European grape growing areas devastated the industry (Corrie et al., 2002). In Australia, phylloxera was first seen in 1875 at Fyansford, near Geelong, in December 1875 but was not positively identified until November 1877 (Boehm, 1996). In response, quarantine zones were rapidly defined to help prevent spread and assist with eradication attempts (Herbert et al., 2008). Currently, phylloxera is confined to the declared Phylloxera Infested Zones (PIZs) in Victoria (North East, Maroondah, Nagambie, Mooroopna, Upton and Whitebridge) and New South Wales (Sydney region and Albury/Corowa).

Management of phylloxera worldwide relies essentially on the use of resistant or tolerant rootstocks derived from native American *Vitis* (Corrie et al., 2002). In South Australia, 74% of commercial winegrape plantings are highly susceptible ungrafted *V. vinifera (*Vinehealth Australia, 2016*)*, and management relies mainly on prevention through quarantine regulations and farm-gate hygiene practices. The proportion of commercial vineyard sin Australia panted to ungrafted material is likely to mirror that of South Australia. To assist with management of phylloxera, in 2009, the Australian National Vine Health Steering Committee documented Australian geographical areas into three types of zones:

1. Phylloxera Infested Zones (PIZs), where phylloxera is known to occur;
2. Phylloxera Exclusion Zones (PEZs), where current testing and historic information provide no evidence of phylloxera infestation; and
3. Phylloxera Risk Zones (PRZs), for which no evidence for phylloxera presence/absence is available.

All Australian grape growing areas are classified within these zones and movement of material between zones is subject to strict quarantine regulations (National Vine Health Steering Committee, 2009).

At present, phylloxera is confined to regions in Victoria and New South Wales (Figure 1). Containing phylloxera within those regions is therefore vital. This containment involves knowledge of where phylloxera is and is not and having a robust surveillance system to determine the limits of these areas and to monitor spread.

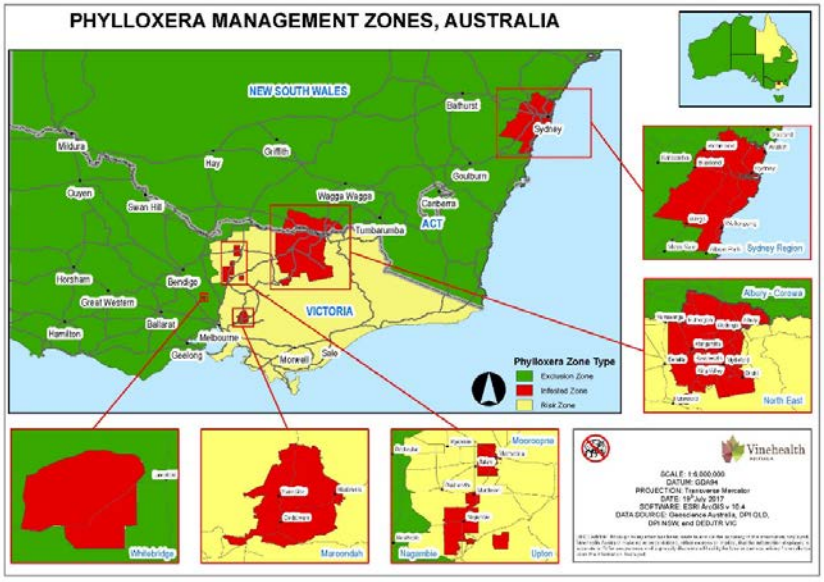

Figure 1. Phylloxera Management Zones map as at 19th July 2017.

Detection is a key component of any management strategy and is of critical importance for the success of surveillance and containment efforts. Typically, phylloxera abundance varies across an infested vineyard; foliar symptoms that can assist in identifying potentially infested areas vary in severity with grapevine pedigree, can even be non-existent in the early years of infestation (Herbert *et al*., 2008, Bruce *et al*., 2011, Benheim *et al*., 2012) and can vary with seasonal climatic conditions. Phylloxera infestations are also characterised by uneven abundance and distribution of insects throughout infested vineyards (Herbert *et al*., 2008, Powell, 2012b). Finally, phylloxera is known for its high genetic diversity, and in Australia alone, at least 83 genotypes have been characterised (Benheim *et al*., 2012). Therefore, detection of phylloxera relies on methods with high sensitivity to give the best possible chance of identifying infestations and limiting further spread. In addition, capability of high throughput to assess large numbers of samples collected across the surveyed area and to account for uneven distribution is of vital importance. Methods that allow for analysis of composite samples without compromising sensitivity have the potential to reduce survey costs. Detection methods also need to be specific for phylloxera while accounting for its genetic diversity and allowing for detection of all genotypes.

A range of detection methods have been developed for phylloxera, including aerial survey, ground survey, emergence traps and molecular techniques (Benheim *et al*., 2012). In Australia, ground survey, in which vine roots are exposed and examined using a magnifying glass (known herein as 'visual root inspection') is the only endorsed method for phylloxera testing (National Vine Health Steering Committee, 2009). A combination of aerial survey, to identify areas of low vigour, followed by a targeted ground survey is often used (Herbert *et al*., 2008). Ground surveys, however, are time consuming, require taxonomic expertise and can fail to detect infested vines (Herbert *et al*., 2008). The emergence trap method, which uses inverted bucket traps, is the most common alternative to ground surveys (Herbert *et al*., 2008), especially undertaken by researchers. Although the emergence trap method has shown greater sensitivity than the ground survey, its efficacy is season-dependent and, like the ground survey method, is time consuming and requires taxonomic expertise in the laboratory. Molecular detection relies on a quantitative PCR (qPCR) assay specific to phylloxera (Herbert *et al*., 2008). Compared with the ground survey and emergence trap methods, qPCR is faster and can detect phylloxera at any life stage (Herbert *et al*. 2008). It has the additional benefits of being quantitative and particularly suitable for automation and high throughput testing (Ophel-Keller *et al*., 2008). Moreover, in previous studies its sensitivity is slightly better than that of the ground survey method and like that of the emergence trap method (Herbert *et al*., 2008, Bruce *et al*., 2011, Powell, 2012a). A potential additional bonus is that the same extracted DNA sample can be used to monitor other soil microbes.

While qPCR has some advantages in terms of sensitivity and throughput compared with more traditional detection methods, its application for detection of soil-borne pests and pathogens in soil requires the development of dedicated protocols for sample collection and handling. Soil moisture is one critical factor to be considered. When assessing methods for detection of root lesion nematode in soil, Hollaway *et al*. (2003) found that in dry soil, DNA did not degrade even though nematodes may have been damaged during sampling, which translated into more consistent quantification by qPCR than by the alternative Whitehead tray method based on sieving soil and recording nematode numbers per gram of soil. Herdina *et al*. (2004a, b) also found that DNA from dead organisms degraded rapidly in moist soil and became undetectable by qPCR in 4–8 days for fungal mycelia DNA and 14 days for nematode DNA. When evaluating sampling strategies for optimal detection of soil-borne pathogens, Heap and McKay (2004) reported that the use of composite samples helped account for the uneven distribution of some pathogens across paddocks and reduced variation in the amount of pathogen DNA per sample compared with single core samples. Based on these reports, the SARDI Molecular Diagnostics Centre (MDC) developed a sampling strategy for detection of soil-borne pathogens by qPCR in soil, which involves collection of composite samples of multiple soil cores and recommends drying samples as soon as possible after collection to help preserve DNA (Ophel-Keller *et al*., 2008).

Traditionally, sampling for surveillance is undertaken by a third party, often regulators and not the growers themselves. The current methods, whilst having proven successful, are expensive and time consuming.

The results of this project will be used to develop a simple, accurate and affordable sampling method that vineyard owners and regulators can use for block level phylloxera surveillance. In addition, suggestions will be put forward for surveillance methodology at a larger than block scale. Findings from this study will contribute to improving protocols for the management of phylloxera, and will in time be reflected in the NPMP.

This project contains some information already reported as part of a smaller project funded by Wine Australia entitled 'Sampling strategies for sensitive, accurate and cost-effective detections of phylloxera for quantifying area freedom status (#PGI1201)'.

3. Aims

The project aims to develop a simple field sampling protocol for the collection of soil cores that facilitates cost-effective, sensitive and accurate detection of grape phylloxera DNA by qPCR. This protocol will be developed with information obtained from the following trials:

1. The effect on phylloxera detection and amount of:
    - Temperature and duration on soil sample storage;
    - Compositing of soil samples;
    - Position, depth, location in a vineyard for soil sampling;
    - Timing of sample collection within and across years; and
    - Sampling density.

2. Comparison of the three primary phylloxera detection methods: DNA, emergence trap and visual root inspection detections methods.

4. Research report

4.1. General materials and methods

4.1.1 Vineyard details for field sampling methodology

Unless otherwise specified, trials undertaken to develop the field sampling methodology for soil sample collection and handling were conducted on three different Victorian vineyards infested with phylloxera (Table 1).

Table 1. Phylloxera infested vineyards used in the soil sampling methodology trials.

Vineyard (also location of vineyard)	Soil type*	Variety	Rootstock	Planted	Phylloxera detection year
Yarra Valley	Restricted duplex soil with hard topsoil (sandy loam to clay loam over clay)	Chardonnay	Own roots	2000	2008
King Valley	Deep well-structured soil (sandy loam to loam topsoil grading to a well-structured subsoil)	Chardonnay	Own roots/ Schwarzmann	1987	1992
Rutherglen	Non-restrictive duplex soil with hard topsoil (hard setting sandy loam to clay loam over well -structured clay subsoil)	Shiraz	Own roots	1975	2013

*Generic soil type descriptions provided only (Dry and Coombe, 2004).

The three vineyards varied in soil type and phylloxera genotype. Phylloxera genotype G1 is predominant in the Yarra Valley and G4 in the King Valley (Powell, 2012b), whereas a mixture of genotypes has been reported in Rutherglen (Powell *et al.*, 2003). Genotyping was not however undertaken for any vineyard blocks included in this study as a verification step.

In the Yarra Valley and Rutherglen vineyards, vines were inspected by the visual root inspection method before the first sampling, and infested grapevines flagged for future use. In the King Valley, vines were initially selected based on their vigour, and then the visual root inspection method conducted post the first sampling.

4.1.2. Sampling procedure

Unless otherwise specified, sampling was undertaken using the current surveillance protocol (NVHSC, 2009), refereed to as the "standard sampling method" where a single vine in every 5th panel in every 3rd row is tested. This frequency equates to approximately 40 vines per hectare with the standard 2.75m row spacing x 1.8m vine spacing. At this sampling density, visual sighting of every vine in the vineyard is expected.

4.1.3. Visual root inspection

Also known as the "dig" method, visual root inspection involves digging under the target vines within 20cm of the trunk or near irrigation drippers to expose the actively growing feeder roots. A piece of root mass containing fibrous roots at least one half of a hand's width wide (or 10cm long) was inspected for root galls and individual insects, using a 10x magnification hand lens. This method is generally conducted in late spring and summer. Refer Appendix 1 for full method.

4.1.4. Emergence traps

Emergence traps (Powell *et al.,* 2000, Herbert *et al.*, 2006) consist of translucent buckets 220 mm diameter and 130 mm depth. The open end is placed onto the flattened soil surface 100 mm from the base of the vine and secured with tent pegs to create an airlock to trap moisture. On emergence from the soil, phylloxera dispersive stages (first instar 'crawlers' and alate or winged adults) are trapped in condensation on the container sides and collected into vials with 70% alcohol. The samples were examined and phylloxera counts recorded at the Agriculture Victoria, Rutherglen laboratories using a low powered microscope (Herbert *et al.*, 2006). Refer Appendix 2 for full method.

4.1.5. Soil sampling for DNA

Soil samples were collected initially using a 15mm diameter dig stick soil corer (Spurr Soil Probes, Adelaide, SA, Australia), which was capable of sampling to depths of 30cm. Once the 10cm sample depth was determined, all soil samples (unless otherwise specified) were collected using a 14mm diameter, 100mm AccuCoreB soil corer (SARDI). All samples collected during this study for DNA testing were triple-bagged, transported into South Australia under quarantine permits and analysed in SARDI MDC. Unless otherwise indicated, soil samples were dried overnight in a dehydration oven at 40°C immediately upon arrival at the laboratory. Sample mass was recorded before and after drying to estimate moisture content.

4.1.6. DNA extraction and quantification

DNA was extracted using the commercial DNA extraction service operated by SARDI, Adelaide (Ophel-Keller *et al*., 2008). The efficiency and consistency of SARDI's method to extract DNA from soil has been shown previously (Haling *et al*., 2011).

Phylloxera DNA was quantified by qPCR using the assay developed by Herbert *et al*. (2008), with forward primer 5'-AATCCGCAGGTTATACGAACATC, reverse primer 5'-CGGTCTCGTCAAATTTCGGA and TaqMan MGB probe 6FAM-CGACCCTCAGACAGG (Thermofisher, Waltham, MA, USA). This assay was previously shown to detect the main Australian phylloxera genotypes (Herbert *et al*., 2008). For further validation, the assay was tested against nine French and 16 American phylloxera genotypes collected between 2010 and 2012 [courtesy of Daciana Papura and François Delmotte, INRA Bordeaux, France]. The results confirmed that a wide range of phylloxera genotypes can be detected, irrespective of their origin, however the genotype is not identified. The DNA extracted from soil samples was diluted 1/5 prior to PCR. All PCR were performed on a ViiA7 real-time PCR system (Applied Biosystems, Foster City, CA, USA), in 10 µL volume containing 4 µL DNA, 200 nmol/L TaqMan probe and 400 nmol/L each primer in 1x Quantitect Probe PCR master mix (Qiagen, Hilden, Germany.) and with the following cycling conditions: 15 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each PCR plate included negative controls as well as calibration standards to calculate the amount of phylloxera DNA/g sample and corresponding detection rates. Results were also converted to equivalent phylloxera number per 200g dry soil (hereafter referred to as phylloxera equivalent) using a calibration derived from Herbert *et al*. (2008).

The amount of phylloxera present was described both qualitatively and quantitatively. The qualitative description of phylloxera was expressed on a detected or non-detected basis as a percentage of samples where some phylloxera was recorded. This detection percentage is not analogous to the accuracy of the qPCR test itself. Due to the ability of the qPCR to accurately detect phylloxera down to a trace level, a low detection rate was less likely due to failure of the qPCR test to detect the phylloxera when present, but rather, absence of phylloxera in the soil sample.

The quantitative amount of phylloxera in a soil sample, tested by qPCR, was typically expressed as phylloxera equivalents per 200g of dry soil. This test quantifies the total phylloxera DNA present, both from whole or partial, alive or dead phylloxera (if not yet degraded), of all growth stages, including eggs.

4.1.7. Statistical analysis

Data were analysed using R software. Specific analysis details are included in the methods for each experiment.

4.2. Effect of temperature and duration of soil sample storage on phylloxera detection

For optimal preservation of DNA, soil samples should be dried as soon as possible after collection, but this is not always practicable. Experiments were undertaken to determine the effect of temperature on the rate of phylloxera DNA degradation in soil samples while in storage between collection and drying. By measuring the rate of decomposition of DNA, the parameters for suitable transport and storage can be established to optimise sample integrity. These trials compared the effect of core types and temperature on the rate of decomposition of phylloxera DNA, including the effect of freezing.

4.2.1. Materials and methods

Samples collected as outlined below were processed for DNA extraction and phylloxera detection by qPCR, as described in 4.1. The results were used to estimate the rate of phylloxera DNA degradation in soil samples.

It was expected that DNA degradation would follow a first order equation:

$$DNA_t = DNA_0 \times \exp(-kt) + error_t \quad (1)$$

where DNA_t is the amount of DNA remaining at time t, and t is the elapsed time between sample receival at the laboratory and drying. Equation 1 can be fitted after taking logarithms:

$$\ln(DNA_t) = \ln(DNA_0) + kt + error_t \quad (2)$$

Separate regressions were initially fitted for each replicate/treatment/temperature combination. It was found that there was no significant difference between slopes (k) within a treatment but that there was a difference between the intercepts reflecting different amounts of DNA between the sets of vines. Separate intercepts were therefore allowed for data from each set of five vines. Half-life estimates were obtained using Equation 3:

$$\text{Half-life} = -\ln(2)/k \quad (3)$$

Rates of decomposition (slopes) were obtained using the regression shown in Equation 2. The slopes were averaged across replicates. The differences in intercepts reflected differences between the sets of five vines.

4.2.1.1. March 2013

Soil samples were collected in the Yarra Valley block in March 2013 from 75 known infested vines (15 sets of five vines). Two sampling tools were used: a 15mm diameter dig stick soil corer (Spurr Soil Probes, Adelaide, SA, Australia); or a >25mm diameter hammer corer, which collected approximately three times the sample volume of the dig stick.

For each sampling tool, four soil cores were collected 10–15cm from the trunk of each of the 75 vines. Then, for each set of five vines, four composite samples were prepared by pooling five cores, one from each vine in the set, giving 60 composite samples per core type.

Samples were immediately stored at 5°C and transported to the laboratory within 2 days of collection. Storage was extended for another 0, 1, 3 or 10 days at 10, 20 or 35°C before drying prior to DNA extraction and qPCR. The four composite samples from any one set of vines were stored at the same temperature (10, 20 or 35°C), and each was retrieved for analysis after 0, 1, 3 or 10 days, respectively. There were five sets of vines for each storage temperature and five replicates per storage duration at each temperature.

4.2.1.2. June 2013

In June 2013, a series of 60 composite samples were collected using the same protocol as in March 2013, but using the dig stick corer only. When they reached the laboratory, half the samples were frozen at -20^{o}C for 24 hours before applying the storage treatments (10, 20 or 35^{o}C for 0, 1, 3 or 10 days), to assess whether killing phylloxera by freezing the soil affected DNA degradation. The other samples were stored in a cool room at 4^{o}C prior to analysis.

4.2.2. Results

Across the two sampling dates, phylloxera was detected in 98% of the samples tested immediately upon arrival at SARDI post-drying (day 0) and in 73% of the samples stored for an extra ten days after receival, irrespective of the storage temperature.

The overall variability in phylloxera number was large with the phylloxera equivalent per 200g dry soil ranging from 0.42 to 8,017 and from 0.08 to 2,308 in March and June, respectively. The average moisture content was 13.4% and 18.6% in March and June, respectively (Tables 2, 5, 6). Decomposition was observed at each temperature and regardless whether the samples had been frozen before storage (Tables 4, 7).

4.2.2.1. March 2013

Phylloxera was detected in 95% of the composite samples (CI 86–99%), irrespective of temperature and duration of storage and in 100% (CI > 95%) of composite samples stored for zero and one day at any temperature (Table 2). Phylloxera was not detected in three samples – one of five replicates stored for ten days at 20°C, one of five replicates stored for three days at 35°C and one of five replicates stored for ten days at 35°C (Table 2).

The mean number of phylloxera per composite sample was not significantly different (P=1) from both corer types (Table 3). The decomposition rate was higher in the samples from the dig stick but the difference was not statistically significant (Figure 2). There was a trend for increasing phylloxera DNA decomposition with increasing storage temperature again but this effect was not statistically significant (p = 0.301) (Table 4, Figure 3).

4.2.2.2. June 2013

Overall phylloxera DNA detection rate and phylloxera equivalent per 200g dry soil were lower in June 2013 (41.33 unfrozen, 116.67 frozen) compared to March 2013 (542.32 unfrozen). Phylloxera was detected in 78.3% (CI 66–88%) of unfrozen soil samples (Table 5) and in 88.3% (CI 77-95%) of frozen samples (Table 6). For both unfrozen and frozen samples, phylloxera DNA detection was highest at 20°C and lowest at 10 days storage (Tables 5, 6). Frozen samples showed a large range of detection of DNA equivalents (Table 6).

Analyses were performed on the means of DNA equivalents (after logging) across composite replicates. Decomposition of phylloxera DNA was higher in frozen compared to unfrozen samples, except when stored at 35°C, resulting in generally a shorter half-life for frozen samples (Table 7). Decomposition increased with increasing storage temperature in unfrozen samples (Table 7).

The amount of phylloxera DNA (on a log scale) initially present prior to exposing the frozen and unfrozen soil samples to the various storage temperatures, is given by the intercepts of the slopes on the y-axes in Figure 4. The intercepts were subject to an analysis of variance (classified by temperature and freezing) and showed no effect of either factor (data not shown). The freezing therefore did not affect the measurement of the initial amount of phylloxera DNA present.

Table 2. Phylloxera DNA detection in soil samples collected in the Yarra Valley in March 2013 and stored at 10, 20 and 35°C for 0, 1, 3 or 10 days after arrival at SARDI laboratory. qPCR results were converted to number of phylloxera equivalents per 200g dry soil.

Replicate	Storage time (days)	Storage temperature (°C)					
		10		20		35	
		Moisture content (%)	Phylloxera equivalent per 200g dry soil	Moisture content (%)	Phylloxera equivalent per 200g dry soil	Moisture content (%)	Phylloxera equivalent per 200g dry soil
1	0	16.7	14	14.5	5216	14.3	4493
2	0	16.4	527	19.1	853	14.0	524
3	0	12.5	166	14.9	4	14.3	436
4	0	12.5	130	11.5	603	12.8	230
5	0	13.7	796	10.6	31	13.5	837
1	1	15.1	18	13.2	38	5.9	41
2	1	12.7	375	16.3	105	14.0	134
3	1	14.3	33	10.6	13	12.8	65
4	1	15.2	1588	9.3	8017	11.4	42
5	1	16.0	47	10.2	825	18.9	288
1	3	15.1	4	16.0	218	13.2	1
2	3	11.8	306	15.2	133	13.7	10
3	3	14.0	34	14.3	283	9.6	138
4	3	13.0	229	10.0	550	14.0	4
5	3	11.8	3583	12.2	16	14.5	0
1	10	14.0	0.42	13.5	0	15.1	0
2	10	11.8	253	15.6	24	14.9	28
3	10	13.7	14	12.8	3	10.4	1
4	10	12.5	10	12.0	91	14.3	66
5	10	12.8	21	13.7	29	13.0	1
AVERAGE		13.8	407	13.3	853	13.2	367

Table 3. Effect of corer type on phylloxera numbers and decomposition rates from samples collected by hammer corer and dig stick in March 2013.

Corer type	Mean number phylloxera per 200g dry soil[1]	Decomposition rate	Half-life (days)
Dig stick	542 ± 195 a	-0.31 ± 0.09	2.26
Hammer corer	547 ± 195 a	-0.21 ± 0.09	3.26

[1] Means with the same letter are not significantly different to each other at the 95% level

Table 4. Effect of storage temperature on decomposition rate of phylloxera DNA pooled across two corer types in March 2013.

Storage temperature (°C)	Decomposition rate[1]	Half-life (days)
10	-0.234 ± 0.07 a	3.0
20	-0.300 ± 0.11 a	2.3
35	-0.385 ± 0.13 a	1.8

[1] Means with the same letter are not significantly different to each other at the 95% level

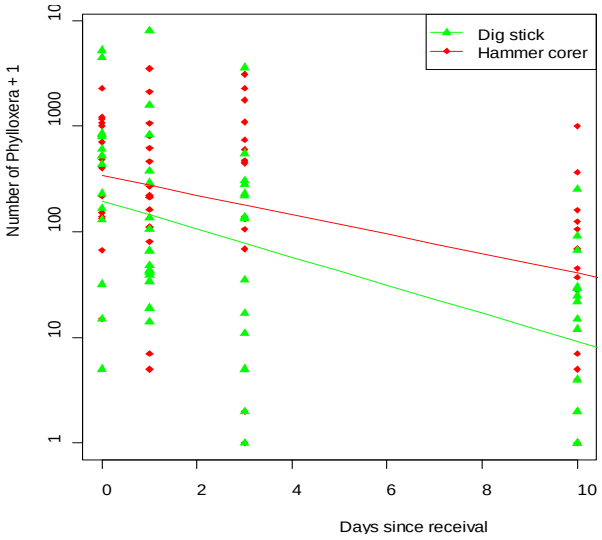

Figure 2. Effect of corer type on decomposition rate of phylloxera DNA in March 2013.

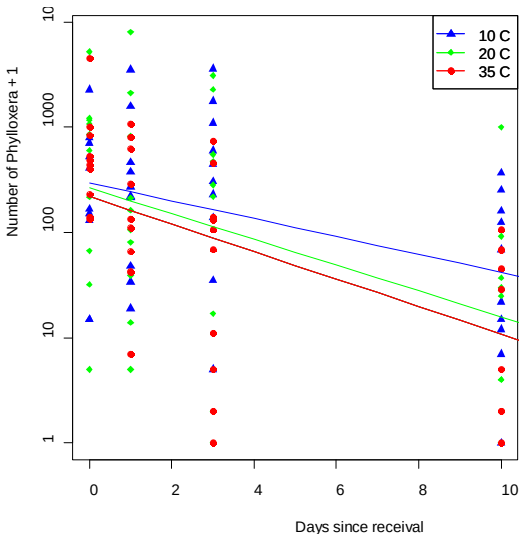

Figure 3. Decomposition rates of phylloxera DNA with different storage temperatures in March 2013. Results from the two corer types have been combined.

Table 5. Phylloxera DNA detection in soil samples collected in the Yarra Valley in June 2013. Samples were stored at 10, 20 and 35°C for 0, 1, 3 or 10 days immediately after arrival at SARDI. qPCR results were converted to number of phylloxera equivalents per 200g dry soil.

Replicate	Storage time (days)	Storage temperature (°C)					
		10		20		35	
		Moisture content (%)	Phylloxera equivalent per 200g dry soil	Moisture content (%)	Phylloxera equivalent per 200g dry soil	Moisture content (%)	Phylloxera equivalent per 200g dry soil
1	0	19.6	3	16.4	829	16.7	3
2	0	17.1	143	18.2	2	20.8	181
3	0	15.7	19	19.7	163	16.7	4
4	0	19.1	0	20.6	113	20.3	37
5	0	16.4	2	19.4	12	19.4	1
1	1	19.2	0.26	14.3	531	19.6	2
2	1	17.6	3	22.2	0	25.0	0.08
3	1	14.9	39	19.1	73	15.2	12
4	1	19.4	0	17.6	1	19.7	6
5	1	15.9	160	19.7	200	19.0	52
1	3	18.2	16	14.7	12	16.7	0
2	3	15.8	1	22.6	15	19.2	0
3	3	20.0	3	19.4	21	15.6	0
4	3	20.3	0	20.0	1	19.2	5
5	3	17.0	27	19.4	170	17.9	3
1	10	26.9	23	16.1	197	9.3	0
2	10	19.4	0	23.8	87	21.4	0.16
3	10	17.6	0	20.7	1	20.0	0
4	10	22.2	0	31.4	1	22.0	0
5	10	18.5	27	15.2	5	22.9	1
AVERAGE		18.5	24	19.7	84	18.9	16

Table 6. Phylloxera DNA detection in soil samples collected in the Yarra Valley in June 2013. Upon arrival at SARDI, samples were frozen at -20°C for 24 hours before being stored at 10, 20 and 35°C for 0, 1, 3 or 10 days. qPCR results were converted to number of phylloxera equivalents per 200g dry soil.

Replicate	Storage time (days)	Storage temperature (˚C)					
		10		20		35	
		Moisture content (%)	Phylloxera equivalent per 200g dry soil	Moisture content (%)	Phylloxera equivalent per 200g dry soil	Moisture content (%)	Phylloxera equivalent per 200g dry soil
1	0	19.2	28	24.0	39	20.8	245
2	0	4.1	288	21.0	1	18.2	43
3	0	21.3	0.28	17.1	1	19.7	527
4	0	12.0	23	19.0	0.48	17.1	0.40
5	0	20.0	4	17.0	2308	11.7	192
1	1	16.7	389	22.6	1453	19.2	26
2	1	18.0	14	20.0	3	18.5	1
3	1	17.0	3	14.7	6	20.0	6
4	1	18.2	0	19.4	36	17.5	23
5	1	23.1	556	17.3	44	20.6	6
1	3	17.9	459	21.4	73	19.6	17
2	3	19.0	8	19.4	0.36	16.7	5
3	3	18.8	6	23.8	3	18.6	0
4	3	15.7	0	20.7	1	17.4	1
5	3	23.3	4	17.4	1	19.7	25
1	10	14.0	0	17.5	14	20.0	7
2	10	18.3	1	16.0	0	14.3	6
3	10	19.3	16	15.6	0.18	18.0	1
4	10	15.7	0	20.7	1	13.3	0
5	10	18.8	7	17.3	23	18.8	11
AVERAGE		17.4	94	18.8	209	17.8	47

Table 7. Average decomposition rate of phylloxera DNA from unfrozen and frozen composite soil samples in June 2013.

Storage temperature (°C)	Unfrozen		Frozen	
	Decomposition rate	Half-life	Decomposition rate	Half-life
10	-0.078 ± 0.09	8.9	-0.193 ± 0.11	3.6
20	-0.116 ± 0.11	6.0	-0.166 ± 0.10	4.2
35	-0.213 ± 0.08	3.3	-0.190 ± 0.09	3.6

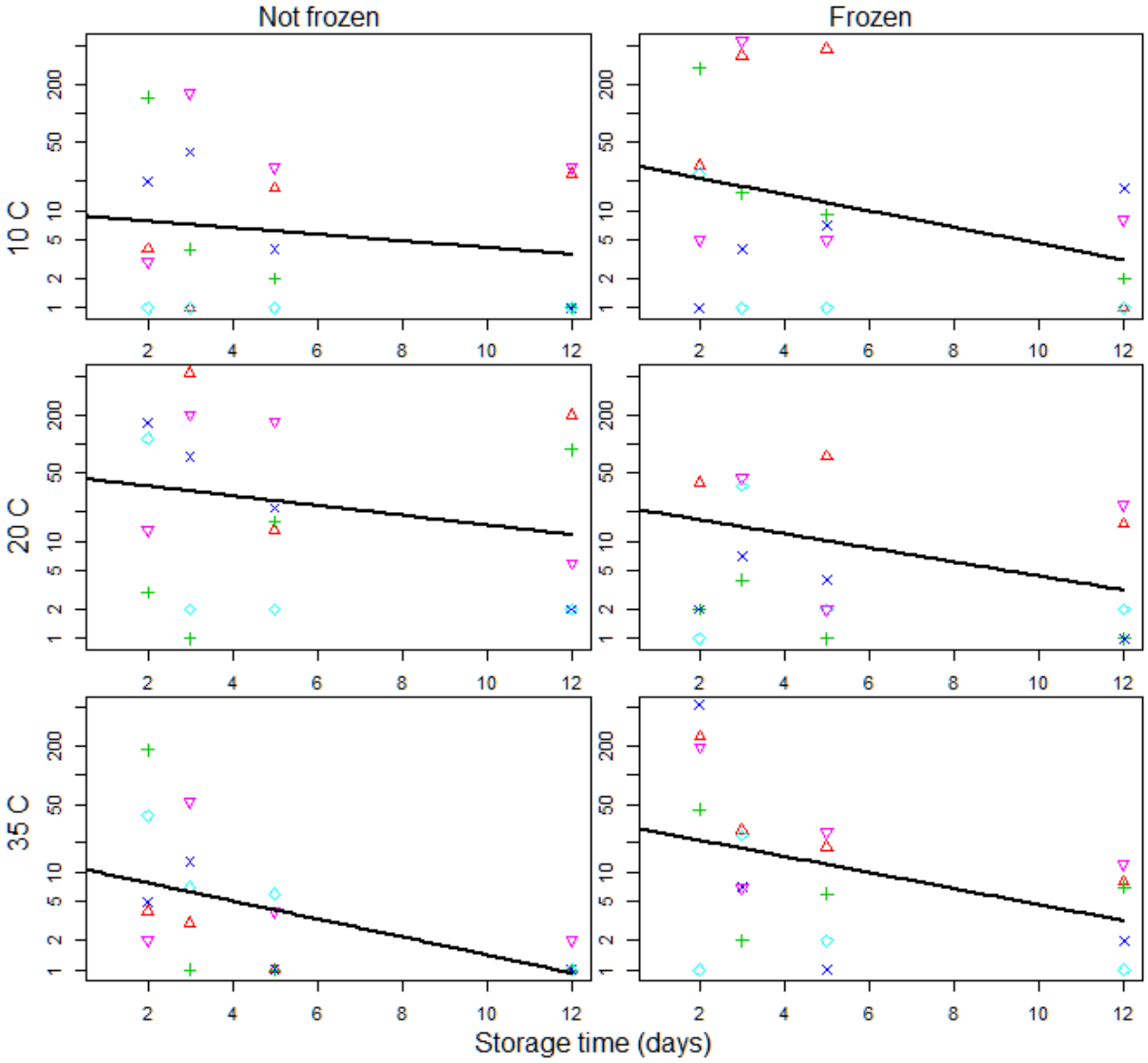

Figure 4. Effect of storage time on the amount of phylloxera DNA for frozen and unfrozen samples in June 2013. The line was fitted to the means of the five replicates.

4.2.3. Discussion and conclusion

Both the March and June 2013 experiments helped to identify suitable field sampling times and storage conditions of the samples both during transportation to the laboratory and at the laboratory prior to analysis. Repeating the March 2013 sampling in June 2013 was useful in being able to compare the relative detection rates and amount of phylloxera DNA. The lower detection rates and amount of phylloxera DNA in June are indicative of the movement of phylloxera down the soil profile in the cooler months and therefore indicative that winter should be identified as a non-preferred sampling time for phylloxera.

The hammer corer was found to be completely impractical in the field for soil sample collection compared to the dig stick due to the sizeable effort required to extract the soil samples. Use of the hammer corer was discontinued beyond the May 2013 sampling time, and due to the relative similarity in the amount of phylloxera DNA obtained using both corer types, was not expected to negatively affect DNA detection thereafter.

Although freezing of soil samples upon arrival at the laboratory did not appear to reduce the amount of phylloxera detected compared to unfrozen samples, freezing did contribute to a higher degradation of phylloxera DNA in the soil samples at the lower storage temperatures. While storage did not increase degradation of DNA following freezing, there was an obvious effect of the storage temperature on unfrozen samples.

The measured half-life of two to three days and the greatest degradation of phylloxera DNA as sample storage temperature increased, are important factors to consider regarding timeliness of sample transport from the field to the laboratory, field sample packaging protocol and capability of laboratory sample throughput.

The integrity of DNA is of paramount importance for detection by qPCR and can be affected by sample handling and storage. Moisture has been shown to be particularly detrimental to the integrity of DNA from dead organisms in soil and can therefore affect PCR detection (Herdina *et al*., 2004a, b). For qPCR testing, the recommendation is to dry soil samples as soon as possible after collection (Ophel-Keller *et al*., 2008) to stabilise the DNA within the sample. In this study, samples were securely packed in the vineyards and sent for analysis without drying to limit handling. The average moisture content of 13.4% recorded in soil collected in March 2013 and 18.6% in July 2013 was likely to affect phylloxera DNA if samples were subjected to an extended period of storage before drying.

The general range of two to four days of phylloxera DNA half-life in soil samples and decreasing pattern with increasing storage temperature is consistent with degradation rates reported for DNA of dead fungal mycelia and nematodes in moist soil (Herdina *et al*., 2004a, b). This finding suggests that in the soil collected for testing, at least some of the phylloxera were dead, possibly because of the soil disturbance caused by coring, or perhaps due to sample packaging. This is further supported by the absence of a significant difference between frozen and unfrozen samples. Despite DNA degradation, the overall detection rate decreased only from 98% within two days of collection to 73% after twelve days, regardless of storage temperature.

Conclusion: To limit phylloxera DNA decomposition, soil samples should ideally remain at no more than 20°C during transport and reach the laboratory within 48 hours of collection. Upon receival at the laboratory, these samples should be dried within approximately 24-48 hours and stored unfrozen at approximately 10-20°C pre-drying. The aim is to get the soil sample to the laboratory and dried as soon as possible to limit DNA degradation.

4.3. Effect of composite samples on phylloxera detection

The soil corers provide approximately 10-50g soil, depending on soil type and moisture status. For improved laboratory efficiency for the DNA test, a minimum of 200g, or preferably 500g of soil is required per sample to analyse. Using multiple cores in one processed sample is the usual way to provide enough soil for testing. Compositing cores from over a number of sampled vines, rather than from individual vines collected over an area, can be used to maximise the coverage of the sample area. This process provides sufficient soil for testing, and minimises the cost by reducing the number of samples being tested for DNA.

In addition, collection of composite samples is recommended for soil-borne pathogen detection as it minimises the effect of spatial variation between samples (Ophel-Keller *et al*. 2008). For phylloxera, the use of composite samples has the potential to increase both the coverage of the survey and its reliability by accounting for the uneven distribution of phylloxera across a vineyard. However, the number of samples that can be composited must be defined to ensure detection is not compromised.

Four trials were undertaken to determine the detection ability of composite soil samples:

1. Laboratory mixing of soil from known infested and non-infested sites;
2. Six single soil cores per vine from a known infested vineyard – this trial was undertaken prior to the start of the current project but the data have not been previously reported; and
3. Two trials comparing single soil cores per vine with composite samples.

The data form an important building block in understanding sampling using soil cores for DNA assessment.

4.3.1. Composite samples – laboratory mixed

4.3.1.1. Materials and methods

Composite samples were made up in the laboratory by mixing phylloxera-free soil with phylloxera-infested soil. Bulk soil samples were taken near the base of trunks of infested vines in Rutherglen (phylloxera-infested soil) and from an adjacent empty paddock (non-infested soil) not previously planted to vines, in March and May 2014. In both instances, soil was transported to the laboratory within 2 days of collection and dried upon arrival. The bulk samples were separately homogenised with a jaw grinder (SP100x100, Labtechnics, Kilkenny, SA, Australia) prior to being used to prepare 400g composite samples. The infested/non-infested soil ratios (mass/mass) were 400/0, 200/200, 100/300, 50/350, 20/380 and 0/400 in March 2014, and 400/0, 200/200, 100/300, 50/350, 20/380, 10/390, 5/395, 1/399 and 0/400 in May 2014, with eight replicates of each. At each sampling time, there were also eight 400g samples with phylloxera-infested soil only and another eight (five in May 2014) 400g samples with non-infested soil only as controls. All samples were processed for DNA extraction and phylloxera detection by qPCR, as described in 4.1.

A generalised linear model with binomial errors and a logistic link function was used to analyse presence/absence data obtained with artificial composite samples.

4.3.1.2. Results and discussion

Mean phylloxera DNA equivalents were higher in March 2014 than in May 2014 for all comparable composite infested samples and no phylloxera was detected in any of the non-infested control soil samples (Table 8). In March 2014, phylloxera was detected in all infested composite samples. In May 2014, phylloxera was detected in all replicates of composite samples containing 50g or more of infested soil, and in 87.5, 50, 50 and 0% of samples containing 20, 10, 5 and 1g of infested soil, respectively (Table 8). For both sampling times, the

phylloxera DNA equivalent per 200g dry soil was directly proportional to the amount of contaminated soil in the composite samples (Table 8). Based on the results obtained in May 2014, it was estimated that 50% detection is likely when there is 0.48 phylloxera equivalent per 200g dry soil, and up to 100% detection when there is 1.5–2 phylloxera equivalents per 200g dry soil (Figure 5).

Table 8. Mean phylloxera equivalent and number of phylloxera positives in composite samples made up by mixing various amounts of phylloxera-infested and non-infested soil collected in Rutherglen vineyard in March and May 2014.

Amount of phylloxera infested soil (g) in 400g composite sample	March 2014		May 2014	
	Phylloxera equivalents per 200g dry soil	Number positive samples out of 8 replicates	Phylloxera equivalents per 200g dry soil	Number positive samples out of 8 replicates
0 (negative control)	0	0	0	0
1			0	0
5			0.34 ± 0.21	4
10			0.26 ± 0.17	4
20	3.6 ± 1.30	8	0.88 ± 0.24	7
50	10.6 ± 2.50	8	7.98 ± 5.05	8
100	36.8 ± 12.00	8	5.44 ± 2.38	8
200	80.4 ± 28.00	8	7.59 ± 1.17	8
400 (positive control)	157.4 ± 37.00	8	16.53 ± 3.13	8

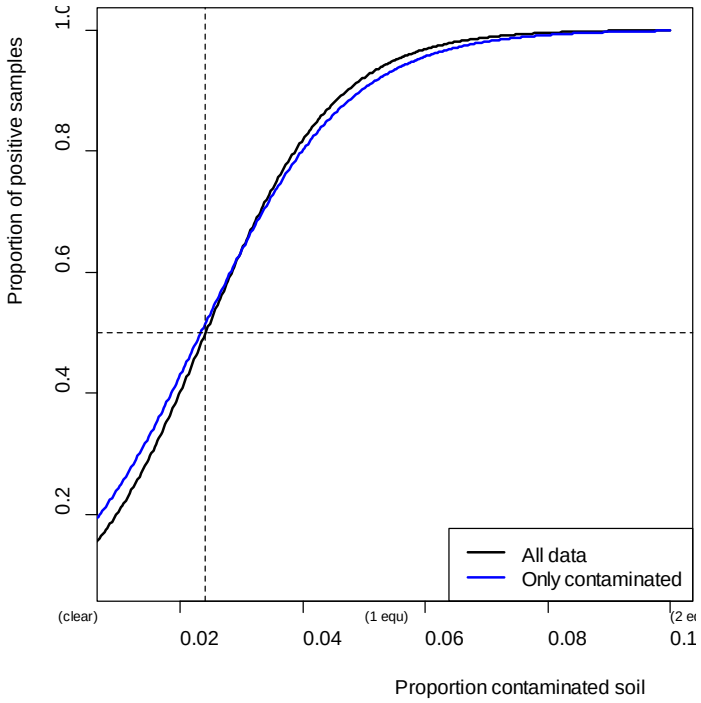

Figure 5. Estimation of the probability of detection of phylloxera in composite soil samples based on the proportion of positives amongst 400g samples made up by mixing various amounts of phylloxera-infested and non-infested soil. Estimation was based on the results obtained from soil collected in May 2014 in the Rutherglen vineyard.

Compositing needs to be implemented carefully to avoid detrimental consequences on detection. The estimate of a limit of detection of 1.5 to 2 phylloxera per 200g composite soil sample from this study indicates that the true limit of detection of the assay is likely lower than the 20 phylloxera per 200g dry soil published previously, based on results using spiked soil samples (Herbert *et al*., 2008).

4.3.2. Single core samples

4.3.2.1. Materials and methods

Forty vines from a known infested vineyard were sampled, each with six soil cores taken radially around each vine trunk within 15cm of the trunk. Each core was processed separately for DNA extraction and phylloxera detection by qPCR, as described in 4.1. Phylloxera equivalents were averaged for each vine based on the results of the six cores.

4.3.2.2. Results and discussion

The sample weight of individual soil cores was small, ranging from 7g to 30g dry weight, with phylloxera DNA concentrations ranging from 0.04 up to 43,000 phylloxera equivalents per 200g dry weight of soil. Once the phylloxera concentration was greater than 10 phylloxera equivalents per 200g dry soil (more than half of the vines sampled), mean detection rate per vine was greater than 90% (Table 9).

Table 9. Mean detection rates per vine based on 6 cores per vine. Concentrations are in phylloxera equivalents per 200g dry weight.

Concentration range	<1	1 to 10	10 to 50	50-100	100-200	>200
Number of vines	11	6	9	4	6	4
Mean detection	42%	72%	93%	96%	97%	92%

The results of the study are shown in Figure 6 with each point representing an average result per vine. A von Bertalanffy type of function was fitted to the data:

$$\text{Proportion of detections} = 1 - \exp(-k \times \text{mean sample DNA})$$

The value of k was estimated to be 0.3610 ± 0.063, and that was used to draw the line shown in Figure 6.

The detection rate of the core samples was 50% when the mean DNA concentration was 2 phylloxera equivalents per 200g dry weight. However, at higher concentrations the detection rate was less than 100% (Table 9), indicating that the fitted von Bertalanffy model was not a good representation at higher phylloxera concentrations, showing 100% detection rate.

The number of detections on a per vine level shows that in 17.5% of cases (7 vines) there were no detections from any of the six soil cores per vine, inferring that those vines were possibly not infested (Figure 7). In 40% of cases (16 vines), all six soil cores per vine detected phylloxera DNA, and in a further 25% of cases (10 vines), there were five positive cores out of six sampled per vine. In the 26 vines with five or six cores out of six per vine for which phylloxera was detected, a single soil core would have had a 94% probability of detecting the presence of phylloxera. Because the proportion of 40 vines showing five or six out of six cores with phylloxera was relatively high, the level of infestation across the 40 vines is presumed to be high.

From all vines where there was evidence of phylloxera being present, there was an 84% detection rate. When including those vines for which no positive cores were detected, the overall detection rate across the 40 vines

was 69%. If the areas that were not infested (true negatives) were removed from the calculations, the detection rate for the actual infested areas would be between those two estimates.

The phylloxera detection rate of soil cores includes the spatial variability around a vine and laboratory variation, whereas the sensitivity of the DNA method is dependent only on the laboratory precision. One of the detections in this study was low at 0.04 phylloxera equivalents per 200g dry soil which indicated the proven sensitivity of the laboratory qPCR test. However, in general, the quantity of phylloxera present in a sample needed to be at 2 phylloxera equivalents per 200g dry soil for the detection rate to be 50%. Some spatial variability of phylloxera around a vine is expected, in terms of both presence and absence of phylloxera and quantity of phylloxera DNA. It is surmised that this variation could be due to uneven vine feeder root distribution and perhaps other factors like placement of the dripper, soil compaction, soil moisture status or even variability in salt/nutrient content which may affect the ability of phylloxera to survive and inhabit close to the soil surface. Where phylloxera infestation levels in a block are expected to be low, collecting more than one soil core per vine may be advantageous in a sampling strategy.

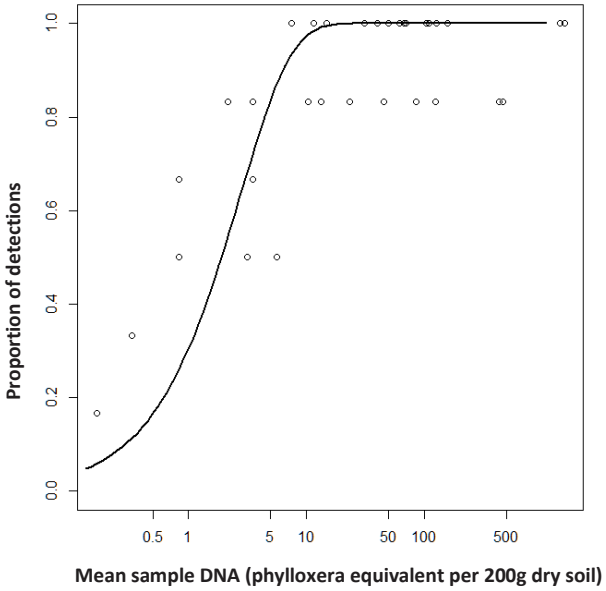

Figure 6. Relationship between detection frequency and mean amount of phylloxera DNA in the soil sample.

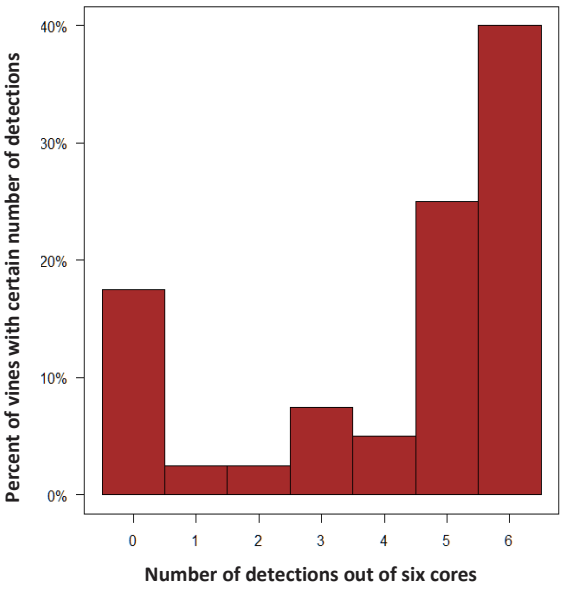

Figure 7. Proportion of detections out of 6 for each of the 40 vines.

4.3.3. Single cores per vine compared to composite samples – part A

4.3.3.1. Materials and methods

Fifty vines from one known phylloxera infested vineyard in the Yarra Valley (Vineyard 2) were sampled in March 2017. Two individual soil cores were taken within 10cm on either side of the vine trunk. One of the two soil cores collected at each vine was processed as an individual core and one was composited with four other individual cores from four vines into a composite sample; with both processed for DNA extraction and phylloxera detection by qPCR, as described in 4.1. Phylloxera detection was analysed as paired samples with detections for each one of the 10 composites compared to the individual soil results from those five vines that comprised each composite sample.

4.3.3.2. Results and discussion

Comparing the relative difference in phylloxera detection from composite samples versus single cores per vine, there was very good correlation with 8 of the 10 composite samples also showing positive detections at the single core level, with at least one of the five individual cores sampled from the same vines as the composite, also testing positive to phylloxera. There was one case where a composite sample did not detect phylloxera and individual cores taken from the same vines as the composite cores also did not detect phylloxera. There was however one discrepancy where the composite sample missed a positive detection found in a single core (Table 10) of 0.4 phylloxera equivalents per 200g dry soil.

The high correlation between the detection rate from the paired composites versus single cores showed that collecting composite cores would have been sufficient to infer the same approximate level of phylloxera infestation across the block. Therefore, the additional laboratory cost of analysing 50 individual soil cores provided little additional insight into the infestation level of the block compared to analysing 10 composite samples. From a whole of vineyard level, the same phylloxera status result would have been achieved for this vineyard, with both sampling methods.

Table 10. Comparison of phylloxera detection rate at Vineyard 2 in March 2017 using five single cores taken from five vines ('Single cores'), compared with a composite of a single core per vine for same five vines ('Composite'). A positive for the single cores was attributed when at least one of the single cores was positive.

	Single (5) cores positive	Single (5) cores negative	Total
Composite (5 cores) positive	8	0	8
Composite (5 cores) negative	1	1	2
Total	9	1	10

The small sample size of this trial negates considerable inference of these results to other situations, however in this instance, analysing individual soil cores for phylloxera did not provide additional benefit to analysing composite samples of five cores per composite. We can infer that the level of infestation in this vineyard sampled was quite high with 8 of 10 composite samples positive for phylloxera and therefore perhaps that there was also relatively low spatial variability in phylloxera at the vine level as demonstrated by the strong correlation in detections between the composite samples and individual cores sampled from the same vines.

4.3.4. Single cores per vine compared to composite samples – part B

4.3.4.1. Materials and methods

One hundred and fifteen were sampled in three vineyard blocks from two known phylloxera infested vineyards in the Yarra Valley (Vineyard 2, Vineyard E-2 and Vineyard E-3) in March 2017. Two individual soil cores were taken within 10cm on either side of the vine trunk. One of the two soil cores collected at each vine was processed as an individual core and one was composited with four other individual cores from four vines into a composite sample, giving a total of 31 composite samples across the three vineyard blocks. Soil samples were processed for DNA extraction and phylloxera detection by qPCR, as described in 4.1. Phylloxera detection was analysed as paired samples with detections for each one of the composites compared to the individual soil results from those five vines that comprised each composite sample.

4.3.4.2. Results and discussion

Overall, there were two thirds (22) of the soil core sets of the 31 for which perfect correlation was found between both the composite samples and the five individual cores per set (Table 11). Importantly though, one third of the sets showed discrepancy between the methods.

The composite samples had a detection rate of 13/31 or 42%, whereas the single five cores per vine had a detection rate of 18/31 or 58%. Quintupling the laboratory costs and adding to the sampling time gave a 16% gain in detection rate across the three vineyard blocks.

There were 7 sets for which the composite sample failed to detect phylloxera but at least one of the five vines of those sets returned a positive result (Table 11). In this situation, there were five sets for which only one of the five cores returned a positive detection and two sets for which two of the five cores returned positives. Eight out of nine of the single core detections were at extremely low levels, with less than 1.8 phylloxera equivalents per 200g dry soil (data not shown). This low level of detection therefore indicates that for a composite sample with five single cores pooled, there is a chance at low levels of phylloxera infestation that the composite could return a false negative detection. These results indicate there is some trade off to be considered between time (cost) of sample collection and processing and sensitivity of the results if analysis of composite soil samples is undertaken rather than single cores.

There were two sets where the composite result returned a positive, but all the single cores for those two sets returned negative phylloxera results (Table 11). The phylloxera levels in these composites were 0.09 and 4 phylloxera equivalents per 200g dry soil – once again low levels of phylloxera. In these two cases with expected low level of phylloxera distribution in the root zone, it could have been purely chance that the soil core taken as part of the composite samples contained the phylloxera present if it was in a small colony, rather than the corresponding core from the same vine which was analysed separately.

Table 11. Comparison of pooled phylloxera detection rate across three vineyard blocks in the Yarra Valley, March 2017, using five single cores taken from five vines ('Single cores'), compared with a composite of a single core per vine for same five vines ('Composite'). A positive for the single cores was attributed when at least one of the single cores was positive.

	Single (5) cores positive	Single (5) cores negative	Total
Composite (5 cores) positive	11	2	13
Composite (5 cores) negative	7	11	18
Total	18	13	31

There was reasonably good correlation between the phylloxera detections from the composite samples versus the single cores. The single cores did have 16% higher detection rate but this result would have been accompanied with five times the laboratory costs and significantly longer field collection time. From a vineyard block level, the phylloxera status would not have changed by implementing a strategy of analysing phylloxera levels in single cores rather than composite cores.

4.3.5. Conclusion

Results in this study demonstrate that a sampling protocol based on composite samples is appropriate for phylloxera detection by qPCR. It also means that the cost of a qPCR-based survey can be significantly reduced without compromising detection by combining soil cores into one sample. This is an important observation as cost is considered by most authors as the main impediment to qPCR use for routine phylloxera surveillance (Herbert *et al*., 2008, Powell, 2012b).

However, the compositing process needs to be implemented carefully to avoid detrimental consequences on detection. Whilst the sensitivity of the DNA method is dependent only on the laboratory precision, some of the detections in this single core study were as low at 0.04 phylloxera equivalents per 200g dry soil which indicated the proven sensitivity of the laboratory qPCR test. However, in general, the quantity of phylloxera present in a sample needed to be at 2 phylloxera equivalents per 200g dry soil for the detection rate to be 50%.

Notwithstanding the low detection potential, in the final trial (4.3.4) there was 7/18 (38.9%) cases of non-detection with composite cores where the single cores detected phylloxera. It is therefore suggested that in situations of likely low infestation, the dilution effect of a single positive core mixed with multiple negative cores as part of a composite sample, could compromise positive detections from a composite level if too many cores are composited.

Of note, there were 2/13 (15%) cases where none of the single cores detected phylloxera, yet the composite did. The very nature of spatial variability of the phylloxera around a vine is expected to contribute to false negatives, especially in situations where phylloxera colonies are quite isolated on the vine roots, in low infestation level situations. As suggested in the discussion, the cores taken for the composite sample may have, by chance, detected the only small colony around that vine. In these situations, sampling more than one core per vine may to increase detection rate at the vine level, however more benefit may be gained by sampling more vines in the area, i.e. increasing the sampling density (refer 4.6), rather than increasing the number of soil cores collected per vine.

Therefore, when developing a sampling strategy for surveillance, a sampling protocol based on composite samples of 5 cores is appropriate for phylloxera detection by qPCR. However, in instances of suspected low incidence and severity of phylloxera in a block, either less compositing of individual cores or higher numbers of cores per block, may be considered to maximise the potential for detection.

4.4. Effect of position, depth, location and time of soil sampling on phylloxera detection

The distribution of phylloxera in the soil around the vine was investigated to optimise the sampling strategy, assessing the effect of sampling depth, position relative to the trunk, location and time on phylloxera detection. These experiments were undertaken over many years in different seasons to provide sampling parameters that ensure the best chance of phylloxera detection.

4.4.1. Materials and methods

Samples were collected on a total of 10 occasions over three consecutive years from March 2013 to March 2015.

Samples were initially collected at three depths (0–10, 10–20 and 20–30cm), three positions (close to trunk, below dripper and between rows) and from the three selected vineyards (King Valley, Rutherglen and Yarra Valley), in March (autumn), July (winter), September (spring) and December (summer) of 2013. In each vineyard, eight groups of five infested vines were identified and tagged so that the same 40 vines were sampled at each sampling date. One 30cm deep core was taken 10–15cm from the trunk of each vine at each position, using a 15mm diameter dig stick soil corer; each core was split into three depth sections (0–10, 10–20, 20–30cm); composite samples were made by pooling the corresponding position/depth sections from each of the five vines within a group, making a total of nine samples per group of five vines and 72 samples per location. Between row samples were collected only in March. In December, sampling was interrupted by heavy rain. As a result, all samples from the Yarra Valley, the King Valley and the first group of vines in Rutherglen (all positions and depths) were taken on the 4 December 2013; all remaining samples from Rutherglen were collected on the 14 January 2014.

To verify the trend observed in the first year of the depth/position/location/time trial, selected sampling was continued for another year. Samples were collected from the three vineyards in March, May, July, October and December of 2014, and March of 2015, 0–10cm deep, close to the trunks of the same infested vines and using the same sampling protocol as described above.

A separate set of samples was also collected in May 2014 to assess the effect of the sampling distance from the vine trunk on phylloxera detection. The vineyard in the King Valley was selected for this purpose as it was postulated that the overall lower incidence of phylloxera in this vineyard, as indicated by previous sampling, was more likely to reveal a distance effect if any existed. Samples were collected from the same eight groups of five vines used for the position/depth/location/time trial. Cores were collected 0–10cm deep at 5, 10, 15, 30 and 45cm from the trunk of each vine in a group. Composite samples were made by pooling the cores taken at the same distance from the trunk from each of the five vines within a group, making a total of eight samples per distance.

All samples were sent to the laboratory within 2 days of collection and processed for DNA extraction and phylloxera detection by qPCR as described in section 4.1.

The sampling depth and position trial was designed with a factorial structure (three locations by three sampling positions by three depths) each with five replicates. The qPCR data were transformed using log transformation $y = \ln(x + 1)$ and ANOVA was performed using the aov or lm functions in R. The results obtained with the samples collected at various distances from the trunk were analysed using a generalised linear model with binomial errors and a logistic link function.

4.4.2. Results

4.4.2.1. Position

Both frequency of detection and the amount of phylloxera (equivalents per 200g dry soil) were significantly lower between rows (mid-row) than close to the trunk and below the dripper (p<0.001), irrespective of depth of sampling and vineyard location (Table 12), for samples collected in March 2013.

Across all sampling times of the year and depths, the frequency of detection was usually higher close to the trunk than below the dripper in the King Valley and the Yarra Valley and the opposite in Rutherglen (Figure 8). In the 0–10cm layer of soil, the difference was not significant (p=0.88) but suggested more detections near the trunk than below the dripper. Moreover, absence of detection across all replicates was observed only with samples collected below the dripper in the King Valley and deeper in the soil profile (Figure 8).

The frequency of detection of phylloxera followed a negative gradient relative to the distance from the trunk, with the proportion of positive samples decreasing from 0.625 to 0.25 between 5 and 45cm from the trunk (data not shown). This gradient was not significant. However, when the phylloxera equivalent was plotted against the distance from the trunk as a boxplot, there were several outliers which contributed to the overall uncertainty (Figure 9). A nonlinear regression of the upper quartile of the form:

$$\text{DNA amount} = 27 \pm 4.02 \times \exp^{(-0.159 \pm 0.0212 \times \text{Distance})}$$

revealed a statistically significant effect of the distance from the trunk on the number of phylloxera detected. The optimal position for sampling is as close to the trunk as possible, but within 10cm (Figure 9).

4.4.2.2. Depth

With soil samples taken close to the trunk, 70 to 80% phylloxera DNA detection was achieved in the 0–10cm surface layer of soil, with little additional detection in the 10–20cm layer and even less in the 20–30cm layer (Figure 10). A similar pattern was observed with soil samples taken below the dripper, with a noticeably lower overall phylloxera DNA detection rate in winter and summer. More detection is therefore likely closer to the trunk than below the dripper. The cumulated DNA phylloxera detection rate with soil samples collected mid-row in March was lower than that of samples taken both close to the trunk and below the dripper (Figure 10) in 2013. Soil samples were more difficult to collect mid-row because the soil profile was drier. Subsequently, mid-row samples were not collected after 2013.

4.4.2.3. Vineyard location

In the 2013 sampling, frequency of detection of phylloxera was consistently and significantly (p<0.001) lower across time of the year, positions and depths of sampling in the King Valley (0–0.5) compared to Rutherglen (0.625–1) and the Yarra Valley (0.5–1 (Figure 8). Phylloxera equivalent per 200g dry soil was also consistently and significantly lower (p<0.001) in the King Valley than in the other two vineyards across all sampling depths and positions except in summer 2013 (Table 13).

The same trend was observed when comparing the three sites over the 10 surveys, with a significant difference (p<0.001) in detection: Yarra Valley had a 99% detection rate compared to 87% at Rutherglen and 57% at King Valley (Tables 14, 15). There were at least two detections in each series of five vines at each of the three locations. Each entry has a maximum of 10 detections, so an entry of two typically implies 20% probability of detection from a composite sample of five cores. Low detection rates (4 or less out of 10) occurred in four of the eight sets of five vines at King Valley, whereas Yarra Valley detection rates were consistently higher, with 10/10 detections at seven of the eight vine sets (Figure 11).

Unlike the detection frequency data, the amount of DNA detected (phylloxera equivalents per 200g dry soil) showed significant differences (P<0.001) between surveys (Table 16).

In the March 2015 samples, there were generally higher levels of DNA detected in all samples compared to October 2014 (Table 17). As in previous sampling times, the highest DNA was found in Yarra Valley (Figure 11).

When a logistic regression was applied to the average detection rate and average amount of DNA, a fair fit was achieved with a residual deviance of 49.1 on 28 degrees of freedom compared to an expected value of 27.3 with an upper 95% limit of 41.34 (Figure 12). All the Yarra Valley points are above the line whereas all but one of the King Valley are below the line. This would suggest that detection is more likely at Yarra Valley than at King Valley for the same concentration of DNA.

4.4.2.4. Time

In 2013, the frequency of detection remained similar across time of the year for a given vineyard, position and depth of soil sampling (Figure 8). In contrast, time of sampling had a significant effect (p<0.001) on the concentration of phylloxera DNA – the highest concentration was observed in March in all three vineyards, the lowest in September in King Valley and Rutherglen and December in the Yarra Valley (Table 13).

While there were variations in detection with time, when a generalised linear model with binomial errors was used to test for differences between surveys and between seasons, no significant differences of detection rates were found (Table 15). Note that there are four degrees of freedom between seasons instead of three because an additional survey was included in late May 2014.

The changes in phylloxera DNA quantity recovered across surveys were plotted on a log scale to account for skewed distribution (Figure 13). The highest densities occurred at Yarra Valley in autumn in March 2013, 2014 and 2015 – there was a similar but not so consistent a pattern of seasonality at the other two sites.

Table 12. Effect of position of sampling on the phylloxera detection rate in composite soil samples collected in the King Valley, Rutherglen and the Yarra Valley vineyards in March 2013.

Location	Position	Depth (cm)		
		0-10	10-20	20-30
King Valley	Trunk	3/8	1/8	3/8
	Dripper	3/8	2/8	4/8
	Mid-row	0/8	2/8	2/8
Rutherglen	Trunk	7/8	8/8	8/8
	Dripper	8/8	7/8	8/8
	Mid-row	3/8	0/8	0/8
Yarra Valley	Trunk	8/8	8/8	8/8
	Dripper	8/8	8/8	8/8
	Mid-row	6/8	4/8	5/8

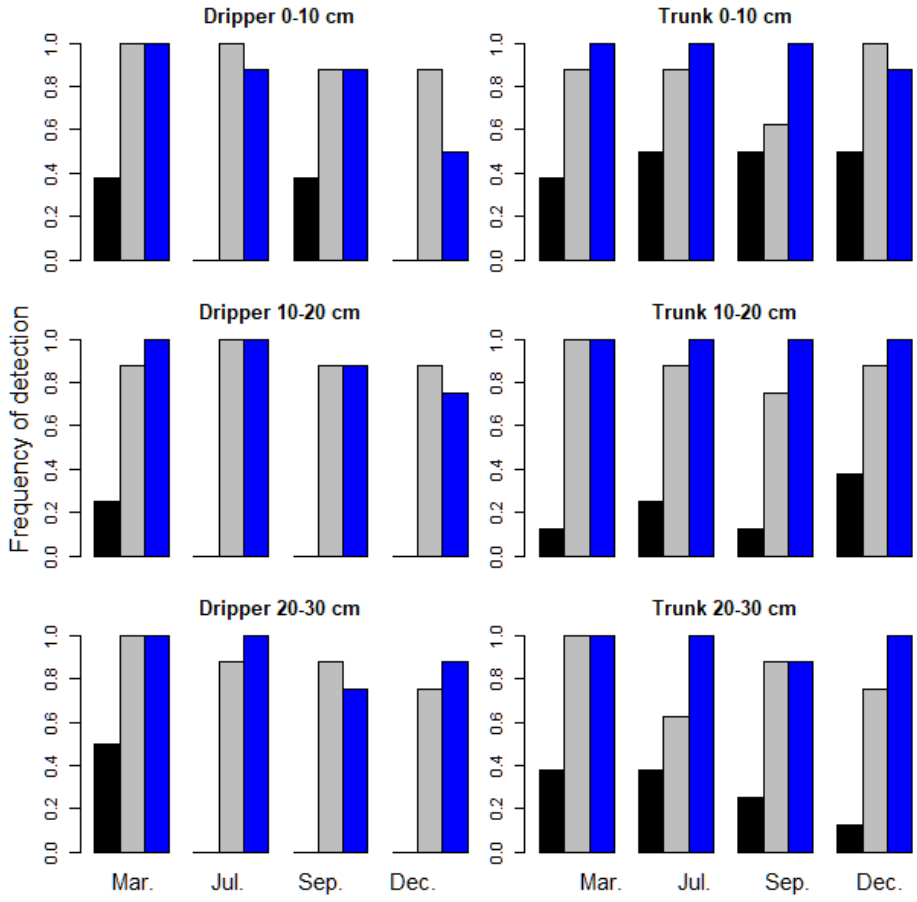

Figure 8. Frequency of detection of phylloxera (assuming all vines are infested) in composite soil samples collected below the dripper and close to the trunk, 0-10, 10-20 and 20-30cm deep, in the King Valley (■), Rutherglen (■) and the Yarra Valley (■) vineyards in March (autumn), July (winter), September (spring) and December (summer) 2013.

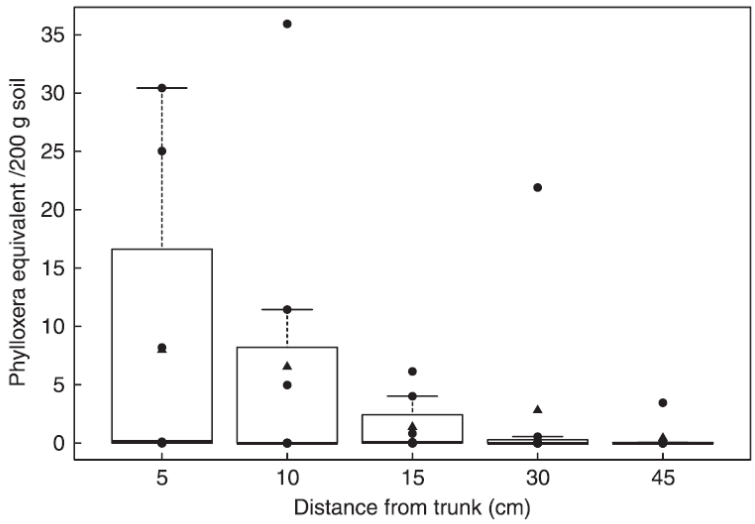

Figure 9. Mean (▲) and observed (●) phylloxera equivalent per 200g dry soil in samples collected 5, 10, 15, 30 and 45cm from the vine trunk, 0-10cm deep in the King Valley vineyard in May 2014.

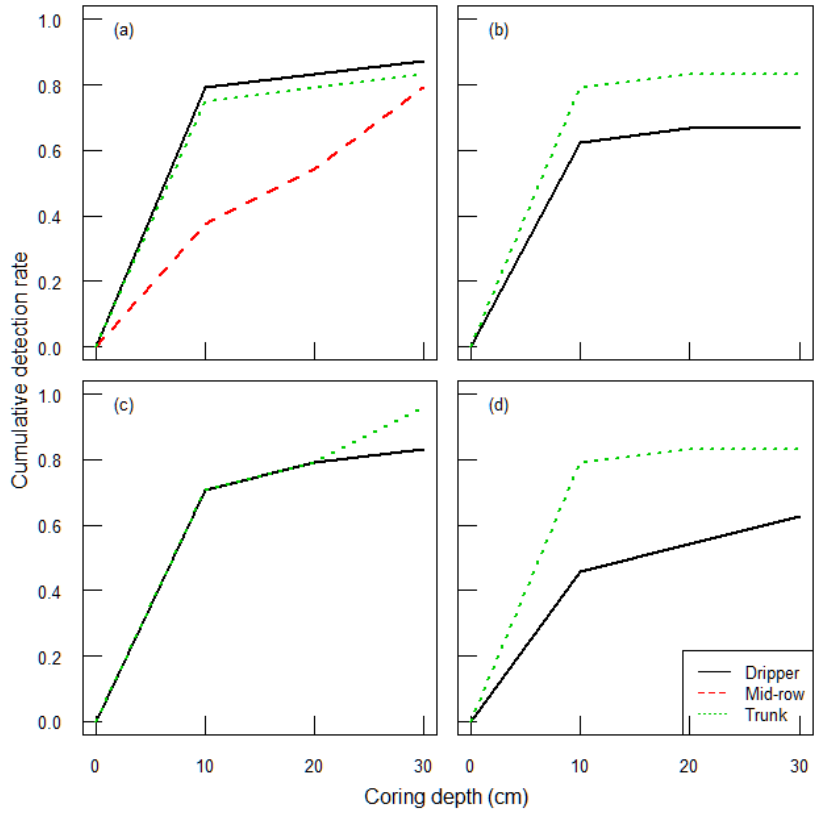

Figure 10. Effect of depth on phylloxera detection rate in soil samples collected under the dripper (—), close to the trunk (---) or between rows (- -) of infected vines in March (a), July (b), September (c) and December (d) 2013. Between row samples were only collected in March. The cumulated detection rate was calculated using results obtained across three infested vineyards in the King Valley, Rutherglen and the Yarra Valley.

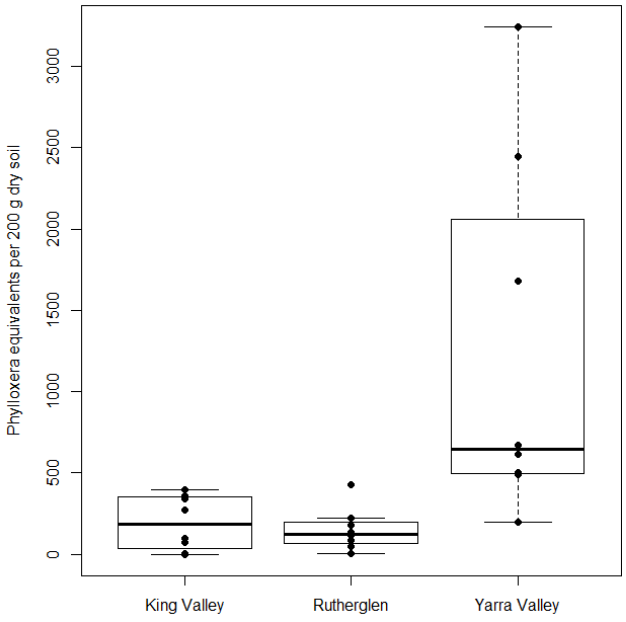

Figure 11. Distribution of concentrations of phylloxera DNA (expressed as phylloxera equivalents per 200g dry soil) at each location, based on 2015 March sampling 0 – 10 cm near trunk samples.

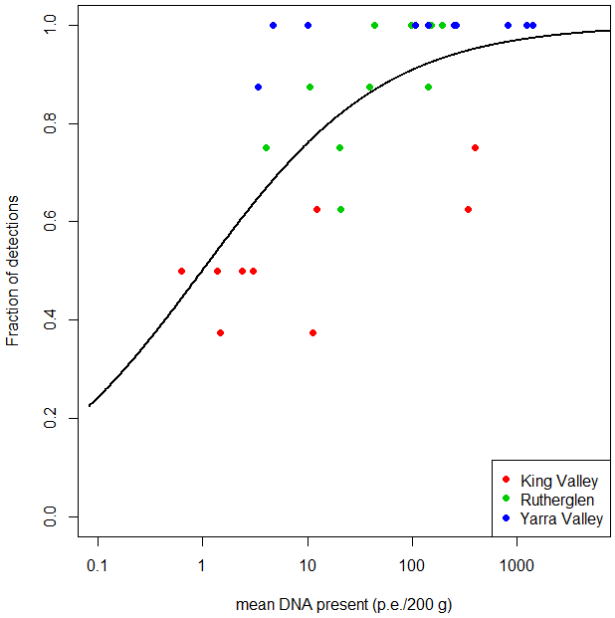

Figure 12. Relationship between mean DNA present in samples and the probability of detection. The units of DNA are phylloxera equivalents per 200g dry soil.

Table 13. Effect of location of sampling on mean phylloxera equivalents per 200g dry soil for composite soil samples collected in the King Valley, Rutherglen and the Yarra Valley vineyards in March (autumn), June (winter), September (spring) 2013 and December 2013/January 2014 (summer).

Location	Mean phylloxera equivalents per 200g dry soil[1]			
	March	June	September	December
King Valley	6.4 ± 5.1 a	4.5 ± 2.2 a	0.2 ± 0.1 b	1.3 ± 0.8 a
Rutherglen	237.0 ± 40.0 a	28.5 ± 6.1 c	15.7 ± 4.4 d	89.0 ± 25.0 (3.2, 102.0)[2] b
Yarra Valley	721.0 ± 140.0 a	94.0 ± 16.0 b	3.4 ± 0.6 c	1.5 ± 0.4 d

[1] Mean phylloxera equivalent values (± error estimates) were estimated means from GenStat ANOVA command using results from composite samples collected across depth and position but excluding between row samples. The same letters represent no significant difference between composite sample means within a vineyard.

[2] First number indicates the overall mean phylloxera equivalent per 200g dry soil in samples collected in December 2013 and January 2014 followed in brackets by the mean phylloxera equivalent per 200g dry soil in samples collected in December 2013 from vines 1-5 and in January 2014 from the other 35 vines.

Table 14. Numbers of phylloxera detections for each group of vines over 10 surveys over three consecutive years from March 2013 – March 2015.

Vine group	King Valley	Rutherglen	Yarra Valley
1-5	4/10	9/10	10/10
6-10	2/10	10/10	9/10
11-15	6/10	10/10	10/10
16-20	3/10	7/10	10/10
21-25	4/10	9/10	10/10
26-30	10/10	8/10	10/10
31-35	9/10	7/10	10/10
36-40	8/10	10/10	10/10
Total	**46**	**70**	**79**
Percent	**57%**	**87%**	**99%**

Table 15. Testing significance of effect of differences between seasons and surveys on detection rates (4 seasons + 1 addition, 10 surveys).

Model	Degrees of freedom	Deviance	Deviance change	Degrees of freedom change	P value
Null	239	231.64	-	-	-
Locations	237	180.13	51.51	2	<0.001
Seasons + Locations	233	175.43	4.70	4	0.319
Surveys + Locations	228	164.16	2.24	9	0.068

Table 16. Testing significance in amount of DNA (log transformed) of effect of differences between seasons and surveys.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	P value
Location	2	535.8	267.9	57.8	<< 0.001
Survey	9	492.6	54.7	11.8	<< 0.001
Location by Survey	18	355.1	19.7	4.25	< 0.001
Residuals	210	974.2	4.64		

Table 17. Concentrations of phylloxera DNA (expressed as phylloxera equivalents per 200g dry soil) at each location, based on October and December 2014 and March 2015 sampling 0 – 10 cm near trunk samples.

Vines	King Valley			Rutherglen			Yarra Valley		
	Oct 14	Dec 14	Mar15	Oct 14	Dec 14	Mar 15	Oct 14	Dec 14	Mar15
0 to 5	0	34	272	10.6	0.35	6.4	0.28	18	1679
6 to 10	0	0	361	92	0.15	83	0.39	136	2446
11 to 15	3.0	183	70	5.9	1.5	428	2.3	78	3243
16 to 20	0	0	95	0	29	221	19	464	502
21 to 25	0.18*	2.6	341	6.2	0	178	49	190	613
26 to 30	7.0	1507	399	0.35	0	136	0.12	660	492
31 to 35	0.46	3.1	0.3	0	1.2	47	9.6	169	196
36 to 40	88	1411	3.1	45	0.17	114	1.0	354	673
Total	**95.5**	**3140.7**	**1541.4**	**160.1**	**32.4**	**1213.4**	**81.7**	**2069**	**9844**

Note * This sample had 5-month interval between collection and analysis

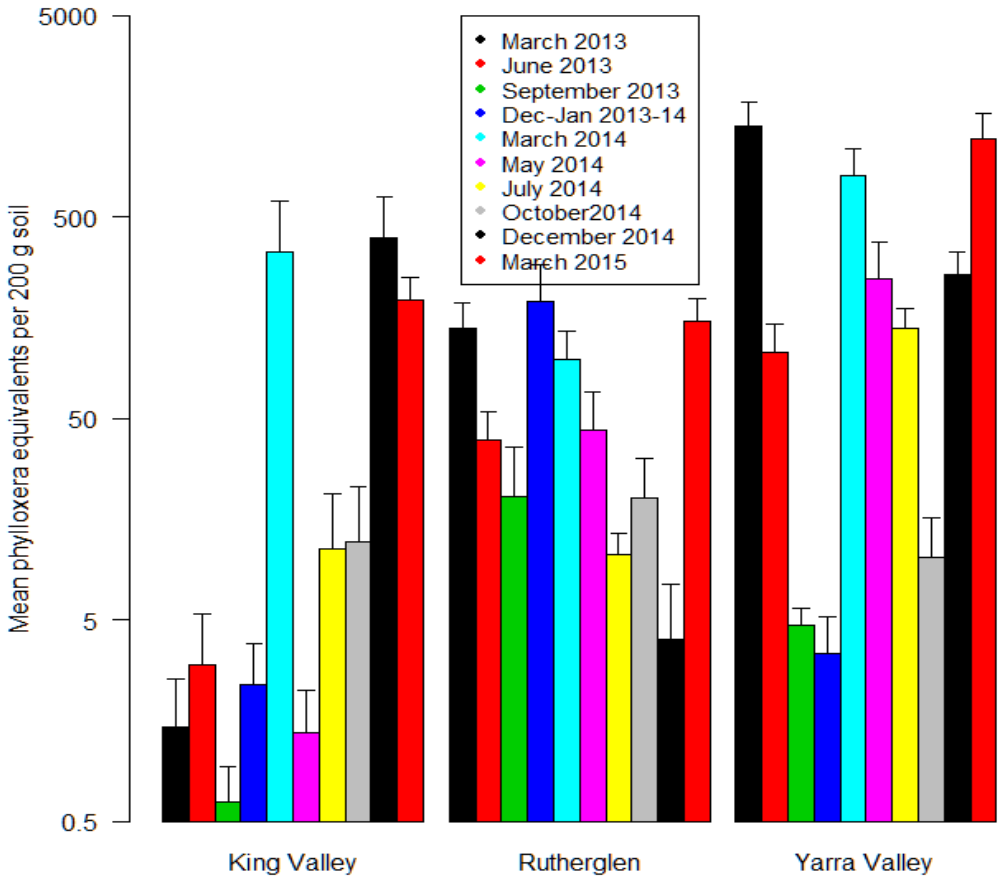

Figure 13. Mean phylloxera equivalents (log y axis) across seasons over a three-year trial in the King Valley, Rutherglen and the Yarra Valley vineyards. Means were calculated across eight composite soil samples each taken close to the trunk of five infected vines, 0-10cm deep. March 2013 (■); June 2013 (■); September 2013 (■); December 2013 (■); March 2014 (■); May 2014 (■); July 2014 (■); October 2014 (■); December 2014 (■); March 2015 (■).

4.4.3. Discussion and conclusion

Detection of phylloxera in soil samples relies on several parameters, among which vineyard location and time of the year have been shown to be important. With the visual root inspection and emergence trap methods, phylloxera detection is highly dependent on seasonal abundance and activity in soil, and both methods have previously shown lower efficiency in winter (Benheim *et al.*, 2012, Powell, 2012a). Results in this study for phylloxera detection by qPCR show that both detection rate and the amount of phylloxera are lower in the King Valley block compared to that in the Yarra Valley and Rutherglen blocks. Such variations between vineyards are not uncommon and could be linked to environmental factors (including soil type), as well as to the phylloxera and vine genotypes (Benheim *et al.*, 2012). As shown in Table 1, phylloxera first detection dates of the sampled vineyards indicated that the King Valley infestation is considerably older than the other two vineyards. This could contribute to a lower detection rate and amount of phylloxera, as vine roots may have been destroyed by phylloxera which have then moved from affected vines in search of fresh vine roots. The most plausible explanation for lower phylloxera levels in the King Valley vineyard, however, is the phylloxera numbers were low (or nil) on the rootstock component of the block, and these rootstock rows were not omitted from the sampling.

Despite these differences in detection rate and amount of phylloxera, the three vineyards showed similar trends in relative frequency of phylloxera detection in terms of sampling time (seasonality), over three years. This contrasts with previous work which reported that qPCR had a lower detection efficiency in winter (Powell, 2012b). The greater detection efficiency observed in our study may be due to the use of composite samples,

which accounts better for uneven distribution across vineyards. Higher phylloxera DNA equivalent were found from late summer to early winter, peaking in autumn, which is consistent with previously described seasonal variations in phylloxera abundance (Benheim *et al*., 2012, Powell, 2012a).

Determining the most likely location of phylloxera in an infested vineyard is an important parameter for designing a sampling strategy for surveillance and is intimately related to phylloxera physiology and life cycle. For instance, because of its feeding habits, phylloxera is usually found on or near vine roots (Benheim *et al*., 2012). In addition, in warm soil (late spring and summer), a higher phylloxera population level is usually found in the top soil (Benheim *et al*., 2012). Phylloxera, however, is also known to move down the soil profile in adverse conditions (Powell, 2012a) and in Australia has been found as deep as one metre below the soil surface (Buchanan, 1990, as cited by Benheim *et al*., 2012).

When comparing sampling position and depth in this study, soil samples collected close to the vine trunk (within 10cm) and below the dripper gave a similar level of phylloxera DNA equivalents, with an overall slightly better detection close to the trunk. Soil samples collected between rows (mid-row) had both a lower frequency of detection and a lower phylloxera DNA equivalent. This difference likely indicates that more vine roots were present in the soil close to the vine trunk and below the dripper than between rows, therefore increasing phylloxera detection. Highest phylloxera detection was achieved within the top 0–10cm of soil. This finding was not affected by time of the year and only slightly by the vineyard location, which suggests that over the survey period, phylloxera position in the soil profile did not vary greatly, even though its abundance changed with the season.

Conclusion: Samples should be taken from the top 10cm of soil and within 10cm of the vine trunk and preferably in autumn.

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4.5. Effect of the presence of vine root on phylloxera detection

A *Vitis* qPCR assay to check for the presence of vine roots in soil became available for use by SARDI MDC in 2015 to test whether this assay could be used as an internal control to add reliability to the phylloxera qPCR test.

4.5.1. *Vitis* qPCR

The *Vitis* qPCR assay measures the amount of vine root DNA in a soil sample, expressed in Cycles to Threshold (Ct) of PCR - a relative value that represents the cycle number (or number of duplications) at which the amount of amplified DNA reaches the threshold level of detection. The higher the Ct value, the greater the amount of duplication of DNA required for detection and therefore the lower the amount of vine root DNA present.

In practice, the duplication is close to but not 100% efficient. Using exact duplication as an approximation, two samples that differ by one Ct unit would therefore differ by a factor of 2 in root DNA concentration. An estimate of the amount of vine root is therefore proportional to:

$$R = 2^{-Ct}$$

The lack of calibration restricts the interpretation of the Ct values – R is therefore in arbitrary units.

4.5.2. 2013/2014

4.5.2.1. Materials and methods

Material analysed was stored DNA extracts collected in 2013/2014 from vineyard soils in the Yarra Valley, King Valley and Rutherglen (4.2, 4.4).

4.5.2.2. Results and discussion

Large variability in the quantity of *Vitis* root DNA was found among the soil samples, with a coefficient of variation of 47% (data not shown). The highest Ct values were found in the Rutherglen soil samples and there was a decline in Ct values in the Yarra Valley samples between March and December (Figure 14). The display of the means based on estimated vine root DNA showed very significant differences ($p<0.0001$) between vineyard locations and between the sampling times in the Yarra Valley (Figure 15). There was a significant effect of distance from the trunk on Ct values, indicative of an exponential decrease in vine root DNA with increasing distance from the vine trunk (Table 18). A large reduction of *Vitis* DNA was found in soil cores taken 30cm and further away from the vine trunk, with significantly lower vine root DNA at the 45cm distance compared to samples taken between 5-15cm from the vine trunk (Table 18). These results validated the distance from the trunk specification for the DNA method field sampling protocol.

For the King Valley, soil samples collected between 5-10cm depth were found to have significantly higher ($p<0.05$) estimated vine root DNA compared to that at the 0-5cm depth (Table 19). This result was not surprising given the relatively common occurrence of surface crusting, higher surface soil temperature and rapid drying out at 0-5cm compared to 5-10cm in vineyards. Therefore, theoretically soil samples for phylloxera DNA analysis could be targeted at 5-10cm soil depth rather than 0-10cm depth. However, this smaller depth is not very practical and would require partitioning cores and more cores to be taken to ensure a minimum sample volume is supplied for analysis. Although an increase in estimated vine root DNA at the 5-10cm was also seen at the Yarra Valley and Rutherglen vineyards, this difference was not significant. The King Valley had

substantially higher estimated vine root DNA levels compared to the Yarra Valley and Rutherglen, but only significant ($p<0.05$) at the 5-10cm sampling depth (Table 19).

The effect of distance from the vine into the headland on the distribution of *Vitis* root DNA was also studied. Ct values were much larger in samples one metre into the headland, indicating an estimated amount of vine root DNA decreased to effectively zero post one metre from the edge of vine plantings (Figures 16, 17). The spread of vine roots into the headland in the Yarra Valley and Rutherglen vineyards was found to be limited to approximately one metre when sampled at a soil depth of 10cm.

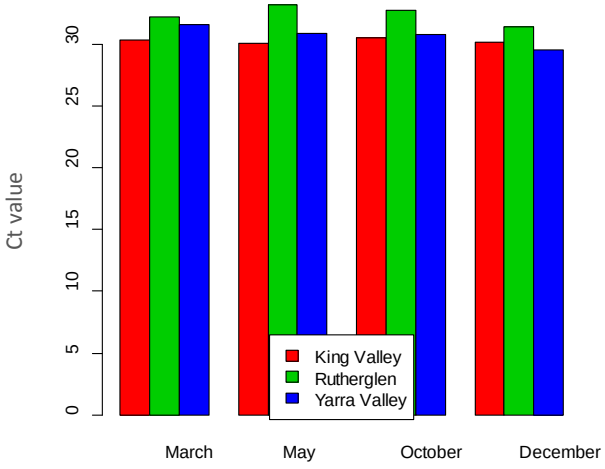

Figure 14. Root DNA Ct values for King Valley, Yarra Valley and Rutherglen vineyards at each sampling time in 2013.

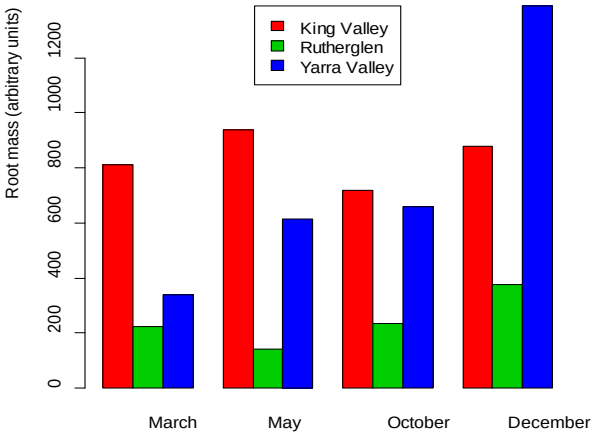

Figure 15. Estimated root DNA (arbitrary units) present in the King Valley, Yarra Valley and Rutherglen vineyards at each sampling time in 2013.

Table 18. Mean estimated amount of root DNA (arbitrary units) in soil samples collected in March 2014 at various distance from the vine trunk in the Yarra Valley, King Valley and Rutherglen. Distances with the same letter are not significantly different at the 95% level.

Distance from vine trunk (cm)	5	10	15	30	45	LSD
Vine root DNA	952 a	971 a	940 a	664 ab	566 b	347

Table 19. Comparison of Ct and estimated vine root DNA (arbitrary units) between 0-5cm and 5-10cm soil depth from samples collected in 2013 in the Yarra Valley, King Valley and Rutherglen.

Vineyard location	Ct at 0-5cm soil depth	Ct at 5-10cm soil depth	Estimated vine root DNA at 0-5cm soil depth	Estimated vine root DNA at 5-10cm soil depth
King Valley	31.61	29.55	337.5	1318.9
Rutherglen	33.30	31.77	119.8	350.5
Yarra Valley	33.41	31.02	95.1	478.3
LSD	1.52		389	

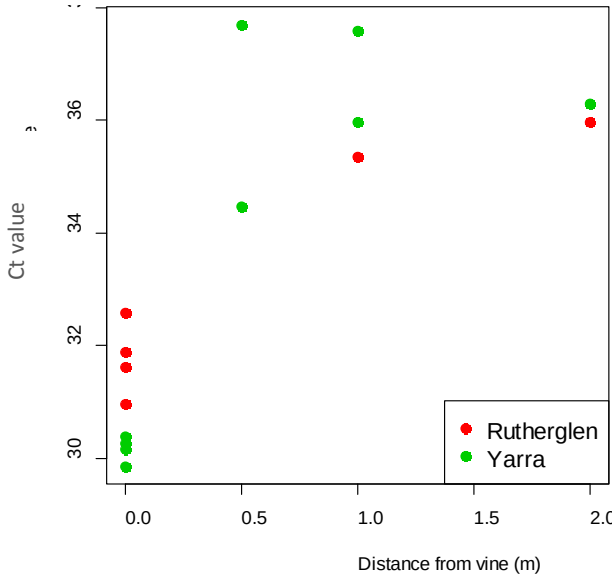

Figure 16. Effect of increasing distance into the headland on root DNA Ct values from soil samples collected in the Yarra Valley and Rutherglen vineyards in 2014.

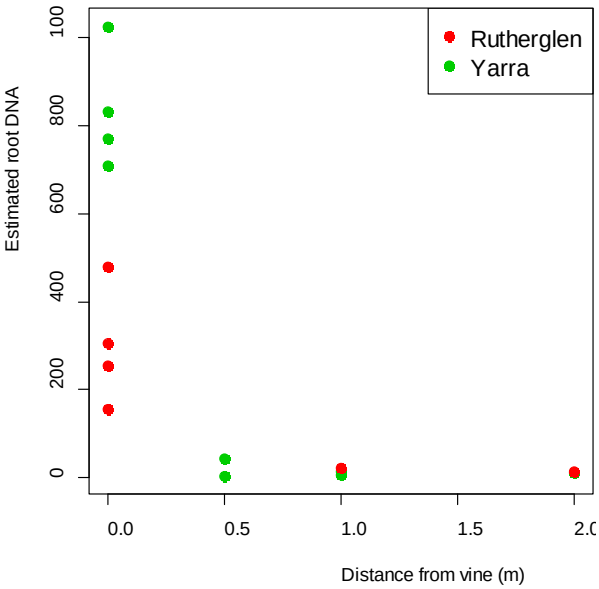

Figure 17. Effect of increasing distance into the headland on estimated root DNA (arbitrary units) from soil samples collected in the Yarra Valley and Rutherglen vineyards in 2014.

Given the requirement of vine roots as a food source for phylloxera, targeting these roots when collecting soil samples for phylloxera testing is likely to increase the chance of detecting phylloxera if present. This trial successfully used the presence of vine root to determine the most likely location around a vine to target core sampling using the DNA method to detect phylloxera, if present. The proposed field sampling protocol of collecting soil samples from within 15cm of the vine trunk and to a depth of 10cm was validated through demonstration of highest vine root density.

4.5.3. February 2015

4.5.3.1. Materials and methods

Composite soil samples collected across 10 vineyard blocks in the Yarra Valley, King Valley and Rutherglen and previously analysed for phylloxera DNA were used (4.9.3). These samples had been collected using the standard sampling method with five single soil cores pooled per composite. As the *Vitis* assay was validated by previous results, SARDI MDC have prepared a calibration standard that was used in the following experiments to convert Ct values into copy numbers of *Vitis* DNA per gram of soil.

4.5.3.2. Results and discussion

The mean *Vitis* copies per gram of soil for composite soil samples collected from the 10 vineyards for which no phylloxera was detected, was 11,726, compared to 22,861 for composite soil samples for which phylloxera was detected (Figure 18). This result presented a significant difference ($p<0.01$). The box plot in Figure 18 shows that there was a considerably larger spread in the number of *Vitis* copies per gram dry soil for the composite soil samples from which phylloxera was not detected of 100 - 35,000 copies (excluding outlier), compared to the relatively tight range for the soils showing positive detections of 4,000 - 40,000 copies (excluding outlier). The presence of vine root in a soil sample is not expected to increase the likelihood of phylloxera detection. However, because of phylloxera's affinity for vine roots, if there is a low level of *Vitis* DNA (as was the case for some of the negative detections in this trial) we can surmise that the sample was not collected within the root zone or did not pick up vine roots and therefore has a lower chance of returning a positive phylloxera detection.

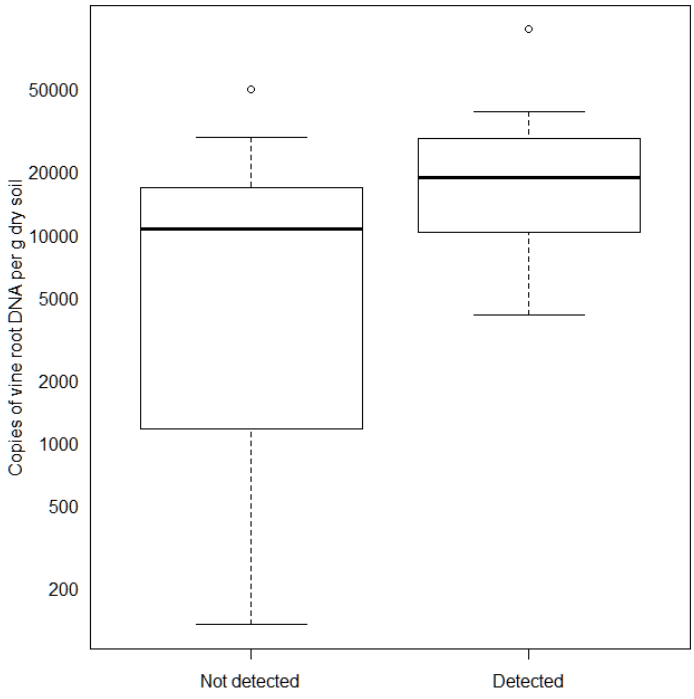

Figure 18. Boxplot showing mean and standard deviation of vine root presence (copies of vine root DNA per g soil). Data represent averaged results for composite samples collected in 2015 across 10 vineyard blocks for which phylloxera was either detected (n = 25) or not detected (n = 25) in each composite.

4.5.4. March 2017

4.5.4.1. Materials and methods

The extracts of soil samples collected composites of five single cores, and single core soil samples, were analysed from three vineyard blocks in the Yarra Valley (Vineyard 2, Vineyards E-2 and E-3) as part of a previous trial (4.3.4).

4.5.4.2. Results and discussion

For the 246 soil samples (a mixture of composite soil samples and single cores) analysed by the DNA method, the mean level of *Vitis* root in the soil samples in which phylloxera was detected was, on average, lower (but not significantly) for two of the three vineyard blocks. This result matched the range of *Vitis* copies reported, which was fewer at the bottom end of the range for the positive detections (Table 20). When the data from the three blocks was pooled, again the mean number of *Vitis* root copies was lower but not significantly, for the soil samples for which phylloxera was detected as opposed to not detected (Table 20). Fewer copies of *Vitis* were also found at the lower end range for the positive detection samples.

Table 20. Comparison of mean vine root copies per g soil and mean phylloxera quantity per 200g dry soil for three vineyards sampled in March 2015. Results are presented as means for the vineyard block across both composite soil samples and individual soil core results. N = number of samples.

	Positive phylloxera detections				**Negative phylloxera detections**			
	N	Phylloxera equivalents per 200g dry soil	*Vitis* copies per g soil	Range *Vitis* copies	N	Phylloxera equivalents per 200g dry soil	*Vitis* copies per g soil	Range *Vitis* copies
Vineyard 2	44	292	12,677	407 - 32,555	70	0	14,803	1,238 - 37,257
Vineyard E-2	22	136	7,961	113 - 30,798	26	0	8,314	676 - 19,221
Vineyard E-3	21	253	14,509	5,144 - 35,782	63	0	12,532	2,567 - 24,150
All data	87	243	11,926	113 - 35,782	159	0	12,813	676 - 37,258

4.5.5. Conclusion

The *Vitis* qPCR assay provides a useful quality control check for soil samples being analysed for phylloxera DNA at the SARDI MDC. If soil samples present with low or no *Vitis* root DNA, the potential for a positive phylloxera detection is reduced and therefore resampling is recommended.

4.6. Effect of sampling density on phylloxera detection

This set of trials were designed to determine whether increasing the density of vines sampled across an area, would also increase the rate of detection of phylloxera. The standard sampling method with a density of approximately 40 vines sampled per hectare was repeated once, twice or three times across a sampled area to provide single, double or triple sampling density comparisons.

4.6.1. Single vs double sampling density trial – July 2014

4.6.1.1. Materials and methods

A single vineyard in the Yarra Valley (Vineyard 1) was sampled as per the standard sampling method previously described (4.1.2) on a total of 40 vines. Individual soil cores were pooled as five cores per composite sample, to make a total of eight composite samples, designated “A series”.

A second set of eight composited samples (“B series”) were collected from 40 vines in the same rows, offset by two panels from the A series (Figure 19). Samples collected therefore enabled the comparison of phylloxera detection from eight and 16 composite samples per hectare.

Samples were processed for DNA extraction and phylloxera detection by qPCR, as described in 4.1.

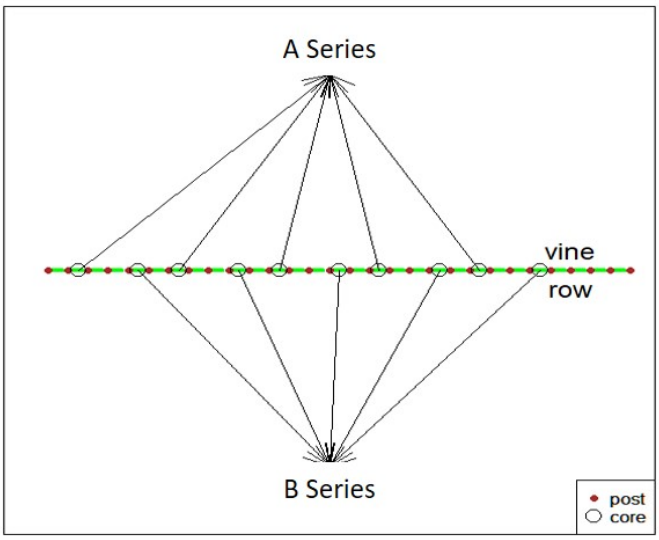

Figure 19. Schematic showing the pattern of sampling for A Series and B Series in July 2014.

4.6.1.2. Results and discussion

There were four composite samples out of eight in which phylloxera was detected in the A series, and three out of eight in the B series (Table 21). There was one composite sample for which phylloxera was detected in the B Series but not in A (Table 22). Conversely, there were two composite samples for which phylloxera was detected in the A series but not in B series (Table 22). More agreement between the two series would have been expected had consecutive panels been infested down a row. The sample size was too small to quantify the degree of concordance. Doubling the sampling density in this single vineyard trial increased the phylloxera detection rate from 44% to 63%.

Table 21. Comparison of phylloxera detections using two different sampling densities in a Yarra Valley vineyard in July 2014.

	A series	B series	Average	A and B series combined
Number cores/composite sample	5	5	5	5
Number of composite samples	8	8	8	16
Number of detections	4	3	3.5	5
Detection %	50%	38%	44%	63%

Table 22. Amount of DNA detected in A and B samples, where samples were offset by two panels. Units are phylloxera equivalents per 200g dry soil.

	Phylloxera DNA equivalents per 200g dry soil	
Set of vines	**A series**	**B series**
Composite 1	0.42	0.00
Composite 2	144.34	0.12
Composite 3	43.86	21.70
Composite 4	0.00	4.33
Composite 5	0.00	0.00
Composite 6	1.31	0.00
Composite 7	0.00	0.00
Composite 8	0.00	0.00

4.6.1.3. Conclusion

In this single vineyard, both the A and B series each detected phylloxera at the vineyard level, therefore doubling the sampling density did not change the phylloxera status at the block level. However, doubling the number of soil cores collected in the sampled area did increase the rate of phylloxera detection from 44 to 63%, providing perhaps a truer indication of the level of infestation across the block, as well as possibly some insights into the pattern of infestation. The small sample size of this trial however, reduces the emphasis that can be placed on the results and therefore this trial provided the basis upon which to run a larger sampling density trial detailed in 4.6.2.

4.6.2. Single versus double versus triple sampling density trial - October 2014

4.6.2.1. Materials and methods

The trial undertaken in July 2014 was repeated in October 2014 on two vineyards in the Yarra Valley (Vineyard 1, Vineyard 2). A larger area was sampled, with 15 composite samples from 75 vines collected rather than eight per sampling time.

Three sets of composite samples were collected from each vineyard. The “A series” was 75 sampled vines at the standard rate of 40 per hectare as previously described in 4.1.2. The “B series” was a second set of 75 sampled vines, each vine offset by two panels from the A series set of vines, and the “C series” collected from

75 vines, also each offset by two panels per vine from the B series set of vines. All series were sampled from the same rows and each set of five single vines composited.

This enabled comparison of 15, 30 and 45 composite samples collected across the same sampling area.

Samples were processed for DNA extraction and phylloxera detection by qPCR, as described in 4.1.

4.6.2.2. Results and discussion

Phylloxera detection rates for Vineyard 1 in October 2014 were lower for all three-series compared to detection rates on the same vineyard in July 2014 (Tables 22, 23). For this vineyard in October 2014, combining the detection rates from all three-series increased the detection rate by almost a factor of three (7% to 20%) (Table 24). However, the actual proportion of composite samples in Vineyard 1 in which phylloxera was detected was very low. Importantly in the C series, phylloxera was not detected in any of the 15 composite samples analysed (Tables 23, 24). The phylloxera detection rates from each of the three series in Vineyard 2 were higher than all series at Vineyard 1 (Table 23). The effect of tripling the sampling density in Vineyard 2 increased the detection rate from 31% to 47% (Table 24).

Combining data from both vineyards indicated that tripling the sampling density increased the phylloxera detection rate from 19% to 33% (Table 24). There was an apparent differential effect of increasing sampling density between the two vineyards.

The impact of increasing sampling density was more pronounced in Vineyard 1 for which phylloxera detection rate was lower across the sampled area and one of the series failed to detect phylloxera. Therefore, in Vineyard 1, had the C series been the only sampling undertaken, the vineyard would have been incorrectly reported as free of phylloxera. In this case, doubling the number of vines from which soil cores were collected by using either of the two series across the sampling area, would have resulted in the vineyard being correctly declared as infested. For Vineyard 1, conducting triple the standard sampling density would not have provided a different whole of vineyard result than doubling the number of vines tested. For Vineyard 2 at the vineyard level, because phylloxera was detected in all three series, sampling double or triple the number of vines would not have changed the block level result and therefore, single sampling density would have been sufficed.

Table 23. Amount of DNA detected in Series A, B and C composite samples across two Yarra Valley vineyards in October 2014. Units are phylloxera equivalents per 200g dry soil.

Vineyard 1					Vineyard 2				
Set of vines	Composite			Average	Set of vines	Composite			Average
	Series A	Series B	Series C			Series A	Series B	Series C	
Composite 1	0.0	0.0	0.0	0.0	Composite 1	0.0	0.0	0.9	0.3
Composite 2	62.8	0.0	0.0	20.9	Composite 2	0.0	0.0	0.0	0.0
Composite 3	0.0	0.0	0.0	0.0	Composite 3	0.0	0.0	0.0	0.0
Composite 4	0.0	1.0	0.0	0.3	Composite 4	0.0	0.0	0.0	0.0
Composite 5	3.7	0.0	0.0	1.2	Composite 5	0.0	0.0	0.0	0.0
Composite 6	0.0	0.0	0.0	0.0	Composite 6	0.0	0.0	0.0	0.0
Composite 7	0.0	0.0	0.0	0.0	Composite 7	0.0	0.0	0.0	0.0
Composite 8	0.0	0.0	0.0	0.0	Composite 8	0.0	0.0	0.0	0.0
Composite 9	0.0	0.0	0.0	0.0	Composite 9	0.0	0.0	0.0	0.0
Composite 10	0.0	0.0	0.0	0.0	Composite 10	0.01	0.0	0.0	0.003
Composite 11	0.0	0.0	0.0	0.0	Composite 11	2.9	0.4	0.0	1.1
Composite 12	0.0	0.0	0.0	0.0	Composite 12	0.9	0.0	16.4	5.8
Composite 13	0.0	0.0	0.0	0.0	Composite 13	0.8	256.9	13.9	90.5
Composite 14	0.0	0.0	0.0	0.0	Composite 14	27.1	63.3	2.0	30.8
Composite 15	0.0	0.0	0.0	0.0	Composite 15	39.7	5.6	7.1	17.5

Table 24. Comparison of phylloxera detections using three sampling densities at two Yarra Valley vineyards (Oct 2014).
Single density = A, B, or C series individually. Double density = A+B series, A+C series, or B+C series. Triple density = A+B+C series.

Vineyard 1	A series	B series	C series	Average	A, B and C series combined
Number cores/composite sample	5	5	5	5	5
Number of composite samples	15	15	15	15	45
Number of detections	2	1	0	1	3
Detection %	13%	7%	0%	7%	20%
Vineyard 2					
Number cores/composite sample	5	5	5	5	5
Number of composite samples	15	15	15	15	45
Number of detections	6	4	4	4. 7	7
Detection %	40%	27%	27%	31%	47%
Combined Vineyard 1 and 2					
Number cores/composite sample	5	5	5	5	5
Number of composite samples	30	30	30	30	90
Number of detections	8	5	4	5. 7	10
Detection %	27%	17%	13%	19%	33%

4.6.2.3. Conclusion

For Vineyard 1, a sample size greater than the single sampling density rate of 40 cores per hectare was required to prevent the vineyard being falsely labelled as phylloxera free. The 'standard' sampling density alone was however sufficient to determine an accurate phylloxera status for Vineyard 2. The variations in these results can be attributed to a likely higher infestation level in terms of both number of infested vines in the sampled area, and that the infestation was relatively contiguous down vine rows in Vineyard 2 compared to Vineyard 1. Increasing the sampling density is therefore a balance between the time (cost) of collecting the extra samples and the benefits the extra samples might deliver in terms of a whole of block phylloxera status determination.

4.6.3. Single versus double sample density trial – March 2015

4.6.3.1. Materials and methods

Nine vineyards across Yarra Valley, King Valley and Rutherglen were selected with presumed low phylloxera infestation levels based on the preliminary survey conducted in 4.9.

Two sets of composite samples were collected from each vineyard. The "A series" was collected at the standard rate of 40 cores per hectare as previously described, with one core collected per vine and 10 cores per composite. A total of 34 composite samples from 340 vines were collected over the nine vineyards.

The "D series" consisted of a resample of the same 340 vines in the A series, plus a second set of 340 vines sampled in the same row but offset by two panels, like the B series described above. This resulted in a total of 680 vines sampled, one core per vine. This double density therefore gave 20 cores for the D series which were equivalent to the 10 cores of the A series and represent the same set of 10 vines. These 20 cores were analysed as two separate samples of 10 cores, both equivalent to half the A series. However, the results of the two separate samples were combined into one result representing the 20 cores so that if either of a pair of D samples was positive, that pair was considered as positive.

For each vineyard in the trial, the data were analysed as a matched pair of A and D samples. The number of phylloxera detections was recorded in each A composite sample and in each pair of the D composite samples. In some cases, where there was variation from the specified sampling protocol, it was not possible to match every A series composite sample with the corresponding two D series samples – in those cases only samples that could be matched were included in the assessment.

4.6.3.2. Results and discussion

Across the landscape level of nine vineyards, the overall detection rate was 12 out of 34 samples from the A series, compared with 14 out of 34 samples for the corresponding D series (Table 25). There was therefore a 6% increase in the detection rate by doubling the sampling intensity. From a landscape level, the fact that phylloxera was detected at least once in the single sampling density (A series), meant the overall phylloxera status of the landscape area did not change with doubling the sampling density.

Doubling the sampling density (D series) did result in phylloxera detections in some composite samples from Vineyards D-2 and E-3 which were not detected in the single sampling density (Table 25). This result could then be important at the individual vineyard level for ascertaining level of infestation. Because the A series at these two vineyards had already detected phylloxera, the additional positive composites at these sites did not change the overall phylloxera status of these vineyards.

Table 25. Comparison of phylloxera detections at the composite sample level using two different sampling densities at nine vineyards in March 2015. Number cores per composite was 10.

	Series A			Series D		
Vineyard	**Number composite samples with positive phylloxera detections**	**Total number composite samples**	**Detection %**	**Number composite paired samples with positive phylloxera detections**	**Total number paired composite samples**	**Detection %**
Vineyard A-1	4	4	100	4	4	100
Vineyard A-3	4	4	100	4	4	100
Vineyard D-1	0	4	0	0	4	0
Vineyard D-2	2	4	50	3	4	75
Vineyard D-3	0	4	0	0	4	0
Vineyard E-3	1	3	33	2	3	66
Vineyard E-4	1	4	25	1	4	25
Vineyard H-1	0	3	0	0	3	0
Vineyard 1	0	4	0	0	4	0
TOTAL	**12**	**34**		**14**	**34**	

4.6.3.3. Conclusion

From a landscape level or block level, doubling the sampling density importantly did not change the phylloxera status result. However, doubling the sampling density for vineyards D-2 and E-3 did result in additional positive detections. In a vineyard with a very low level of infestation, that increased in sensitivity could be important in advising whether the vineyard is considered infested or not.

The small gain in phylloxera detection from a landscape perspective between single and double density sampling across the 9 vineyards sampled, would not have been worth the additional field and laboratory costs for sample collection and processing.

4.6.4. Single versus double versus triple sampling density trial – March 2017

4.6.4.1. Materials and methods

One vineyard in the Yarra Valley (Vineyard 2) was sampled according to the standard sampling density as described in 4.6.2.1. Three sets of composite samples of five cores per composite were collected: A, B and C series, each at the standard sampling density but with sampled vines offset from each other down a row. This enabled comparison of 3, 6 and 9 composite samples collected across the same sampling area, chosen to represent an area of low phylloxera infestation in the vineyard block.

4.6.4.2. Results and discussion

From a whole of sampled area perspective, two of the three series detected phylloxera (Table 26). Had only the vines been sampled as part of the A series; the phylloxera status of that vineyard section would have incorrectly been assigned as phylloxera free. When comparing the composite results across the series which

were offset by two or three panels compared to A series, positive detections were not consistently recorded down each vine row, therefore being indicative of a low infestation (Table 27). In addition, this is coupled with relatively low number of phylloxera equivalents per 200g dry soil for any composites that were positive for phylloxera (Table 27).

As the single density of series A did not detect phylloxera, one of series B or C was required in addition to series A to detect phylloxera. Therefore, in these sampled rows of low phylloxera infestation, double sampling density was required to detect phylloxera. There was no improvement in the phylloxera status level for the sampled area by increasing sampling to triple density as both series B and C detected phylloxera. Triple sample density only gave a slight improvement in the spatial location of the infestation.

Table 26. Comparison of composite sample phylloxera detections across one vineyard in the Yarra Valley at standard sampling density. Single density = A, B, or C series individually. Double density = A+B series, A+C series, or B+C series. Triple density = A+B+C series.

	A series	B series	C series	Average	A, B & C series combined
Number cores/composite sample	5	5	5	5	5
Number of composite samples	3	3	3	3	9
Number of detections	0	1	2	1	2
Detection %	0%	33%	66%	33%	66%

Table 27. Amount of DNA detected in Series A, B and C composite soil sample from Yarra Valley vineyard in March 2017. Units are phylloxera equivalents per 200g dry soil.

	Series A	Series B	Series C
Composite 1	0.00	23.22	9.07
Composite 2	0.00	0.00	0.09
Composite 3	0.00	0.00	0.00

4.6.4.3. Conclusion

With one of the three series not detecting phylloxera, doubling the sampling density was required to record a positive for the sampled area. Tripling the sampling density did not change the phylloxera status of this area but did increase the detection rate, on average, from 33 to 66%, providing perhaps a truer indication of the level of infestation across the sampled area, as well as possibly some insights into the pattern of infestation. The small sample size of this trial reduces the emphasis that can be placed on the results, but the fact that it was conducted on vines of low phylloxera infestation, provides another source of validation that increasing the sampling density above the standard 40 cores per hectare is likely suitable in areas where the chance of a phylloxera detection is low.

4.6.5. Overall conclusion

In the trials conducted, there was no advantage in terms of phylloxera detection at the vineyard level in tripling the sampling density, but importantly, there were instances in two of the four trials where infestation levels were very low, and doubling the sampling density changed the phylloxera status of the vineyard. We cannot rule out the possibility that in other situations of extremely low phylloxera infestation levels, triple the sampling density could be warranted.

This set of trials only considered the DNA method for analysing phylloxera presence. No trials as part of this project were undertaken to analyse the effect of sampling density on either the emergence trap or visual root inspection detection methods. These studies are recommended to provide viable input into surveillance plans for a wine region or state.

4.7. Nematode detection from vineyard soil samples

Given the cost and time to collect and analyse soil samples using the DNA method, the ability to use these soil samples to test for other pests or diseases concurrently, has the potential of returning greater benefit for only a relatively small additional cost.

Root-knot nematode (RKN) are plant-parasitic nematodes, some of which being significant pests in some commercial vineyards, negatively impacting vine health and productivity. The SARDI MDC has pre-existing soil tests (Stirling *et al*., 2004, Hay *et al*., 2016) for some of these nematodes species, and these were used on a series of soil samples collected as part of this project to determine whether the presence of RKN could be verified, despite the samples being collected using a protocol to facilitate phylloxera detection.

4.7.1. Materials and methods

Testing for RKN was undertaken on:

- Eight composite soil samples collected during October 2014 sampling from Yarra Valley, King Valley and Rutherglen vineyard (Table 1);
- 12 composite soil samples from five vineyards each in different South Australian wine regions (Mundulla, McLaren Vale, Langhorne Creek, Limestone Coast, Riverland) in October 2014, collected separately from the phylloxera trials but using the same sampling method; and
- 18 triplicate composite samples (total 54) collected from Vineyard 1, and 12 triplicate composite samples (total 36) collected from Vineyard 2; both in the Yarra Valley in October 2014.

Three tests for root-knot nematodes were used, one for *Meloidogyne fallax,* one for *Meloidogyne hapla* and one for *Meloidogyne* spp that covers *M. javanica, M. incognita* and *M. arenaria*.

Statistical analysis looked at the per cent detection (frequency of detection) and for Vineyards 1 and 2, the consistency of the per cent detection.

4.7.2. Results

4.7.2.1. Frequency of detection

A summary of the detection frequency of each nematode at each sampled vineyard is given in Table 28. *Meloidogyne fallax* was not detected in any location. The number of composite soil samples per vineyard ranged from one at Langhorne Creek to 54 in Vineyard 1, and the low number of samples could have contributed to lack of detection at some vineyards. Average detection rates were not meaningful due to the uneven sample numbers.

Table 28. Frequency of detection of each nematode group in each location.

State	South Australia					Victoria				
Site	**Barossa**	**Langhorne Creek**	**McLaren Vale**	**Limestone Coast**	**Riverland**	**Vineyard 2**	**King Valley**	**Rutherglen**	**Vineyard 1**	**Yarra Valley**
Number of composite samples	3	1	3	3	2	36	8	8	54	8
Taxon	**Frequency of detection**									
Meloidogyne spp.	0%	100%	100%	0.00	100%	3%	0%	0%	19%	0%
Meloidogyne hapla	0%	100%	67%	0%	0%	0%	0%	0%	2%	0%
Meloidogyne fallax	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Meloidogyne. spp includes *M. javanica, M. incognita* and *M. arenaria*

The frequency of detection of *Meloidogyne spp.* across each vineyard was calculated where the frequency was taken as the number of vineyards where that taxon had been detected divided by 10 – the number of vineyards sampled. The frequency ranged from 0 to 50% in all sites, and 0 to 11% in the triplicate sites (Table 29).

Table 29. Frequency of detection (%) of each nematode group across all sampled vineyards and across Vineyards 1 and 2 combined.

Taxon	All vineyards	Vineyards 1 and 2 only for which there was triplicate sampling
Meloidogyne spp.	50%	11%
Meloidogyne hapla	30%	2%
Meloidogyne fallax	0%	0%

Meloidogyne. spp includes *M. javanica, M. incognita* and *M. arenaria*

4.7.2.2. Consistency of detection and quantification

In the triplicate sampling in Vineyards 1 and 2, four of the triplicate composites (2, 6 and 11 in Vineyard 1 and 5 in Vineyard 2) returned inconsistent results within the triplicate (Table 30). *Meloidogyne spp.* was detected in two of three triplicate samples for Composite 2 and 11 in Vineyard 1, and the same species was detected in one of three triplicate samples for Composite 6 in Vineyard 1 and Composite 5 in Vineyard 2. Results for *Meloidogyne fallax* have been excluded due to no detection.

Table 30. Occurrences of each nematode type at each sample site at Vineyard 1 (18 sites) and Vineyard 2 (12 sites).

	Number of detections per triplicate sample for each composite																	
Composite number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Vineyard 1																		
Meloidogyne spp.	3	2	0	0	0	3	0	0	0	0	2	0	0	0	0	0	0	0
Meloidogyne hapla	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Vineyard 2																		
Meloidogyne spp.	0	0	0	0	1	0	0	0	0	0	0	0						
Meloidogyne hapla	0	0	0	0	0	0	0	0	0	0	0	0						

Meloidogyne. spp includes *M. javanica, M. incognita* and *M. arenaria*

4.7.3. Discussion and conclusion

The soil samples used to analyse for the presence of phylloxera were successfully used to effectively detect RKN; although only a few vineyards showed detections. Free-living nematodes, often used as an indicator of soil health, were also detected in most vineyards (data not presented). This trial did not evaluate the vineyard sampling method used for phylloxera as a method of assessing levels of RKN in a vineyard, or the possibility of non-detection. Therefore, while detection in the soil sample is possible, no inferences about level in the vineyard can be made.

RKN and other plant-parasitic nematodes may be the cause of vine weakness, and it would be possible to value add to the phylloxera test by also analysing soil samples for these nematodes as a mechanism to determine the potential causes of the weak vines if not due to phylloxera.

Conclusion: the composite samples collected for phylloxera testing are suitable for nematode detection.

4.8. Detection of phylloxera in headlands

This work was undertaken to determine how far phylloxera could be detected into the headland away from an infected vine at the end of a row.

4.8.1. Materials and methods

Two vineyard blocks were sampled, one in Rutherglen and one in Yarra Valley used in previous experiments (Table 1). To establish that the vines at the end of the rows were infested, two composite samples of 15 cores were taken to a depth of 10cm, one from the end vines and one from the penultimate vines of 15 adjacent rows at either end of each vineyard. Five composite samples were then taken (one core per row), at the distances of 0.5m, 1m, 2m, 4m and 10m outwards from the end strainer post into the headland for each row end of the two vineyards.

Samples were processed for DNA extraction and phylloxera detection by qPCR, as described in 4.1.

The amount of phylloxera DNA was modelled assuming a negative exponential decline with increasing distance from the vineyard using the NLS function in R.

4.8.2. Results

Phylloxera was detected in at least one of the two end vines at either row end of both vineyard blocks (Table 31). There was a detection at the Rutherglen vineyard 0.5m into the headland at one vine end, and in the Yarra Valley vineyard, at 1m into the headland at one vine end. There were no detections at 2m or more into the headland from the end vine at either vineyard.

Table 31. Frequency of detection of phylloxera in headlands of two known infested vineyards.

Distance from end vine (m)	Rutherglen row end 1	Rutherglen row end 2	Yarra Valley row end 1	Yarra Valley row end 2
0 (end 2 vines)[1]	0.5	0.5	0.5	1
0.5	0	1	0	0
1	0	0	0	1
2	0	0	0	0
4	0	0	0	0
10	0	0	0	0

[1]Frequency of detection of 0.5 indicates that phylloxera was detected in either the composite sample from the end vine or the penultimate vine, and 1 indicates phylloxera was detected in both samples.

4.8.3. Discussion and conclusion

Phylloxera was not detected in the headlands at two or more metres past the end vine at a depth of 10cm, in either of the two blocks sampled. As this trial was conducted on two vineyard blocks, it is not representative of all parameters that potentially influence vine root growth, for example, soil type, topography, grape variety, rootstock, soil moisture status, there are no recommended changes to farm-gate hygiene practices or surveillance based on this trial alone. Results do however provide an insight into the potential spread outwards of phylloxera from an infested vineyard block.

4.9. Comparative trials

The currently-accepted and only endorsed practice documented in the NPMP (NVHSC, 2009) to detect phylloxera in a vineyard involves visual inspection of the roots of vines using the standard sampling method of inspection of a single vine in every fifth panel of every 3rd row. This practice also notes the importance of assessing visually weak vines separately from those vines systematically sampled.

Another method largely used by researchers at present to evaluate phylloxera spread on a block basis, includes the trapping of phylloxera in emergence traps (4.1.4). Together with the DNA detection method developed through this project, there is a need for both additional detection methods to become endorsed and included in the NPMP.

To allow these alternative methods to be endorsed as standard methods for detection, they must demonstrate equivalent or better sensitivity to visual root inspection. Results of the comparative trials herein have been undertaken to provide the basis for gaining endorsement for all three methods (visual, trap and DNA) to be included as primary phylloxera detection methods under the NPMP for use by growers and regulators to determine where phylloxera is and is not.

Powell and Bruce (2011) conducted an evaluation of the same three phylloxera detection methods on a single infested vineyard in the Yarra Valley across three successive growing seasons. In the first two seasons of this study, the DNA and emergence trap methods were shown to have higher detection rates on average, than the visual root inspection method. In the third year, however, the visual root inspection method was as effective as the DNA method and more effective than the emergence trap method. The recommendation from this single vineyard study was to validate the DNA and emergence trapping techniques for a range of vineyards of different soil type, at multiple time points in the growing season, on both grafted and ungrafted grapevines with low levels of phylloxera infestation.

As a result, the range of comparative studies reported herein address the following key factors:

- detection rates on vineyards considered to have low levels of phylloxera;
- level of incorrect detections;
- costs associated with each method including field sampling, sample handing and processing;
- turnaround time of results; and
- the stage during the detection method at which expertise is required

Three comparative studies were conducted to compare the different primary detection methods:

(i) DNA compared to visual root inspection
(ii) DNA compared to visual root inspection and emergence traps
(iii) DNA compared to emergence traps

4.9.1. Materials and methods

All three detection methods were carried out at the same sampling density specified in the NPMP as the ‘standard’ sampling density and using the procedures outlined in 4.1.2 to 4.1.5. Composite soil samples were processed for phylloxera by qPCR, as described in 4.1.

In addition, visually weak vines (e.g. poor shoot growth, shortened internode spacing, light leaf colour, thin shoots) in the sampling area were also identified and sampled. Weak vines were analysed on an individual vine basis for the emergence trap and visual root inspection methods but as part of composite samples for the DNA method.

All three detection methods sampled the same set of vines. The visual root inspection results were classified as '0' for no phylloxera detected or indeterminate, '1' for where phylloxera was detected and '0.5' for where root galling was identified but without the presence of phylloxera. As a field sampling method, traditionally once a positive vine is detected, the survey ceases. For these trials, visual root inspection was undertaken at all sampled vines to allow for direct comparison of results with the other two methods.

Emergence traps were placed within 10cm from the base of each sampled vine and the contents recovered approximately 2 weeks after placement.

Where the number of phylloxera detections made by one method and not by another method are presented, the difference between those two numbers was compared using a chi-squared test, McNemar's test (McNemar, 1947).

4.9.2. Comparison of DNA and visual root inspection

4.9.2.1. Materials and methods

Forty vines were inspected by visual root inspection and soil sampled for DNA analysis in a vineyard in King Valley (Table 1) in March 2013 and repeated in March 2015 on the same vines. Soil cores were bulked into 8 composite samples of 5 cores.

Results of the visual root inspection were compared between the two years on a per vine basis. However, to enable direct comparison of phylloxera detections using the DNA method versus the visual root inspection method, the scored results of the visual root inspection were summed across the five vines that were represented by each composite soil sample.

4.9.2.2. Results and discussion

Of the 40 vines sampled, 32 vines were on Schwarzmann rootstock, six were own-rooted and for two vines the rootstock was classified as unknown. Phylloxera was detected on all six own-rooted vines at both sampling times and only on one of the two vines of unknown rootstock (Table 32). Phylloxera detections for the vines planted to rootstock were inconsistent across the two sampling times.

There were 14/32 (44%) rootstock vines with the same rating at both sampling times (Table 32). Ten of the rootstock vines were rated as phylloxera detected in March 2015, after a rating of no phylloxera detected in March 2013 and two rootstocks vines rated as phylloxera detected in March 2015, after being rated as having galls in 2013 without the presence of phylloxera (Table 32). There were six rootstock vines for which the phylloxera rating lowered over time – four of those initially rated as phylloxera infested and then in March 2015 rated as non-infested, and two vines rated as having root galls but no phylloxera in 2013 to being rated as no phylloxera detected in March 2015 (Table 32).

There was agreement in the scorings for only 22/40 vines (55%) across the two sampling times (Table 33). In 2013, phylloxera was detected visually in 11 of the 40 vines (28%) but undetected visually in the remainder of vines whether galling was recorded or not (72%) (Table 33). In March 2015, phylloxera was detected visually in 19 of the 40 vines (48%) but undetected visually in the remainder of vines (52%) (Table 33).

These results are indicative of new infestations, indicating phylloxera spread across the sampled area over time.

When comparing the results of the phylloxera detections using the DNA method versus the visual root inspection method at a composite level in March 2013, four set of comparative vine samples were negative and three positive for both methods in 2013. One composite sample (vines 11-15) did not align (Table 34), with visual root inspection determining the presence of phylloxera at one of the individual vines within the composite where the DNA method did not detect this positive in the analysed composite sample, therefore providing a false negative of the five vines this composite represents. It is likely that at this single infested vine the level of phylloxera presence was low, perhaps confined to an isolated colony, and the soil core collected at this vine by chance did not target the location of this phylloxera colony.

For March 2015, there was full agreement between both the DNA method and the visual root inspection method at a composite level (Table 34).

When comparing the amount of phylloxera DNA present in samples to the visual root inspection score, a poor relationship was found for both sampling times (Figures 20 and 21), especially in March 2015, possibly due to the very large range of phylloxera equivalents found in the composite samples at this sampling time (0.3 to 399 phylloxera equivalents per 200g dry soil).

Table 32. Results of visual root inspections in March 2013 and March 2015. Rating of '0' indicates no phylloxera detected or indeterminate, '1' indicates phylloxera detected and '0.5' indicates the presence of root galling but absence of phylloxera.

Vine	Row	Panel	Stock	March 2013	March 2015
				Rating	Rating
1	40	3	Rootstock	0	0
2	40	4	Rootstock	0	0
3	40	5	Rootstock	0	0
4	40	7	Rootstock	0	0
5	40	8	Rootstock	0	1
6	40	11	Rootstock	0	0
7	41	9	Rootstock	0	0
8	41	7	Rootstock	0	1
9	41	5	Rootstock	0	1
10	41	3	Unknown	0	0
11	42	3	Rootstock	0	1
12	42	4	Rootstock	0.5	0
13	42	6	Rootstock	0.5	1
14	42	7	Unknown	1	1
15	42	8	Rootstock	0.5	1
16	43	9	Rootstock	0	0
17	43	8	Rootstock	0	1
18	43	7	Rootstock	0	1
19	43	4	Rootstock	0	0
20	43	2	Rootstock	0	1
21	44	2	Rootstock	0	1
22	44	4	Rootstock	0	0
23	44	5	Rootstock	0	0
24	44	6	Rootstock	0	0
25	44	8	Rootstock	0	0
26	44	9	Own roots	1	1
27	44	10	Own roots	1	1
28	44	10	Own roots	1	1
29	44	11	Own roots	1	1
30	44	14	Rootstock	1	0
31	45	24	Rootstock	0	0
32	45	18	Own roots	1	1
33	45	18	Rootstock	0	1
34	45	15	Rootstock	0	0
35	45	13	Rootstock	0	1
36	45	9	Rootstock	1	0
37	45	7	Rootstock	0.5	0
38	45	5	Rootstock	1	0
39	45	4	Rootstock	1	0
40	45	2	Own roots	1	1

Table 33. Comparison of detection ratings at the vine level by visual root inspection between March 2013 and March 2015. Rating of '0' indicates no phylloxera detected or indeterminate, '1' indicates phylloxera detected and '0.5' indicates the presence of root galling but absence of phylloxera.

March 2015	March 2013			Total
	Phylloxera detected	No phylloxera detected or indeterminate	No phylloxera but root galling present	
Phylloxera detected	7	10	2	19
No phylloxera detected or indeterminate	4	15	2	21
Total	11	25	4	40

Table 34. Comparison of DNA concentrations (phylloxera equivalents per 200g dry soil) and visual root inspection scores at two sampling times, March 2013 and 2015 based on composite samples.

Vine number as a composite	Number of vines on own roots	March 2013		March 2015	
		Summed visual root inspection score	DNA method (phylloxera equivalents per 200g dry soil)	Summed visual root inspection score	DNA method (phylloxera equivalents per 200g dry soil)
0 to 5	0	0	0	1	272
6 to 10	0	0	0	2	361
11 to 15	0	2.5	0	4	70
16 to 20	0	0	0	3	95
21 to 25	0	0	0	1	341
26 to 30	4	5	1.3	4	399
31 to 35	1	1	8.9	3	0.3
36 to 40	1	4.5	1.5	1	3.1

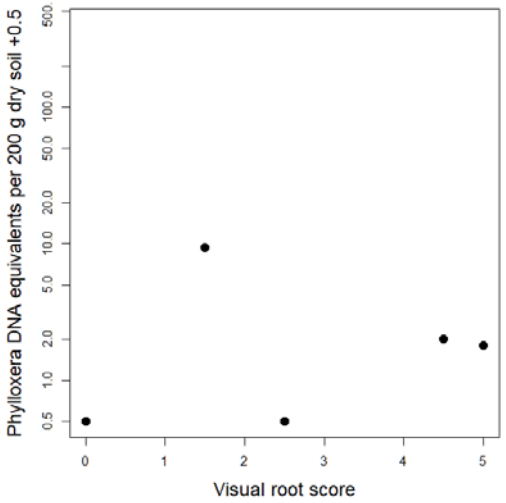

Figure 20. Relationship between visual root inspection score and DNA present (phylloxera equivalents per 200g dry soil) for composite samples assessed in March 2013.

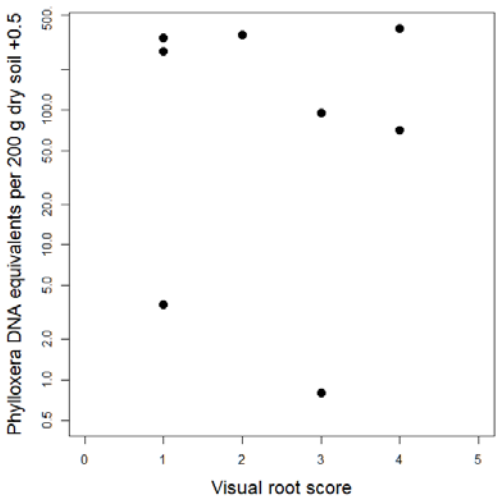

Figure 21. Relationship between visual root inspection score and DNA present (phylloxera equivalents per 200g dry soil) for composite samples assessed in March 2015.

4.9.2.3. Conclusion

An increase in the detection rate of phylloxera at the vine level by the visual root inspection method from 2013 to 2015 was reported and there was strong agreement between phylloxera presence or absence as determined by the visual root inspection and DNA methods at the composite sample level. Visual root inspection results were a poor predictor of the amount of DNA present in a soil sample.

4.9.3. Comparison of DNA, emergence trapping and visual root inspection

Trials conducted as part of this project prior to September 2015 had demonstrated the sensitivity and accuracy of the DNA method to detect phylloxera. However, it was important to show that the DNA method was at least equivalent to, if not more sensitive than the currently endorsed visual root inspection method and the emergence trap method used by researchers. Further to the recommendation of Powell and Bruce (2011), it is important to demonstrate each method's capability for a range of vineyards of different soil type, at multiple time points in the growing season, on both grafted and ungrafted grapevines with low levels of phylloxera infestation.

A preliminary survey was undertaken on 25 vineyard blocks to identify at least 10 blocks to include in a larger trial to compare the three phylloxera detection methods: DNA, emergence trap and visual root inspection.

Three main criteria used to select the ten vineyard blocks were:

(a) low frequency of phylloxera detection based on composite soil core samples;
(b) low density of phylloxera detection averaged across the soil cores collected on each vineyard; and
(c) proximity to other known phylloxera infested blocks on the same vineyard.

There were nine vineyard blocks identified from the suite of 25 sampled using the DNA method, which met the above criteria and another block (Vineyard A-1) chosen for inclusion as a positive control (Table 35).

Table 35. Vineyard blocks evaluated by the DNA method in September 2015 for potential inclusion. Grey = selected.

Vineyard-block	Variety / rootstock	Proportion of composite soil samples with phylloxera detected	Average DNA concentration (phylloxera equivalents per 200g dry soil) across all soil cores collected	Reason for selection
Vineyard A-1	Riesling / Schwarzmann	4/8	1.36	Moderate frequency and low density - to act as a control
Vineyard A-2	Graciano / Schwarzmann	5/8	0.33	
Vineyard A-3	Shiraz/ Schwarzmann	2/8	0.04	Low frequency and very low density
Vineyard A-4	Pinot Gris / Richter 99	0/8	0.00	
Vineyard B-1	Cabernet Sauvignon/ Own roots	0/8	0.00	Adjacent to known infested block – potential for low infestation
Vineyard B-2	Cabernet Sauvignon / AXR	3/3	5.67	
Vineyard C-1	Shiraz / SO4	0/8	0.00	
Vineyard C-2	Shiraz / 101-14	0/8	0.00	
Vineyard C-3	Pinot Noir / Own roots	0/8	0.00	
Vineyard C-4	Chardonnay / Own roots	0/8	0.00	
Vineyard D-1	Merlot/ Own roots	0/8	0.00	Adjacent to known infested block – potential for low infestation
Vineyard D-2	Pinot Noir/ Own roots	1/8	1.49	Low frequency and density
Vineyard D-3	Pinot Noir/ Own roots	0/8	0.00	Adjacent to known infested block – potential for low infestation
Vineyard D-4	Chardonnay / Teleki	0/8	0.00	
Vineyard E-1	Pinot Gris / Own roots	7/8	14.90	
Vineyard E-2	Cabernet Sauvignon / Own roots	4/8	0.91	
Vineyard E-3	Chardonnay/ Own roots	0/8	0.00	Adjacent to known infested block – potential for low infestation
Vineyard E-4	Chardonnay/ Own roots	0/8	0.00	Adjacent to known infested block – potential for low infestation
Vineyard F-1	Sangiovese / Unknown rootstock	8/8	6.30	
Vineyard F-2	Sangiovese / Unknown rootstock	0/8	0.00	
Vineyard F-3	Sangiovese / Unknown rootstock	0/6	0.00	
Vineyard H-1	Tempranillo / 101-14	1/8	0.02	Low frequency and very low density
Vineyard H-2	Mixed / Own roots	0/2	0.00	
Vineyard H-3	Shiraz / Schwarzmann	0/8	0.00	
Vineyard 1	Shiraz / Own roots	11/90	4.28	Low frequency and relatively low density

4.9.3.1. Materials and methods

Ten vineyard blocks identified in the preliminary survey (Vineyards A-1, A-3, B-1, D-1, D-2, D-3, E-3, E-4, H1 and Vineyard 1, Table 35) across King Valley, Rutherglen and Yarra Valley regions were sampled in February-March 2016. The three phylloxera detection methods were undertaken on the same vines (4.1.2 – 4.1.5), with 10 cores from 10 vines composited for the DNA method.

4.9.3.2. Results and discussion

Excessive soil hardness in four of the 10 vineyard blocks precluded visual root inspection on these vineyard blocks, and difficulties were also encountered with others. Gaps in the data resulted. A summary of the phylloxera detections at the 10 vineyard blocks included in this trial is given in Table 36 at both the composite sample level for the DNA and emergence trap methods and at the individual vine level for the emergence trap and visual root inspection methods. Composite sample DNA method results for the preliminary trial are also presented as a reference. There were no phylloxera detections in Vineyard H-1 in the composite samples collected in this trial from either the DNA method or emergence traps, despite detection in a single core by the DNA method in the preliminary trial (Table 36).

Comparison of phylloxera detection rate at whole of vineyard block level between DNA and emergence trap methods over 10 vineyard blocks

For the important vineyard block level, the DNA method failed to detect phylloxera in two vineyard blocks (D-1, D-3) of the nine sampled (excluding H-1 for which no method detected phylloxera), compared to the emergence trap method (Tables 36, 37). In one of these instances, the DNA composites that returned the negative results measured low levels of *Vitis* root DNA (data not shown) which in section 4.5 indicated a potentially lower likelihood of returning a positive for phylloxera by qPCR. Soil hardness at these two vineyards was excessive, with significant difficulty experienced in extracting core samples, and thus likely to have contributed markedly to these results. Retesting of these vineyards is therefore recommended to confirm results. However in lieu of this retesting, current results show that at the whole of vineyard level over the ten vineyard blocks sampled, the emergence trap method was more accurate.

Comparison of phylloxera detection rate at whole of vineyard block level between the three methods over the same six vineyard blocks surveyed

The visual root inspection method detected phylloxera at 5/6 vineyard blocks sampled, compared to 6/6 for the same blocks surveyed by the emergence trap method using the vine level data (Table 36). Vineyard block B-1 which had no phylloxera detected by the visual root inspection, recorded a relatively high frequency of detection at the vine level by the emergence traps compared to other blocks in the trial (Table 36). The DNA method did detect phylloxera at vineyard block B-1 as well, but only detected phylloxera in 5/6 of the same vineyards sampled using the visual root inspection method (Table 36). All six vineyard blocks sampled by the visual root inspection method were classified as ‘suspect positive’ due to the presence of galling but absence of insects (Table 36). At the vineyard level, the detection ability of the emergence trap method was therefore slightly higher than both the DNA and the visual root inspection methods, which were similarly effective at detecting phylloxera to each other. If the ‘suspect positive’ vines had been those deemed positive by the visual root inspection method, the efficacy of classification of vineyard blocks deemed ‘phylloxera infested’ would have been the same for the emergence trap and visual root inspection methods and slightly lower for the DNA method.

Had only the DNA method been used across the 10 vineyard blocks, block D-3 would have been incorrectly classified as phylloxera free, and had only the visual root inspection method been used where positives were classified as having insects, block B-1 would have been incorrectly classified as phylloxera free (Table 36). In this trial, only the emergence trap method would have correctly classified the phylloxera status of each of the vineyard blocks based on appearance of insects. These results are also indicative of the benefits of a multi-method approach across a landscape.

Comparison of phylloxera detection rate between the DNA and emergence trap methods based on composite samples collected at the 10 vineyard blocks

At the composite sample level, the DNA method and emergence trap method detected phylloxera in a total of 25 and 24 composites out of 50 sampled, respectively, across the 10 vineyard blocks (Table 38), and therefore can be said to be equally as accurate on a landscape level. However, there were seven composite sample detections by the DNA method that were not detected in the emergence traps, and six composite sample detections by the emergence traps that were not detected by the DNA method (Table 38). Both detection methods can therefore return false negative results and although may have appeared equally as accurate in terms of overall detection rate, they each returned different results for some of the composite samples.

Comparison of phylloxera detection rate between the DNA and visual root inspection methods based on composite samples from the same six vineyard blocks sampled

When comparing the composite sample detection rate for the DNA method and the calculated composite sample detection rate for the visual root inspection method, the DNA method showed approximately a 20% higher detection rate (Table 39), demonstrating higher accuracy. The visual root inspection method failed to detect phylloxera in seven to eight composite samples in which the DNA method found phylloxera (Table 40). The DNA method failed to detect phylloxera in one composite sample at vineyard block D-3, scored as positive by the visual root inspection method (Tables 39, 40). Based on this trial, the DNA method was more sensitive overall at the composite level than the visual root inspection method.

Comparison of phylloxera detection rate at the individual vine level between the emergence trap and visual root inspection methods over the same six vineyard blocks surveyed

At the individual vine level, the emergence trap method detected phylloxera on 54/430 (13%) vines across the 10 vineyards (Table 36). These data could not be directly compared to the DNA method because individual soil cores were pooled and analysed as composite soil samples. In addition, the visual root inspection method was not carried out on every vine in the trial due to soil hardness leaving less vines sampled than the emergence trap method at some vineyard blocks. To therefore directly compare phylloxera detection rate at the vine level across these two methods, detection rates at vineyard blocks D-2, E-4 and 1 were recalculated and presented according to the same number of sampled vines as the visual root inspection methods at those vineyards (Table 41).

Across the six vineyard blocks sampled, the emergence traps detected phylloxera on 26/215 (12%) of sampled vines, compared to the visual root inspection method which detected phylloxera on 24/215 (11%) sampled vines (Table 41). However, the emergence traps detected phylloxera insects on eight vines which the visual root inspection did not, and the visual root inspection detected phylloxera on a different six vines which the emergence traps did not (Table 42). Considering the presence of phylloxera alone at the vine level, the accuracy of detection was equivalent between these two methods but the sensitivity varied on a vine level basis.

When positive detections in the visual root inspection methods were scored as presence of galling without insects (‘suspect positives’), there were 42/215 (17%) detections (Table 42). This large increase in accuracy of

detection for the visual root inspection method when galling was considered in addition to the presence of insects, resulted in 16 additional vines for which the emergence trap method did not detect phylloxera but the visual root inspection scored as positive (Table 42). According to the NPMP for the visual root inspection method, "inspect for root galls or individual insects". It could then be assumed that the presence of root galls alone without phylloxera would be counted as positive detections. At the vine level for the six vineyards sampled by both methods, we can therefore say that according to the NPMP definition of the visual root inspection method, that the visual root inspection method was of higher accuracy and sensitivity compared to the emergence trap method.

Table 36. Summary of detection rates from DNA cores, emergence traps and visual root inspections in comparison to detection rate from DNA cores in the preliminary survey. Results for emergence traps and visual root inspections are presented on a per composite and per vine basis.

Vineyard-block	Preliminary survey (Sept 2015)	Comparative trial (Feb-Mar 2016)				
	DNA method	DNA method	Emergence trap		Visual root inspection	
	Frequency of detection from composite soil cores	Frequency of detection per composite	Frequency of detection per trap composite	Frequency of detection per trap	Frequency of detection per vine with phylloxera detected	Frequency of detection per vine - root damage (galling) in absence of phylloxera
Vineyard A-1	4/8	5/5	2/5	2/42	*	*
Vineyard A-3	2/8	5/5	3/5	3/42	*	*
Vineyard B-1	0/8	2/4	4/4	8/32	0/32	10/32
Vineyard D-1	0/8	0/5	2/5	3/54	*	*
Vineyard D-2	1/8	3/5	2/5	6/47	6/38	11/38
Vineyard D-3	0/8	0/6	1/6	1/46	1/46	4/46
Vineyard E-3	0/8	4/6	3/6	4/49	6/49	6/49
Vineyard E-4	0/8	1/5	2/5	2/41	4/40	4/40
Vineyard H-1	1/8	0/4	0/4	0/34	*	*
Vineyard 1	11/90	5/5	5/5	25/43	7/10	7/10
Total	19/162	25/50	24/50	54/430	24/215	42/215

* Visual root inspection was not undertaken at these sites due to soil hardness

Table 37. Comparison of frequency of phylloxera detection at the vineyard level by the DNA and emergence trap methods.

	DNA method positive vineyard blocks	DNA method negative vineyard blocks	Total
Emergence trap positive vineyard blocks	7	2	9
Emergence trap negative vineyard blocks	0	1	1
Total	7	3	10

Table 38. Comparison of phylloxera detection frequency of composite samples for the DNA and emergence trap methods across ten vineyard blocks in February-March 2016.

	DNA method positive composite samples	DNA method negative composite samples	Total
Emergence trap positive composite samples	18	6	24
Emergence trap negative composite samples	7	19	26
Total	25	25	50

Table 39. Comparison of phylloxera detection frequency of composite samples for the DNA and visual root inspection methods across the same six vineyard blocks sampled in February-March 2016.

Vineyard-block	DNA method	Visual root inspection method			
	Frequency of detection per composite	Phylloxera detected		No phylloxera detected	
		Frequency of detection per vine	Calculated frequency of detection per composite[1]	Frequency of detection per vine - root damage (galling)	Calculated frequency of detection per composite[1] - root damage (galling
Vineyard B-1	2/4	0/32	0/4	10/32	1/4
Vineyard D-2	3/5	6/38	1/5	11/38	1/5
Vineyard D-3	0/6	1/46	1/6	4/46	1/6
Vineyard E-3	4/6	6/49	1/6	6/49	1/6
Vineyard E-4	1/5	4/40	1/5	4/40	1/5
Vineyard 1	5/5	7/10	4/5	7/10	4/5
Total	15/31	24/215	8/31	42/215	9/31

[1] Estimated by converting the frequency of detection per vine to a per composite basis, based on the same number of composite samples collected per vineyard block with the DNA method. Calculated detection rates at the composite level were rounded up to the nearest whole composite.

Table 40. Comparison of phylloxera detection frequency of composite samples for the DNA and visual root inspection methods across the six vineyard blocks sampled in February-March 2016.

	Visual root inspection method positive[1] composite[2] samples	Visual root inspection method negative[3] composite[2] samples	Total
DNA method positive composite samples	8	7	15
DNA method negative composite samples	1	15	16
Total	9	22	31

[1] Positive phylloxera detections denoted by presence of root galling and absence of phylloxera

[2] Estimated by converting the frequency of detection per vine to a per composite basis, based on the same number of composite samples collected per vineyard block with the DNA method. Calculated detection rates at the composite level were rounded up to the nearest whole composite. The suspect positive results for the visual root inspection method have been presented only for this method.

[3] Negative phylloxera detections denoted by absence of root galling and absence of phylloxera

Table 41. Comparison of single vine phylloxera detection frequency of the emergence trap and visual root inspection methods across the same six vineyard blocks sampled in February-March 2016.

Vineyard-block	Emergence trap	Visual root inspection	
	Frequency of detection per trap[1]	Frequency of detection per vine with phylloxera detected	Frequency of detection per vine - root damage (galling) in absence of phylloxera
Vineyard B-1	8/32	0/32	10/32
Vineyard D-2	6/47 (5/38)	6/38	11/38
Vineyard D-3	1/46	1/46	4/46
Vineyard E-3	4/49	6/49	6/49
Vineyard E-4	2/41 (2/40)	4/40	4/40
Vineyard 1	25/43 (6/10)	7/10	7/10
Total	46/258 (26/215)	24/215	42/215

[1] Single vine detections at vineyard blocks D-2, E-4 and 1 were brought back to the same number of vines sampled as for the visual root inspection method, but retaining the same proportion of detection. These values are presented in brackets.

Table 42. Comparison of phylloxera detection of emergence trap and visual root inspection methods at the vine level in February-March 2016 on the same set of six vineyard blocks.

	Visual root inspection					
	Phylloxera detected	Phylloxera not detected	Total	Galling but no phylloxera	No galling and no phylloxera	Total
Emergence trap positive	18	8	26	26	0	26
Emergence trap negative	6	183	189	16	173	189
Total	24	191	215	42	173	215

Weak vines as a predictor of phylloxera infestation

For two of the three methods, sampling weak vines increased detection above that recorded by the systematically sampled vines when compared at a single vine level (Table 43). For the emergence trap method, there was a three-fold increase in the proportion of phylloxera detections from the systematically sampled vines (10%) compared to the visually weak vines (32%) (Table 43). This result was significant at the $p < 0.001$ level according to a Fisher's exact test.

There was a one and a half-fold increase in the proportion of phylloxera detections at the single vine level from the systematically sampled vines (18%) compared to the visually weak vines (26%) for the visual root inspection method for where a positive phylloxera detection was scored as presence of root galling in absence of insects (Table 42). There was no evidence of a higher infestation rate in weak vines compared to systematically selected vines for the visual root inspection method for where a positive phylloxera detection was scored as presence of phylloxera on vine roots, with only 6% of weak vines compared to 12% of systematically selected vines infested (Table 43).

In this data set alone, weak vines are identified as a potential useful predictor of phylloxera using both the emergence trap and visual root inspection methods, where vines were assigned as phylloxera infested based on the presence of galling rather than the presence of phylloxera in the latter method (which is a valid assumption given the scoring description in the NPMP for the visual root inspection method). Unfortunately, this analysis could not be completed using the DNA method as samples from weak vines were composited together. Future analysis of weak vines by the DNA method as individual soil samples is therefore recommended.

Table 43. Detection rates of phylloxera per vine by emergence trap and visual root inspection methods in 2016 from systematically selected vines and weak vines sampled.

Method		Systematically selected vines		Weak vines	
		Number	**Percent**	**Number**	**Percent**
Emergence traps	Phylloxera trapped	36	10	18	32
	No phylloxera trapped	338	90	38	68
	TOTAL	**374**		**56**	
Visual root inspection	Positive scored as phylloxera found	22	12	2	6
	Negative scored as no phylloxera found	158	88	33	94
	TOTAL	**180**		**35**	
Visual root inspection	Positive scored as root galling in absence of phylloxera	33	18	9	26
	Negative scored as no root galling or phylloxera	147	82	26	74
	TOTAL	**180**		**35**	

Considerations when selecting a phylloxera detection method

Table 44 outlines considerations that end-users should consider when choosing a phylloxera detection method for a purpose. For further discussion on the integration of all three methods for surveillance activities, refer chapter 5.

Table 44. Considerations (positive and negative) associated with sample collection, handling, processing and turnaround time for the three phylloxera detection methods.

Parameter	DNA method	Emergence traps	Visual root inspection
Sample collection	• Minimal training required for sample collection staff • Easy to use sampling equipment (soil corer) • Can be undertaken all year • Excessive soil hardness and stone content may limit/prevent sample collection • Potentially live insects leave the field	• One trap per vine • Fairly simple method of placing and removing traps but requires two separate field visits • Peak usefulness in summer • Samples 'fixed' in ethanol in the field, effectively killing the insects prior to transport to the laboratory	• Requires extensive training of staff to examine roots with hand lens to identify phylloxera and/or root galls • Whole method is manual and reliant on field evaluation • Excessive soil hardness limits sample collection • Success of the method is fully dependent on availability of trained personnel • Potential for false positive if galling not caused by phylloxera
	• Time to collect sample is 59 minutes per 40 vines (one hectare)	• Time to collect sample is 129 minutes per 40 vines (one hectare) including trap placement and collection	• Time to collect and analyse sample is 242 minutes per 40 vines (one hectare) assuming all vines assessed
Sample handling	• Ability to composite samples to reduce laboratory analysis costs • Requirement to organise quarantine permits between PMZs and states • Requirement to send samples from field to laboratory for analysis • Requirement to triple bag the samples prior to shipping • Transport costs from field to laboratory • Samples must reach laboratory within 48 hours • Requirement to minimise excessive heat load on samples during transport • Import Risk Assessment carried out to ascertain the risk of a phylloxera incursion in South Australia as part of moving phylloxera infested soil into the state (being a PEZ) and the measures that need to be put in place to minimise this risk to an acceptable level (Appendix 4)	• Requirement to organise quarantine permits between PMZs and states • Requirement to send samples from field to laboratory for analysis • Transport costs from field to laboratory	• Nil

Sample analysis	• Machine automation - ability to process 500 samples per day • Current cost $55/sample – potential cost efficiencies with increased sample number • Sample processing time efficiencies with increased sample number • Only currently available at a single laboratory • If positive detection, requires collection of sample via emergence trap or visual root inspection to confirm presence of phylloxera and strain	• Requires manual processing by laboratory expert • 36 minutes per 40 vines (one hectare) for trap analysis – limit to how many hours a day a person can do this assessment • Time of processing dependent on availability of trained laboratory technicians • No commercial laboratory setup for analysis (research scale only at time of reporting)	• Nil
Turnaround time	• Transport time of samples from field to laboratory at 1-2 days • Laboratory sample processing time dependent on sample number but likely 2-3 days	• Standing time for traps in field of at least 14 days • Transport time of samples from field to laboratory at 1-2 days • Laboratory sample processing time dependent on sample number	• Field sampling could cease at time of first positive detection

4.9.3.3. Conclusion

Emergence of phylloxera is known to be seasonal (Herbert *et al.*, 2006) and emergence traps have been deemed most effective in late summer. The timing of this trial to compare the ground sampling methods was chosen to be optimal for phylloxera emergence and hence the trapping method; and this may have contributed some bias to the results of this study. The difficulty in using late summer as an ideal time for emergence trapping, was that some vineyard blocks could not be examined using the visual root inspection method because the soil was too hard and dry. In this trial, hard ground, either from stones or from being very dry, placed limitations on visual root inspection, and potentially the DNA method.

For this trial, the DNA method was slightly less accurate at the important vineyard level in detecting phylloxera than the emergence trap and visual root inspection methods. However, on a composite sample level the DNA was equally or more accurate and sensitive than the other two methods. On a per vine basis, the visual root inspection method was equally or more accurate and sensitive than the emergence trap method. These results indicate that all three methods have their weaknesses and therefore a multi-method approach to field surveillance is most likely to best negate the incidence of false negatives. In addition, including the sampling of weak vines into a surveillance strategy appears to increase the chance of detecting phylloxera if present.

4.9.4. Comparison of DNA and emergence trapping

An additional opportunity arose to evaluate effectiveness of phylloxera detection at a landscape level across five vineyards but also at the single vineyard level. This trial involved conducting the three detection methods of DNA, emergence traps and visual root inspection on the same set of vines concurrently. In addition, EM38 (electromagnetic induction) was carried out to map variation in soil moisture, salinity and soil texture for each block. However, at the time of writing this report, the visual root inspection and EM38 data were unavailable and therefore excluded from analysis and reporting.

4.9.4.1. Materials and methods

Five vineyards (I-1, J-1, K-1, L-1 and Vineyard 2) across the Yarra Valley were selected by Agriculture Victoria and sampled in February-March 2017. Over one hectare of each vineyard was sampled using the standard sampling method (4.1.2), and soil cores for DNA analysis (4.1.5) and emergence trap methods (4.1.4) were undertaken on the same vines at each of the five vineyards.

Six soil cores were collected from each sample vine in each vineyard to allow for direct vine comparison between methods as well as comparison at the composite level. Composite soil samples comprised five cores. Where comparisons at the composite level were undertaken, the corresponding five emergence traps per soil composite were compared.

Data were reduced to binary data (phylloxera detected or not detected) and tabulated. The comparison of efficacy of detection was assessed using a McNemar's test (McNemar, 1947) where the off-diagonal counts of a 2x2 contingency table were compared.

4.9.4.2. Results and discussion

From the landscape level with the five sampled vineyards considered as one unit, the phylloxera status would have been assigned as 'infested' given the positive detection at Vineyard 2 from both detection methods (Table 45).

At Vineyard 2, there were 10 composite samples analysed. There was agreeance in phylloxera detection at the composite level for nine of the 10 composite samples between the two methods. Phylloxera was detected in seven of the composite samples using both detection methods (C4 to C10), and phylloxera was not detected in two composite samples (C1, C3) using both detection methods (Table 46). The discrepancy in phylloxera detection was found in composite C2 where the DNA method detected phylloxera but the emergence trap method did not (Table 46). A low level positive in C2 composite sample of 0.3 phylloxera equivalents per 200g dry soil was also reflected at the vine level of a single low level positive of 0.5 phylloxera equivalents per 200g dry soil, with the other four cores negative (Table 47). These low-level detections are perhaps indicative of an isolated phylloxera colony which spatially was not on vine roots above which on the soil the emergence trap was placed, hence explaining the negative emergence trap result.

Collecting soil samples at the vine level allowed for direct comparison of the DNA method with the emergence trap method. At the single vine level for Vineyard 2, the DNA method detected phylloxera on 28/50 (56%) vines compared to the emergence trap method of 16/50 (32%), this was a 24% greater success rate for the DNA method (Table 48). There were 13 vines for which the DNA method detected phylloxera where the emergence trap did not. Seven of those 13 vines for which phylloxera was quantified, reported results of less than 1.5 phylloxera equivalents per 200g dry soil, showing the sensitivity of detection of the DNA method (Table 47). There was only a single vine for which the emergence trap detected phylloxera and the DNA method did not (Table 48). The discrepancy in detection of the two methods (Table 48) was tested using McNemar's test (McNemar, 1947) and found to be significant at the $p < 0.001$ level. For Vineyard 2, the DNA method was significantly more effective at phylloxera detection than the emergence trap method at the single vine level. Had the single vine results from Vineyards I-1, J-1, K-1 and L-1 for both methods also been included in Table 48, only the value in the negative-negative detection quadrant would have been larger. The McNemar test result would not have changed and would still be significant.

Results from the DNA method also allowed comparison of efficacy of phylloxera detection between the composite and single vine levels. For nine of the 10 composite samples, detections at the corresponding single vine level per composite were in agreeance (Table 49). For one composite (C3), the composite soil sample failed to detect phylloxera (Table 49), with a single vine of the five corresponding vines comprising that composite, recording a low detection of 0.4 phylloxera equivalents per 200g dry soil (Table 47).

Table 45. Comparison of phylloxera detection by DNA method and emergence trap method at the vineyard level for five Yarra Valley vineyards in 2017.

Vineyard	Number sampled vines	Number composite samples	Emergence traps	DNA method
Vineyard I-1	60	12	Negative	Negative
Vineyard J-1	50	10	Negative	Negative
Vineyard K-1	68	14	Negative	Negative
Vineyard L-1	55	11	Negative	Negative
Vineyard 2	50	10	Positive	Positive

Table 46. Summary of phylloxera detection at the composite sample level for Vineyard 2 for both the emergence trap method and DNA method.

Composite sample number	Emergence trap result	DNA result
C10	Positive	Positive
C9	Positive	Positive
C8	Positive	Positive
C7	Positive	Positive
C6	Positive	Positive
C5	Positive	Positive
C4	Positive	Positive
C3	Negative	Negative
C2	Negative	Positive
C1	Negative	Negative

Table 47. Number of phylloxera counted in emergence traps and quantity of phylloxera DNA in soil samples for Vineyard 2, presented at both the single vine and composite level for the DNA method.

Position of sampled vine		Emergence trap method			DNA method		
Row number	Panel number	Crawler	Alate	Total phylloxera	Composite sample number	Composite sample (phylloxera equivalents per 200g dry soil)	Single core sample (phylloxera equivalents per 200g dry soil)
5	20	31	8	39	C10	688.7	286.6
5	15	18	0	18			472.1
5	10	508	20	528			305.3
5	5	22	0	22			21.3
8	5	0	0	0			183.3
8	10	216	9	225	C9	601.2	752.4
8	15	0	0	0			14.2

8	20	0	0	0			1.0
11	20	0	0	0			451.2
11	15	335	5	340			357
11	10	1395	5	1400	C8	691.0	38.9
11	5	0	0	0			0.0
14	5	0	0	0			558
14	10	79	10	89	C8	691.0	553.2
14	15	1	0	1			2.2
14	20	293	9	302	C7	471.5	1690.7
17	20	0	0	0			53.7
17	15	0	0	0			0.0
17	10	53	0	53			226.8
17	5	0	0	0			0.6
20	5	67	0	67	C6	255.5	0.0
20	10	22	0	22			656.9
20	15	0	0	0			1.3
20	20	0	0	0			111.8
23	20	8	0	8			356.2
23	15	10	0	10	C5	415.2	306.3
23	10	0	0	0			0.0
23	5	0	0	0			0.0
26	5	0	0	0			0.0
26	10	0	0	0			0.0
26	15	0	0	0	C4	1593.5	0.7
26	20	0	0	0			0.0
29	20	0	0	0			0.0
29	15	8	0	8			292.3
29	10	0	0	0			0.4
29	5	0	0	0	C3	0.0	0.0
32	5	0	0	0			0.0
32	10	0	0	0			0.4
32	15	0	0	0			0.0
32	20	0	0	0			0.0
35	20	0	0	0	C2	0.3	0.0
35	15	0	0	0			0.0
35	10	0	0	0			0.5
35	5	0	0	0			0.0
38	5	0	0	0			0.0
38	10	0	0	0	C1	0.0	0.0
38	15	0	0	0			0.0
41	15	0	0	0			0.0
41	10	0	0	0			0.0
41	5	0	0	0			0.0

Table 48. Comparison of phylloxera detection frequency of the DNA method and emergence trap method at the single vine level for Vineyard 2.

	Emergence trap positive detection	Emergence trap negative detection	Total
DNA positive detection	15	13	28
DNA negative detection	1	21	22
Total	16	34	50

Table 49. Comparison of detection rate of the DNA method for Vineyard 2 between the composite soil samples and the corresponding five cores per vine analysed as a single unit.

	Unit of single cores positive	Unit of single cores negative	Total
Composite soil sample positive	8	0	8
Composite soil sample negative	1	1	2
Total	9	1	10

4.9.4.3. Conclusion

The choice of vineyards in this trial limited the power of comparisons between the sampling methods, as four out of five vineyards tested negative for phylloxera.

This trial presented another opportunity to test the accuracy of phylloxera detection of the DNA method against the emergence trap method at a single vineyard. The DNA method significantly outperformed the emergence trap method for phylloxera detection at the single vine level on this vineyard, demonstrating a greater sensitivity in detecting phylloxera. However, at the composite sample level (of five vines pooled), both methods proved almost equal in ability to detect phylloxera, with the DNA method only detecting phylloxera in one additional composite compared to the emergence traps.

Results from the composite samples compared to single vine samples for the DNA method alone showed that the composite samples were sensitive enough to detect phylloxera, with only one composite not detecting phylloxera which was detected at the single vine level. With the single vine analysis at a five-fold laboratory cost of the composite samples for the same sampled area in Vineyard 2, compositing the soil cores through the DNA method in this trial represents a significant cost saving to the user compared to single vine analysis and only a marginal reduction in sensitivity.

4.9.5. Overall conclusion

The current method of vine selection for phylloxera surveillance according to the NPMP is a mixture of a systematic sample and targeting of weak vines. This systematic strategy ensures that all vines are within one row and two panels from an inspected vine (if a grid pattern is employed). Consequently, any area of infestation greater than two rows wide and five panels long will include one sample vine, which gives good coverage at that level of infestation. In the early stages of an infestation however, when only single vines or a few vines in a row are infested, this systematic selection and associated sampling density may not detect phylloxera even if present. The identification and sampling of weak vines may in general increase the probability of a phylloxera detection.

One of the clear findings from this comparative trial chapter is that each of the detection methods varied in their accuracy and sensitivity to detect phylloxera, but most importantly, they all failed on occasion to detect phylloxera when it was found by one of the other methods. The occurrence of a proportion of false negative results highlights the difficulty in declaring that a vineyard is phylloxera free, based on a single detection method conducted at one point in time only. The implications for these failures of detection will however, depend on the level of the failure. The failure to detect phylloxera at a vine or composite sample level if the block is already declared as phylloxera infested, is not highly important, as management changes such as quarantine or replanting to rootstock most often occur at the vineyard block level. A failure of a phylloxera detection at the whole of vineyard level could have much larger operational ramifications in terms of biosecurity management and the prevention of an incursion and management of spread to other vineyards. For the same reasons, a failure of detection across a landscape level can have huge implications. The reasons for the failures in detection by all three detection methods in these studies are not immediately apparent. However, each method only samples a very small proportion of a vine's rootzone on which to decide about phylloxera presence or absence - either on the soil surface (emergence trap method) or below the soil surface (DNA and visual root inspection methods). It therefore follows, that the more isolated phylloxera colonies are by chance, the greater the likelihood they will be missed by the relatively small volume of soil targeted by all three detection methods. Soil hardness (as noted in 4.9.3.2), the fact that phylloxera was not at a mobile stage or that the soil conditions did not permit emergence could also be plausible explanations for failures to detect phylloxera.

There is also potential for any of these three detection methods to incorrectly identify the presence or absence of phylloxera due to, amongst other things, field or laboratory sampling errors/issues, misidentification of insects, and the fact that only a small proportion of vines in an area is ever sampled as part of a surveillance activity. It is therefore vital that vineyard blocks are resampled over time to verify phylloxera status.

Given the variation in accuracy and sensitivity of the three detection methods in these trials, an integrated approach to phylloxera surveillance where all three detection methods are used, is recommended for the greatest chance of limiting the occurrence of false negatives and false positives and returning a cost effect result within the required timeframe for the end user.

5. Towards an integrated toolkit for phylloxera surveillance

With the lack of effective eradication measures for phylloxera, prevention of infection through maintenance of regional and state quarantine and farm gate hygiene procedures are the main line of defence. Thus, knowing where phylloxera is and is not and having a robust surveillance system to determine the limits of these areas and to monitor spread is vital.

Surveillance for phylloxera is required for many different purposes. These include:

- To determine whether an area (block or vineyard) has phylloxera;
- Delineating a known infestation of phylloxera;
- Supporting phylloxera freedom status at a region or state level; and
- Supporting a proposal to upgrade a PRZ or PIZ status.

Each of these may require different strategies, but all must consider the possibility of missed detections and put in place strategies to reduce the occurrence of these misses.

Through this project we have successfully validated the DNA method for detecting phylloxera in soil samples and confirmed the sample collection and handling methods for these soil samples destined for DNA analysis. Further, the studies in this report have significantly increased our knowledge and understanding of the effectiveness of the various phylloxera detection methods (emergence trapping, visual root inspection and DNA testing of soil). Sampling methods have been developed to enhance the detection probability of an infestation for the DNA method. In addition, there is awareness of the differing requirements of each detection method in terms of resources, expertise, cost, sample throughput and turnaround time of results.

This project is working towards endorsement of the DNA and emergence trap methods though the SPHD. These methods, along with the currently approved visual root inspection method, can then be used individually or in conjunction with each other, considering known limitations such as time of year, accessibility of trained personnel and relative soil hardness. This project has shown that each of these three sampling methods are capable of detecting phylloxera and warrant being part of an integrated toolkit. This toolkit will vastly improve our ability to manage the devastating impacts of this pest on the wine industry.

However, this project has also highlighted the significant limitations of all three detection methods, where each method failed in some cases to detect phylloxera even though phylloxera was present. None of the methods could guarantee detection of early infestations, so it is therefore impractical to give a guarantee that an area is phylloxera-free based on a single detection method conducted at one point in time only.

As discussed in the recommendations, further research is needed to elucidate additional data to inform an effective surveillance plan, including aspects such as improving ground detection methods; understanding the risk factors to predict where to sample, translation of block-level surveillance to landscape-scale surveillance and developing rigorous protocols to detect low or isolated infestations. This is critical given that most vineyards in Australia are in PEZs and a high portion on susceptible own-rooted *Vitis vinifera*.

However, surveillance is needed now, and with our current knowledge, it is still possible to develop an informed integrated surveillance plan, while being mindful of the limitations of the current detection methods.

5.1. Sampling scenarios

If there were a guarantee of an even spread of an infestation across a block, the number of samples required to give an assurance would be little affected by the size of the area sampled. The current standard sampling method assumes that no area within a vineyard is more likely to be infested than any other by using a grid or

regular sampling strategy. However, it also recommended that weak vines be targeted, which has been shown to increase the likelihood of detection.

While it is well known that phylloxera infestations are clustered, there is little consistent information about the shape of clusters. However, with the results of these studies, the detection probability for various infestation scenarios is better understood, and can be incorporated into a surveillance strategy.

Single vine infestation/new infestation in a vineyard: the standard sampling method sampling one vine in every 5th panel in every 3rd vine row instigates a 1/45 chance of sampling any particular vine in the sampled area. Even if the only infested vine in a vineyard was that sampled, there would be less than 100% chance of the soil core collected from that vine being positive for phylloxera. Unfortunately, the probability of a detection will be very low, and it would appear almost impossible to detect a very early infestation by any of the methods considered in this project. For the DNA method, the detection probability in this scenario could be increased by sampling more vines in the area (increased sampling density) and/or by sampling more cores at each vine, and/or by only analysing single cores not composite soil samples.

Multiple isolated infested vines/cluster of infested vines in a vineyard: the phylloxera detection rate of the three detection methods in this trial were based on these scenarios. The probability of detection of an infested vine in this scenario could be best improved by doubling the sampling density to double the detection probability.

Identification of weak vines in a vineyard: one of the key findings from this study was that phylloxera was detected more frequently by the emergence trap and visual root inspection methods from visually 'weak' vines compared to visually 'healthy' vines (not assessed for DNA method). However, inspection of weak vines alone should not be used in isolation, but rather, incorporated into a broader sampling plan.

5.2. The value of aerial imagery

Since the mid-1990s, remote sensing technologies have advanced significantly. These advances have improved our ability to conduct accurate and timely plant-based surveillance, at both a block and regional (landscape) level to detect subtle variations in factors such as nutrition and water status (Pagay *et al*., 2016), biomass (yield), relative vigour, maturity, stress and specific pests and diseases (de Castro *et al*., 2015; MacDonald *et al*., 2016; Dix *et al*., 2017; Psirofonia *et al*., 2017).

More recently, research has focussed on developing spectral signatures for plant based stress (Kancheva and Borisova, 2006), to determine crop type (Nidamanuri and Zbell, 2012) and pests and diseases (Mahlein *et al*., 2010; Mahlein *et al*., 2013; Wahabzada *et al*., 2015; AL-Saddik, *et al*., 2017), including phylloxera (Vanegas *et al*., 2018). This area of research we feel has the greatest scope for early detection of phylloxera (and other pest and disease infections) in leading to the identification of weak vines prior to the development of obvious visual symptoms, and enabling discrimination of stress symptoms attributed to phylloxera as opposed to other biotic and abiotic factors.

To demonstrate the value of aerial imagery from a landscape (multiple block level) to a block level, Vinehealth Australia undertook a mapping activity to define vineyard boundaries for all vineyards in the Yarra Valley Geographic Indicator (GI) region, including those vineyards in the current Maroondah PIZ as at 19 July 2017 (Figure 22). This exercise involved the sourcing from an Australian provider two sets of true colour (RGB) imagery (each taken in February and April 2017) at high spatial resolution of 10cm and 7.5cm respectively, to ensure the desired extent of the landscape was captured. Using the sourced aerial imagery as a base map loaded into ArcMap 10.5.1, a fishnet (grid) of 10km x 6km tiles was laid over the capture. Each tile was manually inspected and vineyards identified from other attributes in the landscape. Tracing was then undertaken around the edges of every planted area of vineyard. This also included tracing of vineyards where

trellising systems were in place but vines had not yet been planted. The vineyard map layers were saved in various formats – shapefiles, kml and pdf.

The use of this mapping exercise as part of on-going surveillance activities currently and into the future facilitates the viewing of individual vineyards or groups of vineyards across the region to look for variations in vine growth both at one time and over time with a series of annual images. This could be used to indicate weak vines, possibly attributed to phylloxera infestation, which could then be used to drive the potential ground sampling strategy for those blocks.

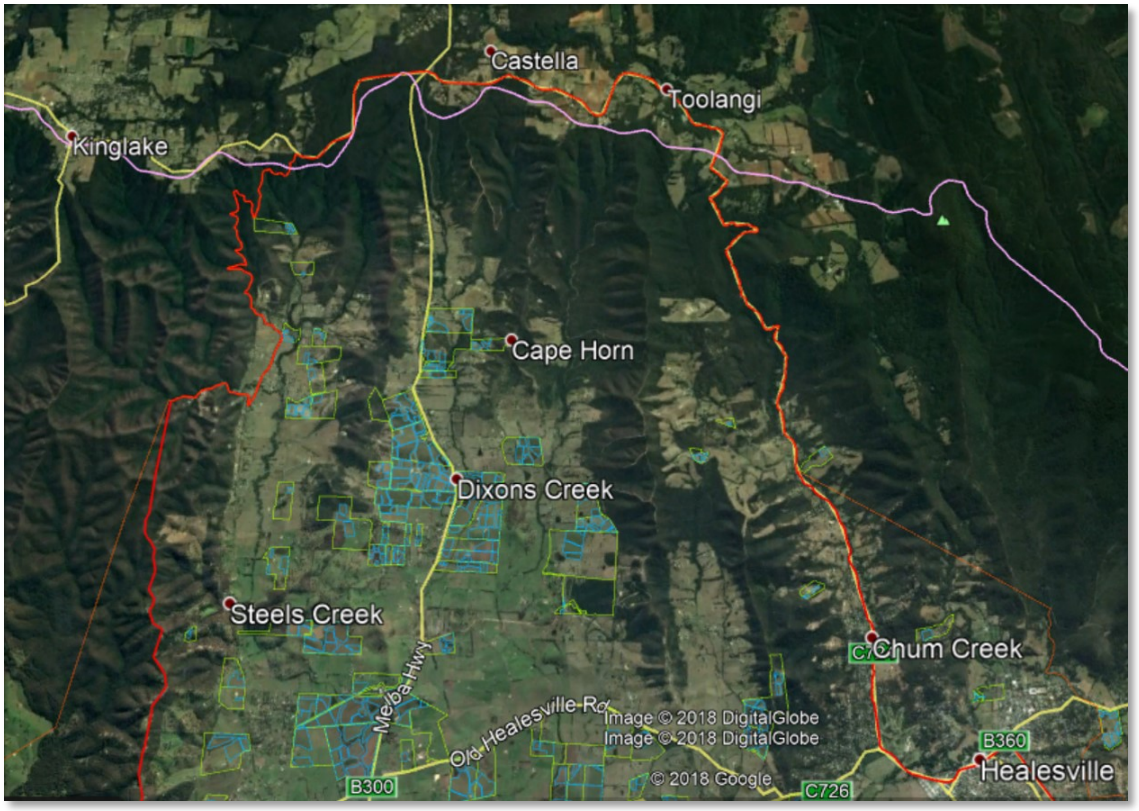

Figure 22. Snapshot of a portion of vineyard block boundaries (blue), parcel boundaries (green), Maroondah PIZ boundary (red) and Yarra Valley GI boundary (purple).

Individual vineyard blocks used as part of various studies conducted in this project have not been identified in Figure 22 for reasons of privacy. However, the potential for aerial imagery to be used to assist in the visualisation of patterns of weakness in a block, which can then be targeted at a high sampling density for phylloxera ground surveillance, are shown by Figures 23, 24. These true colour image examples are however not indicative of 'early' infestations. Such levels of infestation which would potentially require the use of spectral imaging to identify patterns of weakness not necessarily visible to the human eye. In addition, the value of imaging for surveillance will depend on factors such as the resolution of the capture, electromagnetic spectrum covered by the cameras at time of capture and the relative aggressiveness of the infestation to show visually as weak vines.

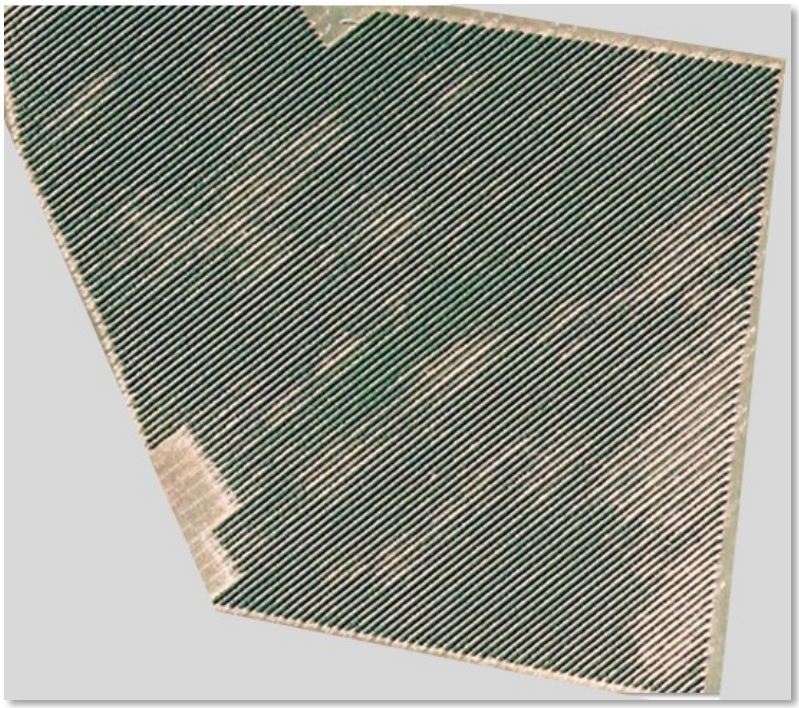

Figure 23. Vineyard in Yarra Valley: true colour aerial image from January 2014 capture. Block used as part of sample collection and handling studies (Table 1).

Figure 24. Vineyard 1: true colour aerial image from 8 January 2017 capture from Google Earth. Block used as part of surveillance sample density studies (4.6).

5.3. Developing surveillance strategies

While this report is not about developing or proving surveillance strategies, the results provide us with a way forward to provide sampling advice to inform surveillance though on ground sampling. Most of this study has focussed on sampling at a block level. However, upscaling from a single vineyard block level, to a whole vineyard, or a wine region, or even a state, would require a combination of primary and secondary detection methods, alongside risk assessment. For example, using landscape spectral imagery, which could be utilised on a single occasion or a series of annual images, overlaid with identification of areas of high risk. This could be followed by targeted on-ground surveillance of particular vines with a possibly higher potential of being infested with phylloxera.

6. Recommendations

This project has developed clear recommendations outlined herein:

6.1. Sampling recommendations

1. That the qPCR method to detect phylloxera DNA delivered by SARDI MDC, and validated through this trial, be offered as a commercial service to industry and regulators.

2. That the draft protocol (Appendix 3) for soil sampling to detect phylloxera DNA using the qPCR be adopted, which includes the following recommendations:

    a. To limit DNA degradation, soil samples for DNA extraction should be:
        i. stored at no more than 20°C during transport and reach the laboratory within 48 hours of collection; and
        ii. dried within approximately 24-48 hours of receival at laboratory and stored unfrozen at approximately 10-20°C pre-drying.

    b. Samples should be taken from the top 10cm of soil and within 10cm of the vine trunk.

    c. A sampling protocol based on composite samples of 5 cores is appropriate for phylloxera detection by qPCR. However, in instances of suspected low incidence and severity of phylloxera in a block, either less compositing of individual cores or higher numbers of cores per block (rather than per vine) may be considered in the surveillance plan to avoid phylloxera being diluted below the point of detection with the compositing process.

    d. The current field sampling density outlined in the NPMP of sampling one vine in every 3rd row in every 5th panel has been shown to be suitable for the DNA method. However, higher sampling density above this standard recommendation may be warranted for any blocks suspected to have a low or isolated phylloxera infestation.

    e. Samples can be collected successfully throughout the year for analysis given no significant difference in the frequency of detection across all seasons and sites. However, with a trend of higher quantities of phylloxera DNA in autumn (March), it is preferable to sample around this time if possible.

3. Continue evaluation of weak vines as part of any phylloxera surveillance strategy.

4. Integrate recommendations 1-3 into a submission for the SPHD to update the National Diagnostic Protocol for grape phylloxera.

5. SARDI MDC to undertake *Vitis* qPCR assay as an internal quality control on all samples analysed for phylloxera DNA.

6. Actively facilitate the endorsement of the DNA and emergence trap method by Plant Health Committee, and their integration into the NPMP as alternate phylloxera detection methods.

7. The three detection methods evaluated gave different rates of detection of phylloxera, and each method failed in some cases to detect phylloxera even though phylloxera was present. It is therefore impractical to give a guarantee that an area is phylloxera-free based on a single detection method conducted at one point in time only. Therefore, need to develop a national phylloxera surveillance

plan which appropriately utilises the three detection methods (DNA, emergence trap, visual root inspection) as well as secondary surveillance methods for area freedom maintenance, delimiting of new incursions and zone status upgrades. This surveillance plan must ensure repetitive surveillance over time, particularly with respect to upgrading phylloxera management zone status.

8. If the DNA method is utilised as part of a surveillance plan, an additional detection method will need to be employed thereafter if positives are detected, to collect insects for identification and strain typing.

9. Potential for the soil samples collected for the DNA method to be value-added through testing of other soil-borne pathogens, e.g. root-knot nematodes.

6.2. Future research

Through the implementation of this project, project team members have identified various items for future research focussed on the areas of improving the understanding of the phylloxera biology as well as improving surveillance strategies.

1. Life table studies on seven key endemic strains under different environmental conditions with and without food.

2. Greater understanding on how phylloxera moves and survives.

3. Rapid laboratory identification of phylloxera in emergence traps
    a. Use of scanning and shape recognition systems to confirm phylloxera identification and trap counts.

4. Strain identification of phylloxera
    a. Interaction of strains with environmental conditions.
    b. Competition between strains of phylloxera - is it possible to inoculated with a benign strain?
    c. Rootstock-strain interactions.

5. Translation of block-level surveillance to landscape-scale surveillance with protocols for situations of varying incidence and severity levels, as indicated by secondary surveillance methods. Options evaluated side by side, over years and in different locations.

6. Use of existing and emerging secondary detection methods to identify potentially phylloxera infested vines to help target areas as part of a surveillance plan.

7. Development of a unique ‘spectral fingerprint’ for phylloxera to assist in facilitating the identification of weak vines associated with phylloxera infestation prior to visual cues becoming evident.

8. Development of a model using a range of risk factors to predict where to sample in a proposed landscape-scale situation, for example:
    a. Vineyards adjacent to cellar doors attracting tourist visitation;
    b. Blocks that share machinery, equipment and contractor movement;
    c. Own rooted versus rootstock blocks;
    d. Frequency near gates, wineries etc;
    e. Blocks not planted with propagation material from certified sources;
    f. Vineyards that have changed ownership frequently.

9. Development of strain typing capability for the DNA method.

10. Improving ground detection methods:
    a. Evaluate the effect of sampling density on detection rates across methods (DNA, emergence trap, visual root inspection).
    b. Can a food-based lure be used as an attractant for phylloxera inside emergence traps when surveying blocks with potentially low infestation rates to try to 'draw out' phylloxera from the soil if present?
    c. Is there a nutrient or level of a nutrient that phylloxera must have from vine roots to survive?
    d. What is the frequency of false positives from 'damage' using dig sampling method where phylloxera is absent:
        i. Especially in soils where nematodes are present;
        ii. Could a stain be used in-field to distinguish nematode from phylloxera galls?
    e. Is there potential for an in-field molecular test?

7. Communication and engagement plan

An important component of this project has been to ensure ongoing engagement with industry and regulators regarding the development of the DNA method. An overarching communication and engagement plan was developed, the main components of which follow. Importantly, insights from the PBCRC project (PBCRC4004) titled 'Advancing collaborative knowledge systems for plant biosecurity surveillance' were integrated as they became available to ensure that the outcomes of the project had the best chance of being integrated into a toolkit for active surveillance of phylloxera and adopted by end users.

7.1. Barriers to adoption

Key to successful adoption by end users is to first understand potential barriers to adoption. These include:

Vineyard owners / regulators

- Perceived relevance of the method to all potential end users
- Preference for other phylloxera detection methods
- Lack of understanding of impact of phylloxera incursions and need to conduct surveillance
- Time required for end users to engage
- Ability to disseminate training for method to end users
- Costs associated with method
- The need to purchase a soil corer

New research

- Development of other diagnostic tools that supersede the DNA method

Strain typing

- Inability of current DNA method to determine strain of phylloxera

Biosecurity framework

- Not endorsed by the relevant subcommittee of the Plant Health Committee
- Not endorsed by National Viticulture Biosecurity Committee
- No review of NPMP and therefore DNA method not incorporated
- Poor knowledge management of results from the DNA method

Surveillance methodology

- Perceived accuracy by end users of method to detect phylloxera
- The difficulty to detect a phylloxera infestation when it first occurs and before it has been spread further within the block or to other vineyards
- Gaps in scientific knowledge to underpin the surveillance methods (e.g. life-table studies under different conditions with/without food; how phylloxera moves and survives)

A single South Australian laboratory undertaking the qPCR analysis

- Requirement for SARDI MDC to report positive results for interstate samples to Biosecurity SA first (refer to Appendix 5 for communication flowchart)
- Perceived threat of sending diagnostic samples into South Australia for analysis (refer to Appendix 4 for Import Risk Analysis)

7.2. Objectives and strategic priorities

Objective

Establish and defend area freedom status for phylloxera. To know where phylloxera is and is not.

Key success measures

- Endorsement of these protocols by industry and Plant Health Committee
- Integration of the DNA method into the NPMP
- Adoption of the DNA method by state regulators for state surveillance plans
- 5% increase year on year for the first 3 years following endorsement, in the number of samples sent to SARDI MDC for analysis by growers

Strategic priorities

Three strategic priorities were identified; being endorsement by Plant Health Committee and industry groups, and engagement and investment by end users and stakeholders. The achievement of these priorities of the communications and engagement plan will be enabled by ten guiding principles listed in Figure 25.

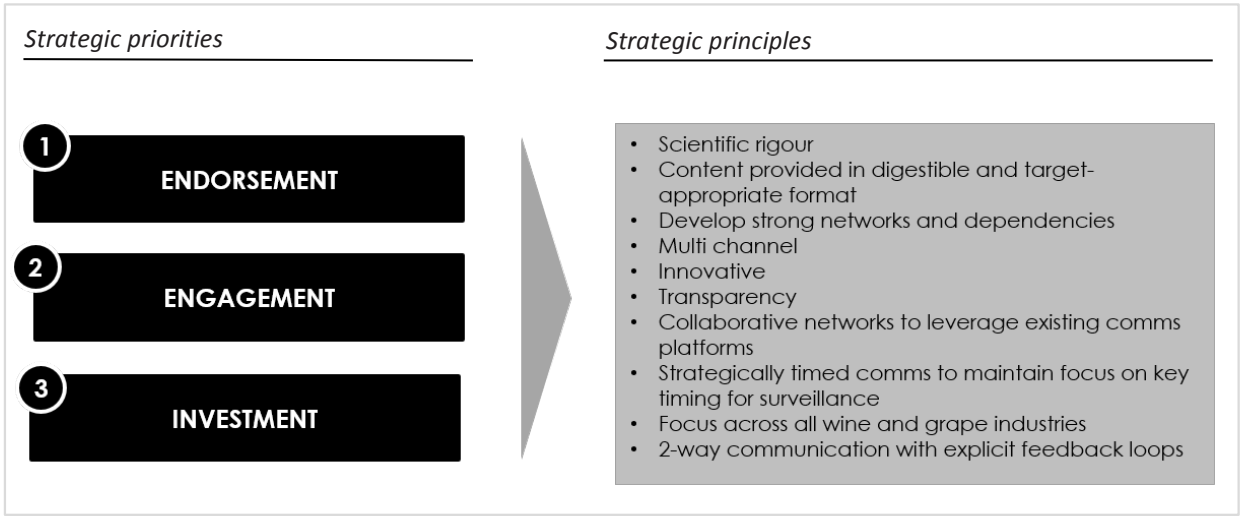

Figure 25. Strategic priorities and principles underpinning the communication and engagement plan.

7.3. Timeline

The communications and engagement plan includes initiatives that have been ongoing as part of the project, and initiatives that are phased over the next three years following endorsement of the method. Importantly, three key phases of (1) building awareness, (2) accelerating adoption, and (3) sustaining adoption were identified. The timing of this phasing is shown in Figure 26.

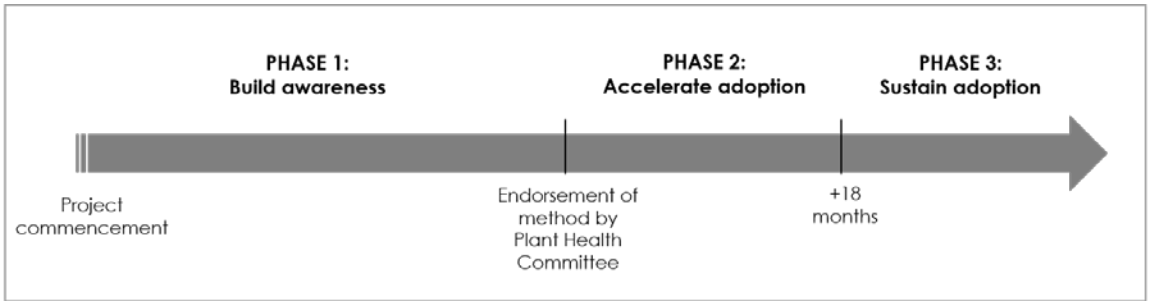

Figure 26. Timing for delivery of communications and engagement plan.

7.4. Stakeholder analysis

The target stakeholders for the communications and engagement plan were categorised into ten categories (Figure 27). Each category requires a customised approach to ensure optimal engagement and investment in the DNA method.

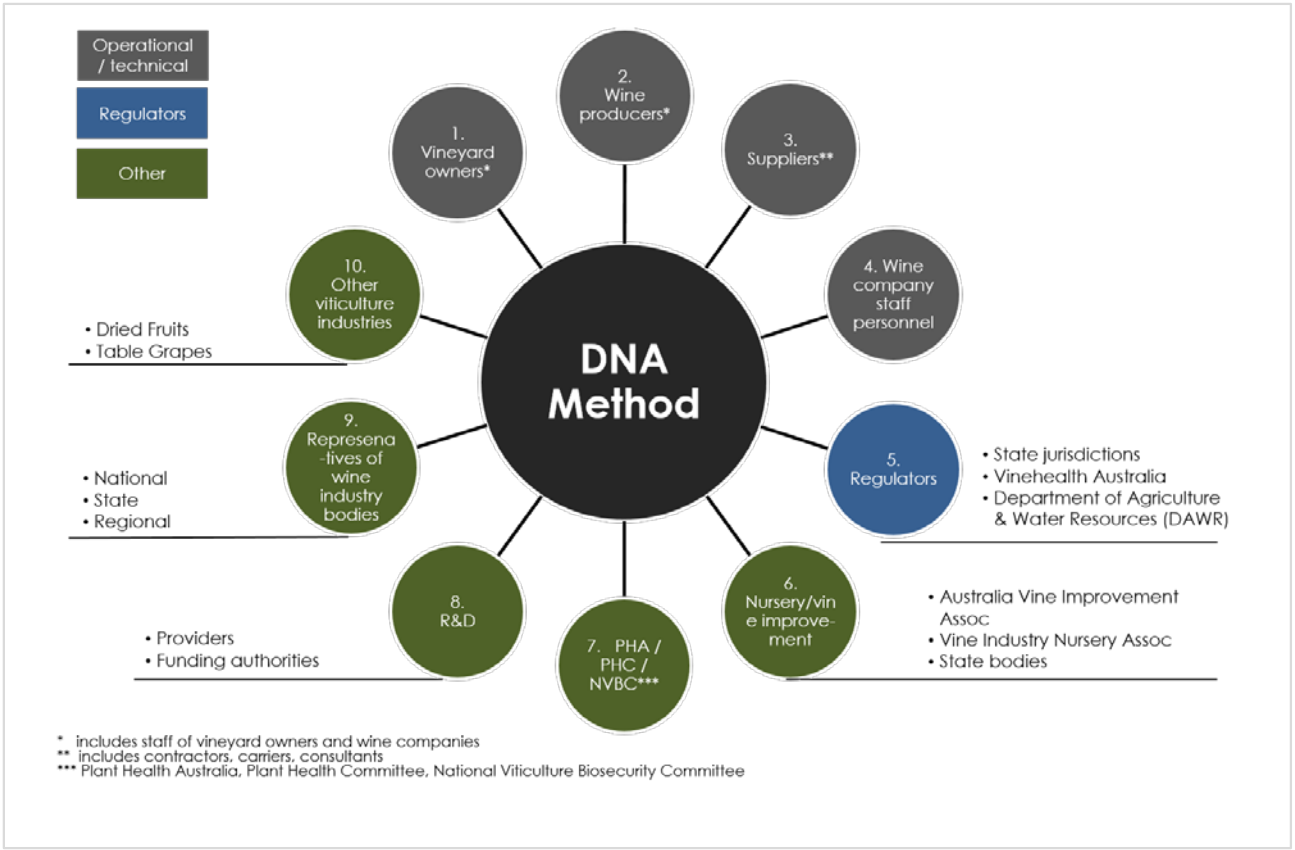

Figure 27. Categorisation of target stakeholders for the communications and engagement plan

7.5. Initiatives to be implemented

A list of detailed communications initiatives to ensure adoption of the DNA method is outlined in Table 50. Work has been ongoing for Phase 1 (Figure 26) initiatives during the life of the project with presentations, articles, discussions, membership of biosecurity committees and work on specific projects enabling the project team to advance the understanding of the DNA method and work towards its formal integration into national protocols and systems. Many of these outward facing activities are reported on Vinehealth Australia's website (http://vinehealth.com.au/).

In the final 18 months of the project, considerable time has been invested in:

- Assisting a desktop review to inform a national approach to grape phylloxera management – a final copy of this document is available from the NVBC.
- Developing a Digital Biosecurity Platform – this Platform is a lead practice digital knowledge management system that empowers decision making for biosecurity preparedness, prevention and incursion management activities. Importantly, for the DNA method, it will allow end users to GPS locate points of soil sample collection, and enable machine to machine connectivity to transfer results from the diagnostic laboratory directly into the system. All results can then be geospatially presented and information appropriately managed to support phylloxera management zone status or incursion management. Without a knowledge management system to collate sampling information and diagnostic results for the DNA method, the ability to inform decision making will be markedly reduced. Refer to http://www.vinehealth.com.au/media/Digital-Biosecurity-Platform-Snapshot-LR.pdf for snapshot of the Digital Biosecurity Platform that has been developed during 2016-17. A comprehensive business case for the platform can be obtained from Vinehealth Australia.
- Assisting the development of a prioritised R&D plan for phylloxera, including phylloxera detection and surveillance, to inform the development of a new phylloxera R&D program funded by Wine Australia and Agriculture Victoria.
- Meeting with regional and state grape and wine industry representative bodies to discuss the application of the DNA method in context of the current NPMP and likely review of the NPMP.
- Discussing a state PEZ surveillance strategy to maintain area freedom status by utilising an integrated approach to phylloxera detection, with the DNA method being an integral part of this strategy.

Table 50. Communication and engagement initiatives to ensure widespread adoption of the DNA method.

#	Initiative	Actions	Audience[1]	Timing phase[2]
ENDORSEMENT				
1	Endorsement for biosecurity committees	Provide brief and present to SPHD and SPHS regarding endorsement of DNA method and incorporation into National Diagnostic Protocol for phylloxera	7	1
2	Industry endorsement	Present to National Viticulture Biosecurity Committee (NVBC) seeking endorsement of DNA method as an alternate primary detection method for phylloxera (in addition to the currently endorsed 'dig' method)	7	1
		Advocate to the NVBC that the NPMP be comprehensively reviewed and methods for area freedom establishment and maintenance updated to incorporate DNA method	7	1
		Present to the relevant state grape and wine representative bodies on the DNA method	9	2
ENGAGEMENT				
3	Engagement with regulators	Provide training programs for state regulators if necessary	5	1
		Advocate to state regulators to incorporate DNA method into state surveillance plans	5	1,2
		Advocate for continual review of sampling methodology associated with DNA method	5	1
		Ensure state plant quarantine standards / regulations are updated		2,3
4	User-friendly testing kit	Support the testing laboratory to develop a user-friendly and practical sample collection and handling kit for use by industry	1,2,3,4,5,6,10	1,2
		Explore opportunity to have real-time tracking mechanisms from field to laboratory (e.g. RFID) as part the sampling kit		
5	Engage other viticulture industries	Brief Australian Tablegrapes (ATG) and Dried Fruit Australia (DFA) on the outcomes of the research and next steps for implementing DNA method for phylloxera detection	10	1
		Present at ATG and DFA convened workshops and seminars on the DNA method	10	2
6	Educate wine industry and other researchers regarding new DNA method	Presentations at the International Phylloxera Symposium	1,2,3,4,6,8,9,10	1,2,3
		Articles in Vinehealth Australia e-news and annual reporting to industry		
		Feature articles in wine industry technical and business magazines		
		Incorporate 'hands-on' training of how to collect samples in viticulture roadshows / seminars		
		Brief regional technical groups on new method and how it can be used by regulators and growers		

		Targeted briefing for large corporate wine businesses		
		Information on Vinehealth Australia website		
		Youtube videos demonstrating collection of samples		
		Biosecurity workshop at Australian Wine Industry Technical Conference to include discussion of this method		3
7	Improve general awareness of phylloxera impacts	Continue to communicate the impact of phylloxera on vines and the wine industry to heighten industry adoption of phylloxera detection methods	1,2,4,6,9,10	1,2,3
		Conduct phylloxera 'immersion' tours to encourage behaviour change towards proactive farm-gate hygiene and regular vine health monitoring for phylloxera		
INVESTMENT				
8	Knowledge management – value adding results	Develop a Digital Biosecurity Platform to house all DNA method results to enable geospatial mapping of surveillance data at a landscape level	1,2,5,6,8,10	1,2,3
		Develop governance rules for analysis and interpretation of surveillance results by stakeholders		
9	Investment in R&D	Ensure the inclusion of phylloxera research in the wine industry's R, D&E plan to enable new knowledge on phylloxera biology, spread and survival to optimise surveillance sampling strategies	8	2,3
		Promote the development of technology to better target high risk areas for on ground surveillance		
		Prospect for opportunities to translate the current DNA method to an in-field test		3

1 – the numbers refer to the stakeholder category in Figure 27

2 – the numbers refer to the three phases detailed in Figure 26

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9. List of Appendices

These five documents were developed as part of this project but are not suitable for public release at this stage.

1. Surveillance of grape phylloxera in vineyards – Visual root inspection method
2. Surveillance of grape phylloxera in vineyards – Emergence trap method
3. Draft DNA coring protocol for growers
4. Import risk assessment for movement of infected soil samples
5. Draft communication flow for phylloxera results from SARDI MDC

10. Abbreviations/glossary

ABBREVIATION	FULL TITLE
ATG	Australian Tablegrape Association
DFA	Dried Fruit Association
DNA	Deoxyribonucleic acid
GPS	Global Positioning System
MDC	SARDI Molecular Diagnostics Centre
NPMP	National Phylloxera Management Protocol
NVBC	National Viticulture Biosecurity Committee
NVHSC	National Vine Health Steering Committee
PBCRC	Plant Biosecurity Cooperative Research Centre
PEZ	Phylloxera Exclusion Zone
PIZ	Phylloxera Infested Zone
PMZ	Phylloxera Management Zone
PRZ	Phylloxera Risk Zone
RKN	Root-knot nematode
SARDI	South Australian Research and Development Institute
SPHD	Subcommittee on Plant Health Diagnostics

11. Plain English website summary

CRC project no:	PBCRC2061
Project title:	Sampling strategies for sensitive, accurate cost-effective detection of grape phylloxera for quantifying area freedom status
Project leader:	Mrs Inca Pearce (Vinehealth Australia)
Project team:	**Vinehealth Australia** Ms Suzanne McLoughlin (2015-2018) Mr Brendan Tully (2013-2018) Mr Alan Nankivell (2013-2015) Mr Matthew Edge (2013-2017) Mr Andrew Downs (2013-2014) Ms Rachel Innes (2013-2014) **Rho Environmetrics** Dr Ray Correll **SARDI** Dr Kathy Ophel- Keller Dr Alan McKay Dr Danièle Giblot-Ducray Mrs Barbara Hall **University of Adelaide** Dr Cassandra Collins **PIRSA (Biosecurity SA)** Mr Geoff Raven **DEDJTR** Mr Greg King **DPI NSW** Mr Chris Anderson
Research outcomes:	This project has successfully developed a field sampling protocol for collecting soil cores and validated a diagnostic protocol using qPCR for the detection and quantification of phylloxera ('DNA method'). Endorsement of the DNA method alongside the emergence trap and visual root inspection phylloxera detection methods, will provide growers and regulators with an integrated toolkit of field sampling and detection options to utilise as part of a national surveillance plan.
Research implications:	This project has delivered a significant advancement in the ability of industry and regulators to detect phylloxera, and therefore manage the impact of this devastating insect on vines, through: • Developing a test that can be readily adopted by growers to sample and test their blocks for phylloxera,

	• Providing regulators with a method that enables rapid collection and analysis of samples which will greatly assist not only general surveillance activities, but also management in the event of an incursion, and • Developing an additional primary phylloxera detection method that can form part of an integrated national surveillance plan. The capability to conduct extensive ground surveillance using the DNA method will enable greater confidence in area freedom status, delimiting of incursions and upgrading phylloxera management zone status from a PIZ or PRZ to a PEZ. Ultimately, the DNA method will improve our ability to establish where phylloxera is and is not in Australia, and thus enable protection of vineyards from phylloxera.
Research publications:	Giblot-Ducray, D., Correll, R., Collins, C., Nankivell, A., Downs, A., Pearce, I., Mckay, A. C., and Ophel-Keller, K. M. (2016) Detection of grape phylloxera (*Daktulosphaira vitifoliae* Fitch) by real-time quantitative PCR: development of a soil sampling protocol. Australian Journal of Grape and Wine Research, 22: 469–477.
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