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MULCHING TRIALS:

To identify an effective means of reducing the Psa-V inoculum source from cut-out vine material

VLS Project No E2012-09/11

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Executive Summary

MULCHING TRIALS: To identify an effective means of reducing the Psa-V inoculum source from cut-out vine material

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Mulching has been a key management practice used by the Kiwifruit industry for many years to recycle prunings however, with the widespread infection of Psa-V in vines, there is concern that mulching could significantly increase the inoculum level in orchards and consequently contribute to the cycle of infection and vine loss.

This project set out to try and quantify the levels of Psa-V in mulched plant material originating from heavily infected Hort16A vines. qPCR employed a so-called *third generation* Taqman test and for plating we employed a new selective media which proved excellent under the difficult conditions i.e. high levels of non-target micro-fauna.

For logistical reasons the work was divided among 3 field trials in conjunction with several lab experiments. In the first trial we looked at a range of treatments applied to the mulch: Copper, Nitrogen, chlorine dioxide and an organic "digester" product called PS1+Biostart. The second trial removed the mulch by raking before spraying with copper and in the third trial we applied compost to the mulch. Apart from these field trials we also swabbed the mulcher for Psa to determine the risk of spreading Psa through orchard equipment. In the lab we undertook specific calibrations on mulch and compost for qPCR as well as looking at the survival of Psa on mulch and compost under different temperature and humidity conditions.

The mulcher was swabbed 18 days after use during which time it had been housed in a dry shed. Psa-V was detected by PCR on all swabs however none produced viable colonies. Given the duration since use and the dry conditions the lack of viability is not surprising however there is a clearly a very significant risk of infection from recently used equipment.

Mulch from each field trial was sampled weekly for 5 weeks. Generally Psa levels increased for about 2 weeks and then declined. qPCR results declined more slowly than our enumerations from plating and we attribute this to the difference between live (plated) and PCR total (live + dead) populations. We attribute the initial increase in numbers to the rapid exposure of Psa to extensive substrate through the act of mulching. Generally however, we expect the mulched material to be unsuitable for the long term survival of Psa (hot, dry, sunny, dead food source, competing micro-fauna) so we expect numbers to decline in the medium term as we found. We might expect the *rate* of decline to depend on weather conditions and perhaps factors like the fineness of the mulch.

Application of Chlorine dioxide or copper seemed to have minimal effect on the decay of Psa-V. Nitrogen and perhaps PS1+Biostart, may have hastened Psa loss a little. Psa levels determined by PCR declined at a rate of about 1 decade (1/10 x) per 20 days whereas levels from plating and enumeration dropped at a rate of about 1 decade per 5 days. At the levels of inoculum on our first trial (10^5 – 10^6 cfu/mL) this meant that Psa was detectable in the field for 7-8 weeks. Examining the results from treatments applied at different times after mulching, the pattern of decline was determined by time-after-mulching rather than by weather or time of treatment application. We note that the significant and continuing Psa-V population drop is quite different from previous long term monitoring within orchards where Psa could be found for months (15 weeks) on undisturbed litter beneath vines.

The final compost trial showed some different characteristics – a lower initial level of inoculum, no period of increase following mulching, and a greater difference between plating and qPCR. In the other trials plating and qPCR showed close agreement at the start of each trial whereas in the case of compost the initial plating population estimates were considerably lower than the qPCR estimates. Compost also required a new calibration for qPCR. It appears there were considerably more inhibitors present in the

compost treatment. The compost results were intriguing enough that we regret this treatment was not part of the initial trial and consider further work would be worthwhile.

When tested in lab conditions we found that Psa-V levels on mulch maintained in high humidity conditions dropped rapidly to below reliable detection levels (qPCR) whereas mulch kept at the same temperature under conditions of low humidity was unchanged after 5 weeks. We attribute this to the rapid growth of other bacteria, more suited to humid conditions, on the decaying substrate.

Besides the immediate results this work has enabled us to develop techniques to examine and track field populations of Psa-V with some confidence even in the presence of overwhelming numbers of competing micro-fauna. These results would not have been possible without the excellent performance of both the PCR Taqman test and the new selective media we were trialling.

MULCHING TRIALS

VLS Project No: E2012-09/11

Aim

To determine whether mulching is an appropriate method for disposal of infected vine material and to identify an effective means of reducing *Pseudomonas syringae* pv *actinidiae* (Psa-V) inoculum source from mulched infected vine material.

Background

Mulching has been a key management practice used by the Kiwifruit industry for many years. It is an effective way to recycle prunings from both vines and shelter while improving organic matter and consequently soil structure and the kiwifruit root zone. However, with the widespread infection of Psa-V in vines, mulching practices could significantly increase the inoculum level in orchards and consequently contribute to the cycle of infection and vine loss.

One of our areas of uncertainty regarding Psa is its ability to survive in, or on the soil. It appears some P syringae species can survive for long periods in, or on, the soil whereas others may be rapidly destroyed by soil micro-fauna. Organic amendments such as compost can modify the composition of the bacterial taxa in the rhizosphere as well as increasing the general level of microbial activity resulting in increased competition and/or antagonism among microbes and perhaps decreasing the activity of plant pathogens.

This project aims to examine a range of chemical/biological treatments, including compost, for efficacy in hastening the destruction of Psa-V on mulched canopy on the ground with the aim of eliminating the inoculum source and consequently mitigating the risk of re-infection events that may overload vine defences and drive progressive systemic infection. This risk may be exacerbated where entire sections are being removed and mulched prior to re-grafting in a new (Psa free) variety.

Field trials

Three field trials were undertaken with different treatments as summarised in Table 1.

The first field trial was undertaken on a Hort16A orchard which was heavily infected with Psa-V and in the process of being mulched. The canopy was completely cut out and then double mulched using 1 pass in each direction (Photos 1-4). 25 plots of about 1m width were set up across the width of a row (spread across 2 rows) allowing 5 plots for each of 5 treatments including a control set. Initial samples were collected immediately prior to the treatments being applied and then tested weekly for 5 weeks post treatment application.

A second trial uses a series of samples taken from a different site where the canopy was removed (25th May) and then buckraked out of the orchard before any remaining material was mulched. The surface was then sprayed with Nordox (27th May 52.5 g Cu/100L at 1000L/Ha) and our first samples were taken on the 1st June. There were no control plots for this trail since the treatment was applied to the entire block prior to our involvement. Understandably there was very little residual mulch left on the ground in this orchard. Two sample types were collected:

- Leafy mulch – more mulched debris
- Grassy mulch – less mulched debris

A third field trial was undertaken using an application of compost following mulching. Since this took some time to arrange it was undertaken about a month later at a different orchard which was being removed at the time(again Hort16A and strongly infected with Psa). Compost (Provided by Revital Fertilisers) was applied at a rate of 10 tonnes per Ha i.e.1 kg/m^2. 12 plots laid out 6 with compost and 6 without as controls. The sampling regime was the same as for the first field trial with samples collected and tested over 5 weeks period post treatment application.

- 6 control with no treatments
- 6 plots with compost treatment

Table 1: Summary of field trial details treatment applied and rate of application.

Field Trial	Orchard Name	KPIN	Mulch date	Application (Treatment)	Application rate	No Plots	Application date	Sampling Start date
1	Lake	4675	18-May-12	Nitrogen (Tech Urea)	115g N /10L	5	25-May-12	30-May-12
			18-May-12	Nordox	8.25gCu/10L	5	25-May-12	30-May-12
			18-May-12	Chlorine Dioxide	10g/10L	5	29-May-12	30-May-12
			18-May-12	PS1 + Biostart (digester)	10g/10L + 120mL/20L	5	29-May-12	30-May-12
			18-May-12	Control (Mulch only)	N/A	5	N/A	25-May-12
2	Fourth Estate Orchard		25-May-12	canopy cut, raked out, mulched then sprayed with Nordox	5.25gCu/10L 1000L/Ha	2	27-May-12	01-Jun-12
3	Transpack	1227	11-Jul-12	Compost	10t/Ha	6	12-Jul-12	16-Jul-12
			11-Jul-12	Control (Mulch only)	N/A	6	N/A	12-Jul-12

Lab trials

Several ancillary laboratory experiments were also undertaken:

1) To determine the effect of temperature and relative humidity on survival of Psa-V in mulch.
2) To determine the risk of spread of Psa-V around the orchard using mulching equipment
3) To undertake the "threshold of detection" through PCR by spiking clean mulch with Psa-V inoculum.
4) To determine the survival of Psa-V in spiked mulch/compost mix together with the limit of detection using PCR.
5) To determine the efficacy of copper and chlorine dioxide under varying temperature and humidity conditions

Methodology

Media

Other than during explicit laboratory testing, this trial is our first use of some *improved* Psa-V selective media. One of the known issues with evaluating treatments which endeavour to alter the microbiology of field trials is the difficulty of plating field samples (and in our case including compost material) and then finding the desired species in the resulting forest. The new media we used in these trials performed extremely well and made possible the results we present here.

Sampling

Weekly samples from all plots (treated and controls) were collected, bagged and labelled individually for all three field trials.

Analysis

Because of the difficulties in evaluating changes in surface biota associated with experimental treatments in the field we will endeavour to use both qPCR and plating to indicate results. This means we will consistently compare the Cq value from qPCR with our media enumeration results i.e. we are interested in examining the utility of qPCR as a quantification tool as well as a detection tool for Psa-V. Here Cq denotes the PCR quantification cycle: the cycle number at which the fluorescent signal crosses the pre-determined threshold. It is inversely correlated to the logarithm of the initial copy number.

Validation of our implementation of the Psa-V test has shown that the reliable detection threshold for Psa-V occurs at Cq≈35 however, the effect of substrate may change this threshold and calibration.

Sample processing

About 5g of each mulch sample was weighed and approximately 50 mL of 0.85% bacteriological saline was added to it. The tubes were shaken thoroughly to allow mixing and left for at least 5 minutes and then centrifuged for 3-5 minutes and the supernatant tested for Psa-V in PCR and cultures.

Isolation, Quantification and Identification by qPCR

At each weekly sampling, isolation of viable Psa-V was made from the supernatant on two highly selective Psa-V media. An attempt was also made at quantification of one sample per treatment weekly in cfu/mL through serial dilution and plating on selective media. Growth of any Psa-like colonies was checked in qPCR using our Psa-V Rapid test.

DNA extractions were also conducted on a 500 µL aliquot of the supernatant and tested in qPCR using a Psa-V Rapid test. qPCR results are given as the replication value (Cq). Our "standard" detection range for Psa-V detection is taken as Cq ≤ 35 i.e above that value we expect qPCR results to be unreliable and potentially contaminated by fragments of DNA (primer dimer). However other trials have shown "continuous" looking trends for Cq values greater than 35 i.e. we see steadily increasing Cq values greater than 35 in situations where we expect Psa-V populations to be decreasing (e.g. Figure 18 in this report). For this reason we will include Cq values wherever they were determined. In cases where **no** Psa was detected we will specify Cq=ND (not detected).

We will often use a calibration of qPCR to present Cq values in terms of cfu/mL for comparison with enumeration data from plating. Since we use 100µL aliquots for plating we will ***assign*** (and colour red) a value of 10 cfu/mL if no colonies were detected i.e. about the lowest detectable limit using these laboratory parameters.

Detection threshold for Psa-V colonies in mulch.

1. Clean mulch was obtained from an uninfected orchard in Katikati.
2. Psa-V solution was made by the re-suspension method and quantified as 1×10^7 cfu/mL.
3. 50 mL of this solution was serially diluted down to 1×10^1 cfu/mL.
4. 5 g of clean mulch was then re-suspended in 50 mL of each dilution, mixed thoroughly and allowed to stand for 30 minutes.
5. It was then centrifuged for 5 minutes and the supernatant was tested in PCR and cultured on selective Psa-V media to determine the threshold limit for isolation and detection of Psa-V in mulch.

Detection threshold for Psa-V colonies in an infected mulch compost mixture

1. Psa-V solution was made by re-suspension method and quantified as 1 x 10^7 cfu/mL.
2. This suspension was then serially diluted in 50 mL aliquots down to a minimum concentration of 1 x 10^1 cfu/mL.
3. 1.5g of mulch was mixed with 1.5g of compost and suspended in 50 mL of each solution, mixed thoroughly and allowed to stand for 30 minutes.
4. It was then centrifuged for 5 minutes and the supernatant was tested in PCR and cultured on selective Psa-V media to determine the threshold limit for isolation and detection of Psa-V in mulch.
5. The tubes were also kept at 25°C for a period of 25 days post spiking and checked at interval to check survival of Psa –V in the much/compost mix

Determination of Psa-V on mulching equipment.

After mulching infected vines swabs were taken from different parts of the mulching equipment to determine the risk of contamination (and consequently Psa spread). The swabs were carried out as per Table 2. Swabs were re-suspended in 500 µL of 0.85 % normal saline and tested for growth of viable Psa-V colonies. The supernatant was also tested in qPCR using Psa-V rapid test.

Table 2: Swabs conducted on dirty mulching equipment post use in infected orchard

Test no	Date	Samples	ID
1	12-Jun-12	Under mulcher (1)	Sw 1
2	12-Jun-12	Under mulcher (2)	Sw2
3	12-Jun-12	Roller	Sw3
4	12-Jun-12	Above coller	Sw 4
5	12-Jun-12	Under	Sw5
6	12-Jun-12	under back (1)	Sw 6
7	12-Jun-12	under back (2)	Sw 7
8	12-Jun-12	top	Sw 8

The effect of temperature and relative humidity on survival of Psa-V in mulch under laboratory conditions

Fresh mulch obtained from the first trial site was kept in 3 different conditions and monitored for 35 days for survival of Psa-V.

The following conditions were imposed and temperature and RH were monitored using data loggers:

1. Dry (low humidity) at 25°C
2. Wet conditions kept humid by spraying water every second day at 26°C
3. Ambient temperature in a sealed plastic bag

A sample of 5g from each condition was tested for the presence of viable Psa-V colonies (and identification through qPCR) at intervals over a period of 35 days.

Results

Detection limit for isolation and identification of Psa-V colonies in mulch

The lowest detected dilution was 1 x 10^4 cfu/mL for both plating and qPCR meaning that the lower detection was between 1 x 10^4 and 1 x 10^3 cfu/mL (Table 3, Fig 1). Using this data as a calibration for qPCR gave an excellent linear relationship between Cq and log (concentration) with a slope of -3.462 (Fig 2). i.e.

we can say the sensitivity of the qPCR is such that a reduction of 3.46 in Cq corresponds to an increase of 1 decade in Psa concentration.

Table 3: Summary of threshold detection limit for spiked mulch with Psa-V

Date	Sample ID	dilution	Concentration	Direct PCR on samples (Cq)	Growth on Psa-V media	calc from calibration cfu/mL
22-Jun-12	T1	Neat	1×10^7	22.92	G	9.00E+06
22-Jun-12	T2	1/10	1×10^6	26.06	G	1.11E+06
22-Jun-12	T3	1/100	1×10^5	29.53	G	1.11E+05
22-Jun-12	T4	1/1000	1×10^4	33.3	G	9.02E+03
22-Jun-12	T5	1/10000	1×10^3	0	NG	N/A
22-Jun-12	T6	1/100000	1×10^2	0	NG	N/A
22-Jun-12	T7	1/1000000	1×10^1	0	NG	N/A

Key: G= Growth NG= No Growth

Figure 1. Cq values down a dilution series using a neat solution of 1×10^7 cfu/mL until reaching the non-detected stage.

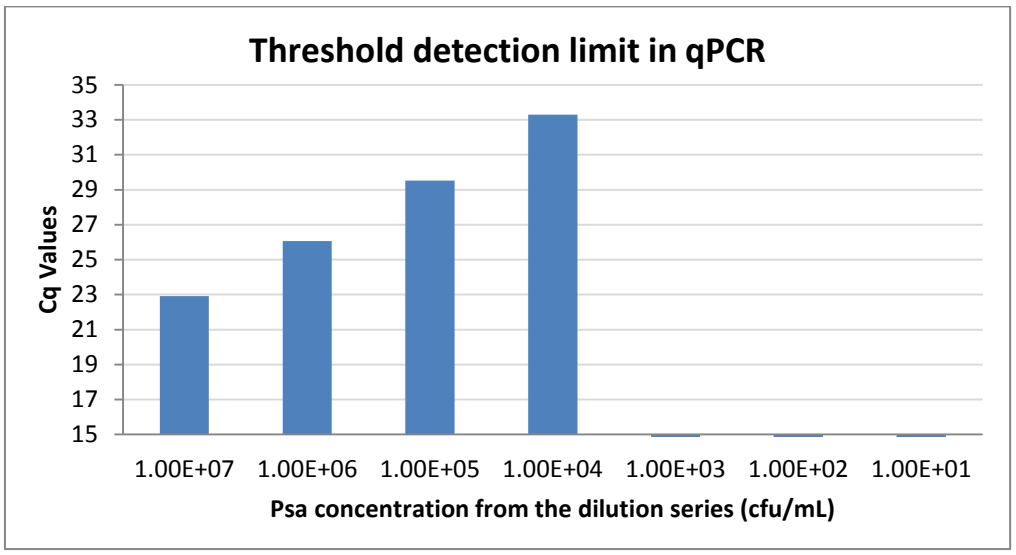
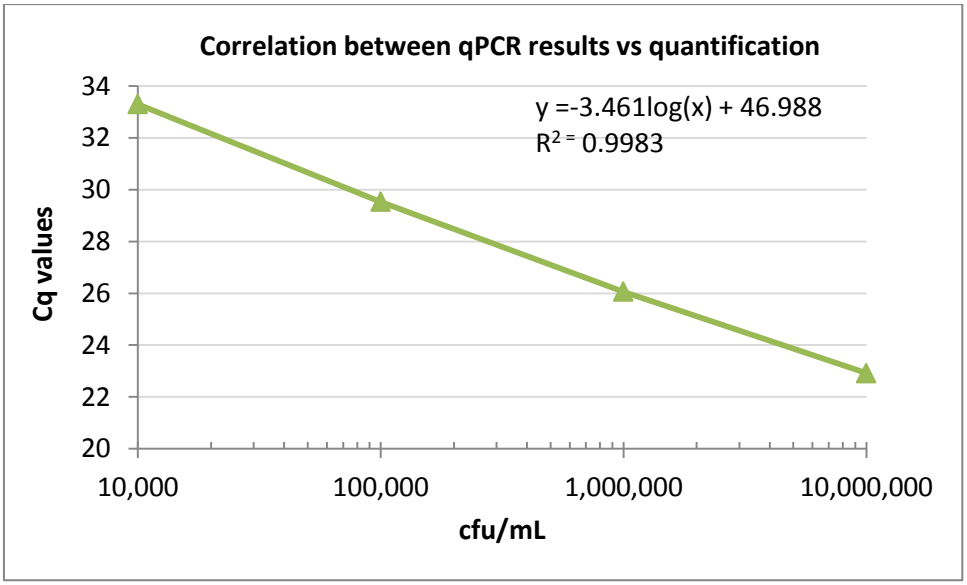


Figure 2. Cq vs concentration (on a log scale) of points on the dilution series.



This calibration implies that our lower detection limit (Cq ≈35) corresponds to a concentration of 2.9 x 10^3 cfu/mL.

Detection and survival of Psa-V colonies in infected mulch with compost

We undertook this determination since we were unsure of the effects of adding compost to mulch both in terms of the effect of available substrate on qPCR and also the effects of "enhanced" bacterial and fungal populations on Psa-V survival.

Starting with a quantified solution of Psa (1 x 10^7 cfu/mL) we constructed a dilution series down to 1 cfu/mL and added 50 mL of each concentration to 1.5 g mulch + 1.5 g compost in falcon tubes. Each concentration was then sampled at intervals (table 2) and analysed by qPCR and plated. At this stage of the trial we did not have any of our selective media available and the subsequent plating consistently gave colonies too numerous to count. This meant we do not have independent enumerations to use to construct a calibration for this compost mixture. Figure 3 shows the series dilution after various time intervals. Clearly our lowest detectable Psa-V concentration in this mixture is about 100 cfu/mL – below this concentration Cq remains at ≈35.

Figure 3. PCR results for serial dilutions of mulch + compost at time intervals.

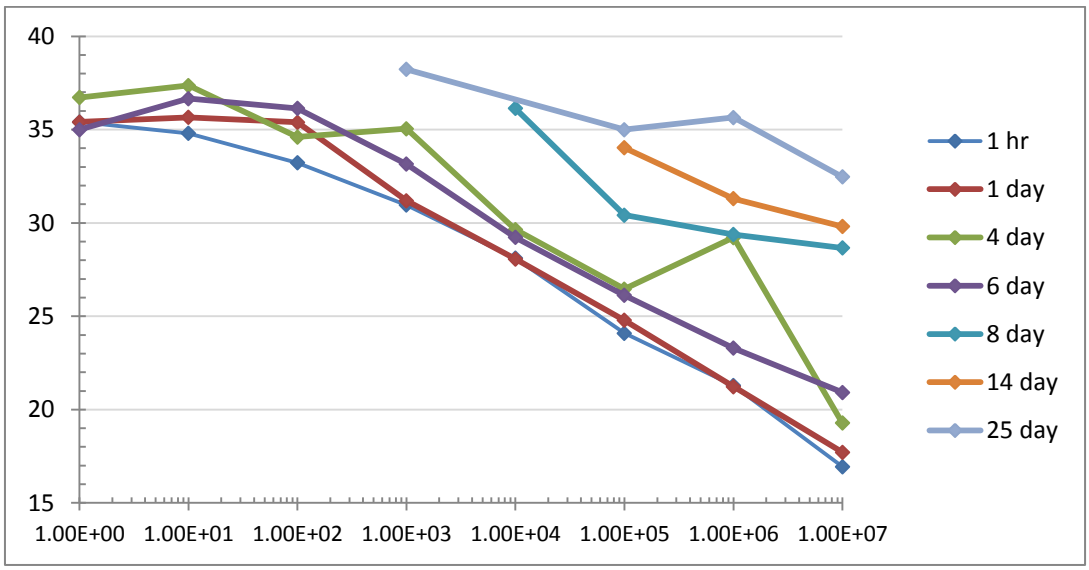


Table 4. qPCR data from laboratory dilutions of mulch+compost samples taken at intervals after mixing.

Spiking date	Sampling date	DAT	Sample ID	Psa Concentration in saline	Sample Type	qPCR (Cq)
12-Jul	12-Jul	1 hr	XN	10,000,000	M+C	16.94
12-Jul	12-Jul	1 hr	XN/10	1,000,000	M+C	21.29
12-Jul	12-Jul	1 hr	XN/100	100,000	M+C	24.09
12-Jul	12-Jul	1 hr	XN/1000	10,000	M+C	28.11
12-Jul	12-Jul	1 hr	XN/10000	1,000	M+C	30.96
12-Jul	12-Jul	1 hr	XN/100000	100	M+C	33.22
12-Jul	12-Jul	1 hr	XN/1000000	10	M+C	34.8
12-Jul	12-Jul	1 hr	XN/10000000	1	M+C	35.41
12-Jul	13-Jul	1 day	XN	10,000,000	M+C	17.71
12-Jul	13-Jul	1 day	X1/10	1,000,000	M+C	21.23
12-Jul	13-Jul	1 day	X1/100	100,000	M+C	24.79
12-Jul	13-Jul	1 day	X1/1000	10,000	M+C	28.07
12-Jul	13-Jul	1 day	X1/10000	1,000	M+C	31.19
12-Jul	13-Jul	1 day	X1/100000	100	M+C	35.39
12-Jul	13-Jul	1 day	X1/1000000	10	M+C	35.66
12-Jul	13-Jul	1 day	X1/10000000	1	M+C	35.41
12-Jul	16-Jul	4 day	XN	10,000,000	M+C	19.28
12-Jul	16-Jul	4 day	X1/10	1,000,000	M+C	29.22
12-Jul	16-Jul	4 day	X1/100	100,000	M+C	26.46
12-Jul	16-Jul	4 day	X1/1000	10,000	M+C	29.65
12-Jul	16-Jul	4 day	X1/10000	1,000	M+C	35.05
12-Jul	16-Jul	4 day	X1/100000	100	M+C	34.6
12-Jul	16-Jul	4 day	X1/1000000	10	M+C	37.36
12-Jul	16-Jul	4 day	X1/10000000	1	M+C	36.72
12-Jul	18-Jul	6 day	XN	10,000,000	M+C	20.92
12-Jul	18-Jul	6 day	X1/10	1,000,000	M+C	23.29
12-Jul	18-Jul	6 day	X1/100	100,000	M+C	26.12
12-Jul	18-Jul	6 day	X1/1000	10,000	M+C	29.23
12-Jul	18-Jul	6 day	X1/10000	1,000	M+C	33.15
12-Jul	18-Jul	6 day	X1/100000	100	M+C	36.14
12-Jul	18-Jul	6 day	X1/1000000	10	M+C	36.66
12-Jul	18-Jul	6 day	X1/10000000	1	M+C	35
12-Jul	20-Jul	8 day	XN	10,000,000	M+C	28.66
12-Jul	20-Jul	8 day	X1/10	1,000,000	M+C	29.38
12-Jul	20-Jul	8 day	X1/100	100,000	M+C	30.42
12-Jul	20-Jul	8 day	X1/1000	10,000	M+C	36.14
12-Jul	20-Jul	8 day	X1/10000	1,000	M+C	ND
12-Jul	20-Jul	8 day	X1/100000	100	M+C	ND
12-Jul	20-Jul	8 day	X1/1000000	10	M+C	ND
12-Jul	20-Jul	8 day	X1/10000000	1	M+C	ND
12-Jul	26-Jul	14 day	XN	10,000,000	M+C	29.81
12-Jul	26-Jul	14 day	X1/10	1,000,000	M+C	31.3
12-Jul	26-Jul	14 day	X1/100	100,000	M+C	34.03
12-Jul	26-Jul	14 day	X1/1000	10,000	M+C	ND
12-Jul	26-Jul	14 day	X1/10000	1,000	M+C	ND

12-Jul	26-Jul	14 day	X1/100000	100	M+C	ND
12-Jul	26-Jul	14 day	X1/1000000	10	M+C	ND
12-Jul	26-Jul	14 day	X1/10000000	1	M+C	ND
12-Jul	06-Aug		XN	10,000,000	M+C	32.47
12-Jul	06-Aug		X1/10	1,000,000	M+C	35.65
12-Jul	06-Aug		X1/100	100,000	M+C	35
12-Jul	06-Aug		X1/1000	10,000	M+C	ND
12-Jul	06-Aug		X1/10000	1,000	M+C	38.23
12-Jul	06-Aug		X1/100000	100	M+C	ND
12-Jul	06-Aug		X1/1000000	10	M+C	ND
12-Jul	06-Aug		X1/10000000	1	M+C	ND

M=Mulch, C=Compost, M+C=mulch+compost, ND=Not detected

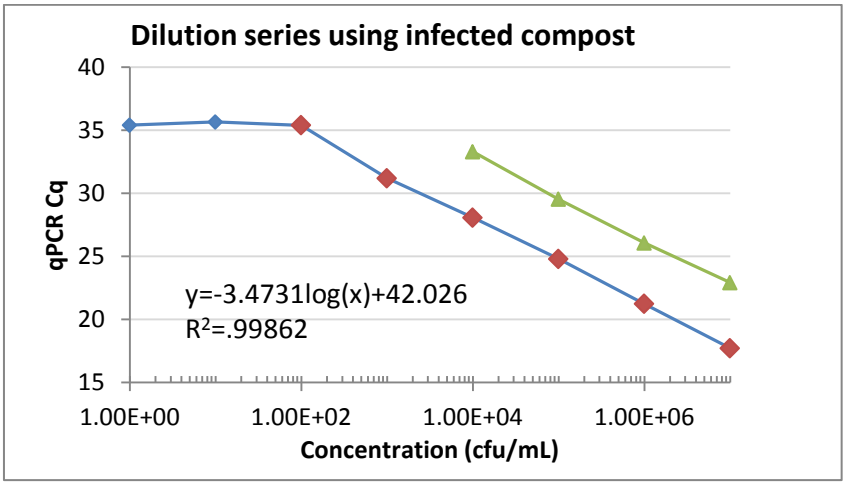
As time progresses the amount of Psa-V at any initial concentration (X axis, Figure 3) reduced (Cq increases). The straight line decline part of each curve *rises* and the *undetermined* plateau (Cq ≥ 35) extends further to the right (i.e. higher starting concentrations have declined to the point where they become undetermined). The plateau disappears (Cq=ND) as populations get too low and we are left with curve *fragments* on the right (from day 8 onwards).

These curves generally show more variability than our other graphs – for example the points at 10^6 and 10^3 cfu/mL after 4 days are both anomalously high. Whether this is lack of replication or the effect of compost we are unsure.

The reason for the reduction in Psa-V populations may be lack of substrate or it may be that the compost has anti-bacterial activity. If it was simply a lack of substrate we might expect the higher concentrations to decline first – which we did not observe.

Given the consistency of the first 2 curves (1 hr and 24 hrs) we will use this data as a calibration curve i.e. the fact that we see no change in the curves means that it is reasonable to assume that all the original Psa-V added to the solution is still present so we can use the original (neat) Psa-V concentration, along with the dilution ratios, to provide the cfu/mL for each solution at these times. Clearly this is no longer true as the Psa-V populations' declines – since we then no longer know the concentration of Psa-V in solution.

Figure 4. Dilution graph for Psa in infected compost after 24 hours: Cq vs concentration. Red points are used for the PCR calibration. Green points are those from the previous calibration above.



Using only concentrations > 10^2 cfu/mL (i.e. the linear region) we define a relationship between Cq and log[concentration] (Figure 4). The previous calibration line is also shown on the figure and it is apparent that the presence of compost has shifted this line significantly compared with the earlier calibration for mulch alone. This effect may be caused by the inhibitory effect of humic substances on PCR determinations (Matheson et. al., 2010). The slope is very similar (-3.4731) compared with the previous non-compost

correlation (-3.4619) however the line is *shifted* significantly to the left i.e. the same Cq will correspond to a significantly lower Psa concentration.

Psa-V risk from mulching equipment

18 days after the grower had finished mulching swabs were taken from the mulcher used in Trial 1 from the positions listed in table 5.

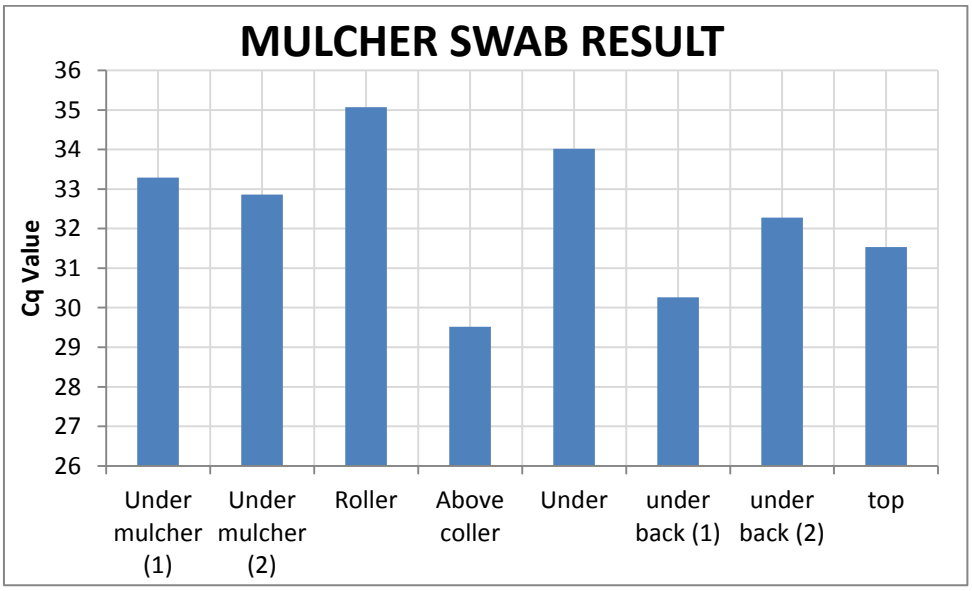
Psa-V was detected at every position using qPCR (Table 5 and Figure 5) however none of the swabs yielded any growth of viable Psa-V when cultured on media i.e. the bacteria were present on the mulching equipment post-mulching but were not viable by the time we swabbed. This points to the importance of cleaning and sanitisation being essential to limit the spread of Psa-V for equipment in constant use. It does not tell us the Psa viability time since we did not undertake a time series of swabs from the equipment. Through the experimental circumstances we know that after 18 days all Psa was dead.

Table 5: Summary of results of swabs on mulcher

Test No	Date	Sampling position	ID	Media B	Media H	Psa-V media	Cq	Overall result
1	12-Jun-12	Under mulcher (1)	Sw 1	NG	NG	NG	33.29	Detected, non-viable
2	12-Jun-12	Under mulcher (2)	Sw 2	NG	NG	NG	32.86	Detected, non-viable
3	12-Jun-12	Roller	Sw 3	NG	NG	NG	35.07	Detected, non-viable
4	12-Jun-12	Above collar	Sw 4	NG	NG	NG	29.52	Detected , non-viable
5	12-Jun-12	Under	Sw 5	NG	NG	NG	34.02	Detected, non-viable
6	12-Jun-12	under back (1)	Sw 6	NG	NG	NG	30.26	Detected, non-viable
7	12-Jun-12	under back (2)	Sw 7	NG	NG	NG	32.28	Detected, non-viable
8	12-Jun-12	top	Sw 8	NG	NG	NG	31.53	Detected, non-viable

(1). Closer to tractor; (2) further back.

Figure 5. Results of swabs on mulching equipment using qPCR



Trial 1

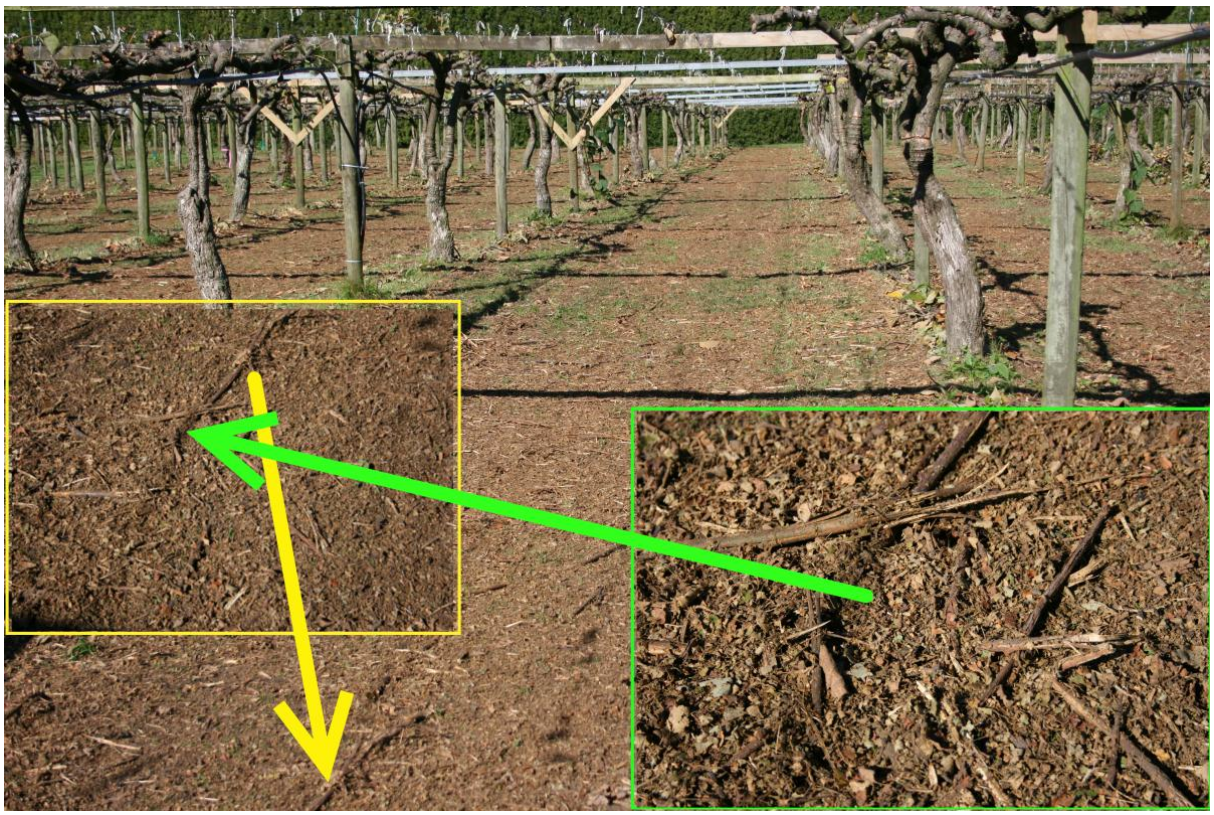
Photos 1 - 4 from the site of the first trial show the process of canopy removal and mulching.

Photo 1. Psa infected Hort16A orchard in the process of being pruned and mulched on a fine warm day after harvest.

Photo 2. The volume of material to be mulched on the orchard floor.

Photo 3.The first mulch of 2 passes (one each way) each biased to one side

Photo 4. Final result after two passes of the mulcher with 2 enlargements showing the granularity and degree of ground cover.



Prior to the application of any treatments all plots were sampled. The same day the Nordox and nitrogen (Tec Urea) treatments were applied (Trial 1: Part A). Four days later the chlorine dioxide (ClO_2) and PS1+BioStart treatments were applied (Trial 1: Part B).

Isolation, Identification and Quantification of Psa-V from mulch infected samples

Trial 1 part A: Nordox and Nitrogen

The first weekly sample was taken 5 days after the Part 1 treatments were applied (1 day after Part B treatments were applied). Psa-V was detected on all samples (treated and untreated) by qPCR and all samples grew viable colonies on plates (Tables 6 and 7) i.e. there were no ND values and only 1 sample was >35 (see below). Subsequent qPCR tests identified Psa-V in every determination from this trial but it was not found in all plating enumerations.

Figures 6 and 7 show the ***individual plot data*** from each replicate for the control and Nordox treatments respectively. The one extreme outlier in the control treatment (Cq≈40, Fig 6) was the only such outlier seen throughout this trial and will be excluded from further analyses. The data ***excluding*** this outlier are shown in brackets in Table 6. All other points showed consistent data and trends.

Figure 6. Time series of Cq values from each of the control plots

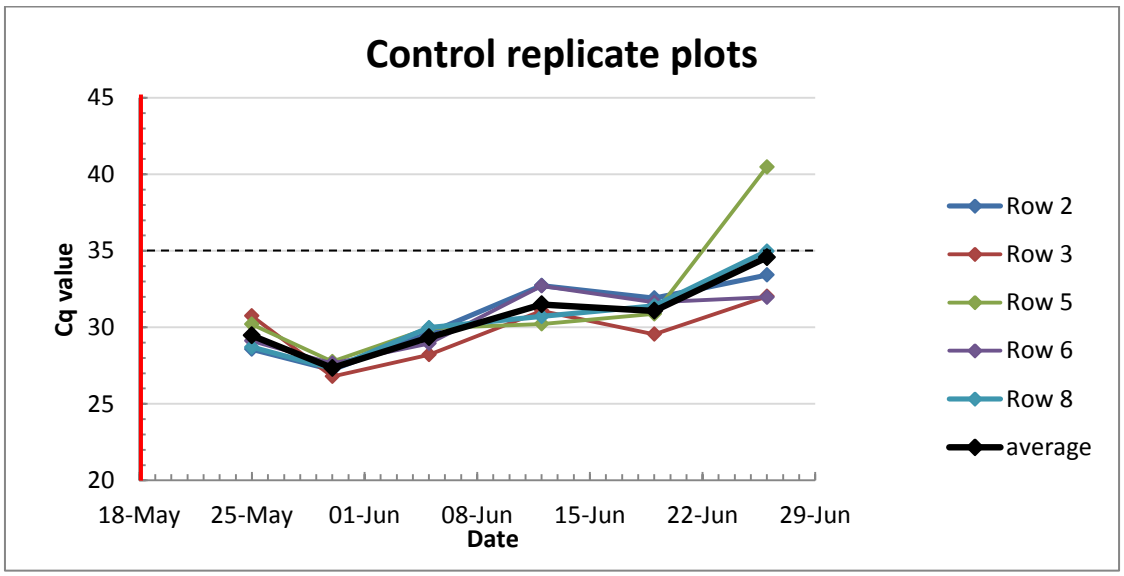


Table 6: Summary of results for trial 1A: average Cq, standard error, and presence of plate growth (viability).

Treatment applied	Sample date	Control Av Cq	Control S.E	Nordox Av Cq	Nordox S.E	Nitrogen Av Cq	Nitrogen S.E	Plate growth of colonies
Pre-Trt sample	25-May-12	29.46	0.43	30.37	0.36	29.87	0.56	Growth
25-May-12	30-May-12	27.35	0.17	27.24	0.54	27.52	0.37	Growth
25-May-12	5-Jun-12	29.34	0.34	29.5	0.26	28.95	0.52	Growth
25-May-12	12-Jun-12	31.49	0.52	29.54	0.40	32.13	0.65	Growth
25-May-12	19-Jun-12	31.07	0.42	31.47	0.67	31.51	0.24	Growth
25-May-12	26-Jun-12	34.6(33.1)	1.58(0.71)	32.84	0.46	33.71	0.62	Growth

Given this level of data consistency we will henceforth present ***treatment means*** from each date along with standard errors (standard deviation in the means; Table 6). Graphs will include the modified average shown in brackets in table 6.

The treatment level qPCR data is shown in Table 6 and graphed in Figure 8. The data looks remarkably consistent however we must remember that this is a log scale (inverted) of bacterial populations and log

scales tend to reduce visual differences. The general trend is for the population to increase to a maximum after 1 week (lowest Cq) and then for bacterial numbers to drop away from this maximum value by almost 2 decades by 5 weeks after mulching. There appears little to pick between treatments – nothing between the control and nitrogen treatments and only one week when the Nordox was slightly worse (higher Psa).

Figure 7. Time series of Cq values from each of the Nordox plots. The vertical dashed line shows the treatment application date.

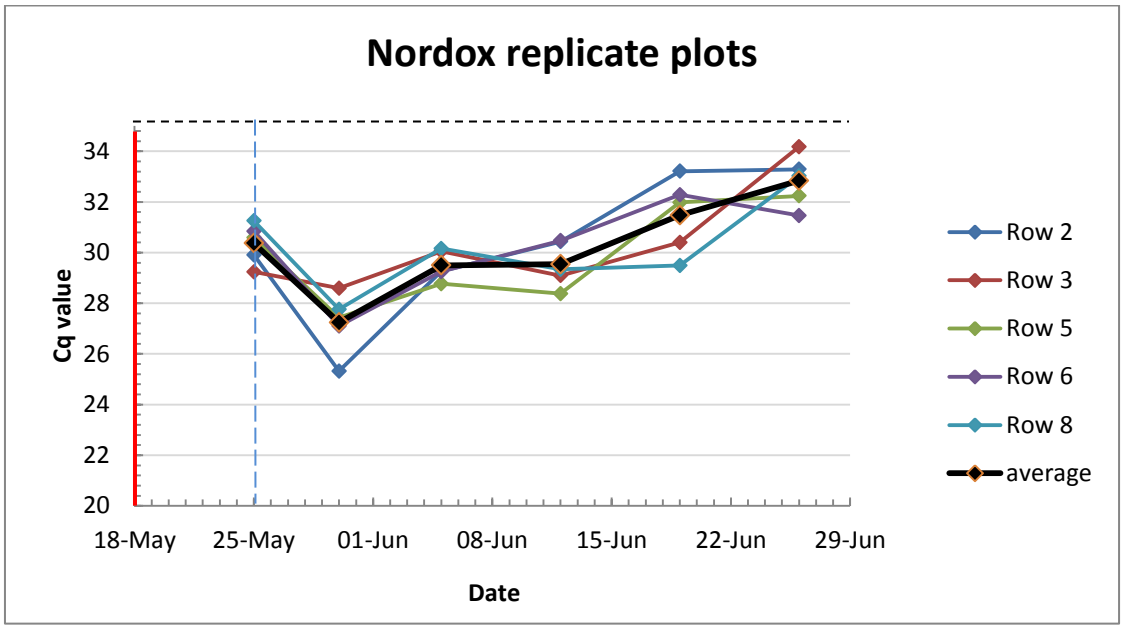


Figure 8. Mean values (and their standard errors) of Cq for each treatment and time from Trial 1A.

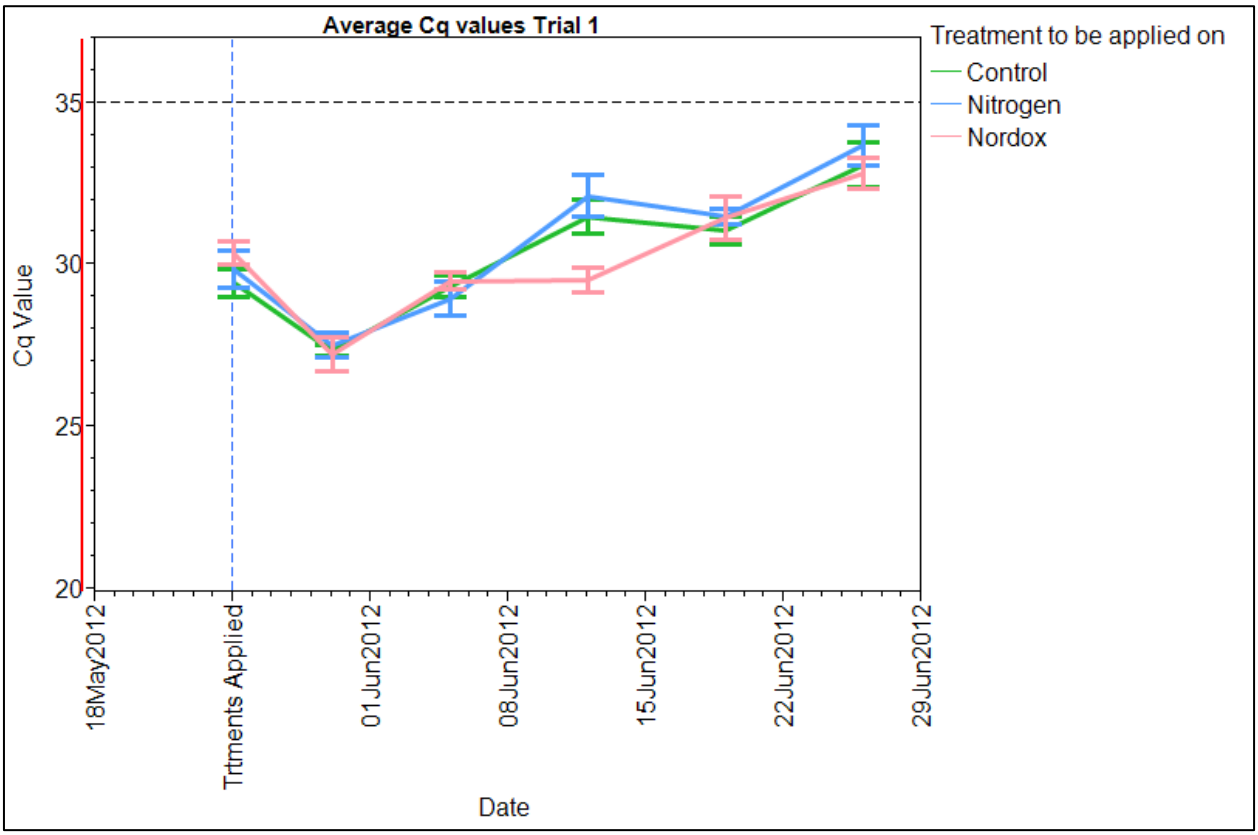


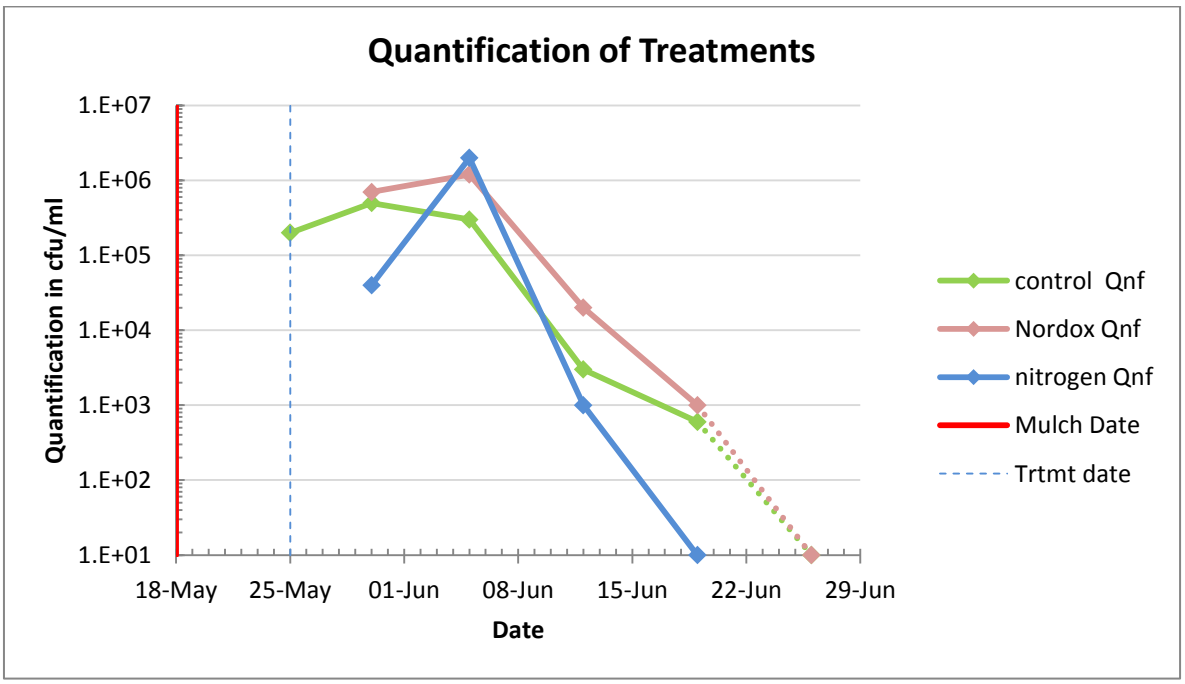
Table 7 and Fig 9 show the quantification results (cfu/m) for Part A of the trial. Both treatments show an increase in numbers at the second sampling after treatment followed by an abrupt decrease. While trends are the same as seen in the qPCR results, somewhat curiously the peaks for both treatments from this determination occurred on the 5th Jun for both treatments which is a week later than observed in the qPCR data. We note that we expect greater sampling errors in the quantification results since there was no replication (cost savings) unlike the PCR where every plot was sampled and a Cq determined.

Again the Nordox treatment showed ***no*** inhibitory activity against Psa-V compared with the untreated control however the nitrogen data show a more abrupt decrease, after a higher peak, in the last 2 samples. We also note that ***all*** samples were zero (no live Psa found) at any dilution at the last sampling.

Table 7: Summary of results in cfu/mL post treatment v/s no treatment on mulch samples

Treatment	Sampling date	Control	Nordox	Nitrogen
pre-trtment	25-May-12	200,000		
25-May-12	30-May-12	500,000	700,000	40,000
25-May-12	5-Jun-12	300,000	1,200,000	2,000,000
25-May-12	12-Jun-12	3,000	20,000	1,000
25-May-12	19-Jun-12	600	1,000	10
25-May-12	26-Jun-12	10	10	

Figure 9. Quantification of Psa (cfu/mL). ND values are replaced by 10 only on their first occurrence – further ND's are left blank.

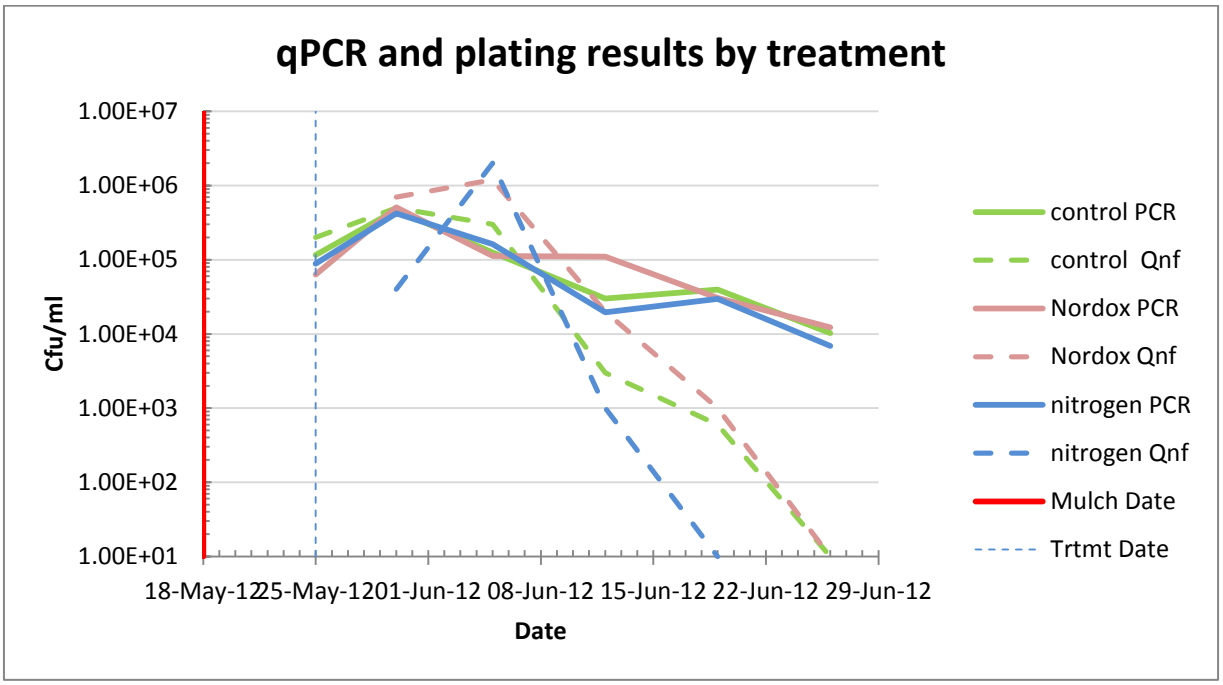


If we use the calibration between Cq and the plating enumeration shown earlier (Figure 4) we can plot both datasets on the same graph as cfu/mL (Figure 10). The starting absolute values are gratifyingly similar and the two datasets follow the same general form. The plating estimates for both treatments peaked a week later than the qPCR data and then show a more rapid fall-off to much lower levels. At least part of this divergence may reflect the difference between dead and alive bacteria. At the start we might expect most bacteria to be alive and multiplying on the newly exposed substrate. In the longer term we expect the mulch environment to be much less hospitable for Psa-V since the surface will be dry, and hot (under direct sunlight) as well as dead and decaying i.e. the food source will dissipate more rapidly than within a cane and the environment should be much less hospitable for Psa survival - and that's apart from the effect of any applied treatment). This may explain the rapid drop of the plated (live) determination finishing at zero.

It does not explain the higher peak found by plating a week after the peak in the qPCR data since both live and dead DNA should be detected by qPCR.

The nitrogen treatment appears to hasten the loss in Psa numbers.

Figure 10. qPCR and plating results for Nordox and nitrogen. Cq values converted to cfu/mL. Final points for the plate enumerations for all treatments were zero and are not plotted



Trial 1 Part B: chlorine dioxide and PS1+BioStart

Because the same control plots were used as in Part A pre-treatment samples were taken 4 days before these treatments were applied and the first post-treatment sample was taken 1 day after their application.

Again Psa-V was detected in all samples (treated and untreated) by qPCR but viable colonies were not always found on plates. Table 8 and Figure 11 show a summary of the qPCR results. The two treatments show similar trends to those already seen in Part A: maximum numbers were found on the 30th May followed by a decline through the remainder of the trial. All treatments (and the control) showed remarkably close agreement throughout and again there is no difference between the control and the treatments.

Table 8: Summary of results of treatment Field trial 1 continued (Average Cq values)

Treatment applied	Sampling date	Control Avg Cq	Control S.E	chlorine dioxide Av Cq	chlorine dioxide S.E	PS1 Biostart Av Cq	PS1 Biostart S.E	Plate growth of colonies
pre trtment	25-May-12	29.46	0.43	29.07	0.46	29.82	0.22	Growth
29-May-12	30-May-12	27.35	0.17	27.29	0.33	27.13	0.27	Growth
29-May-12	5-Jun-12	29.34	0.34	29.69	0.48	29.47	0.37	Growth
29-May-12	12-Jun-12	31.49	0.52	30.97	0.30	32.04	0.50	Growth
29-May-12	19-Jun-12	31.07	0.42	33.13	0.47	31.22	0.21	Growth
29-May-12	26-Jun-12	33.1[1]	0.71[1]	32.96	0.15	31.53	0.72	Growth

1. The one outlier rep has been deleted as discussed above.

Figure 11. Mean values (and their standard errors) of Cq for each treatment and time from Trial 1B

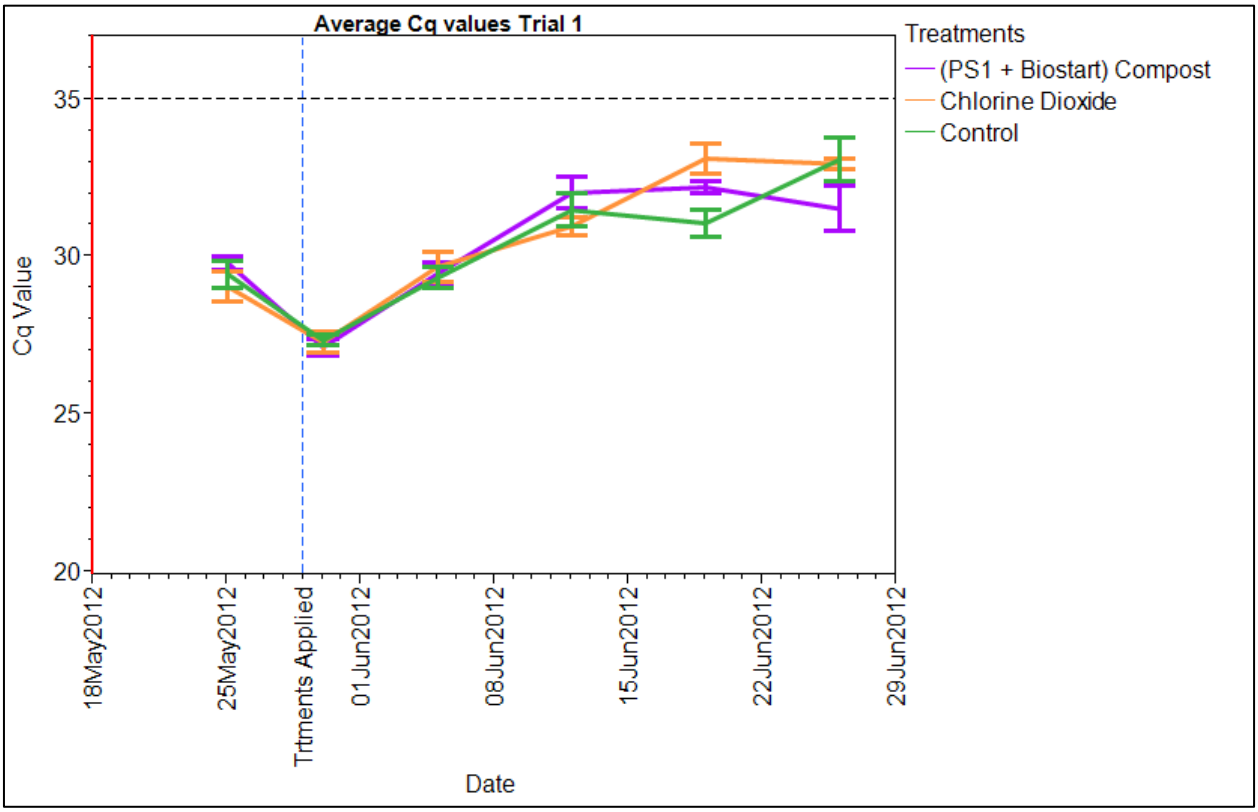


Table 9 and Figure 12 show the enumeration results from plating for these two treatments and the control.

Figure 12. Quantification of Psa (cfu/mL.

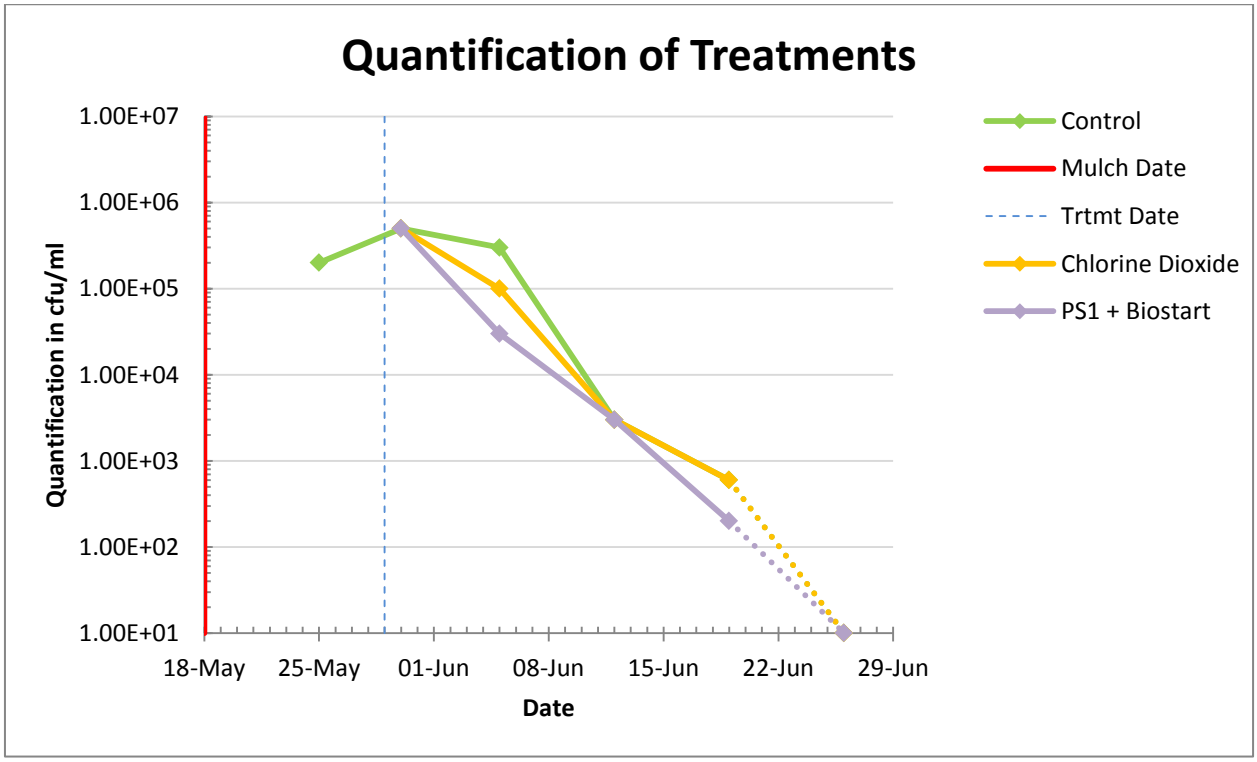
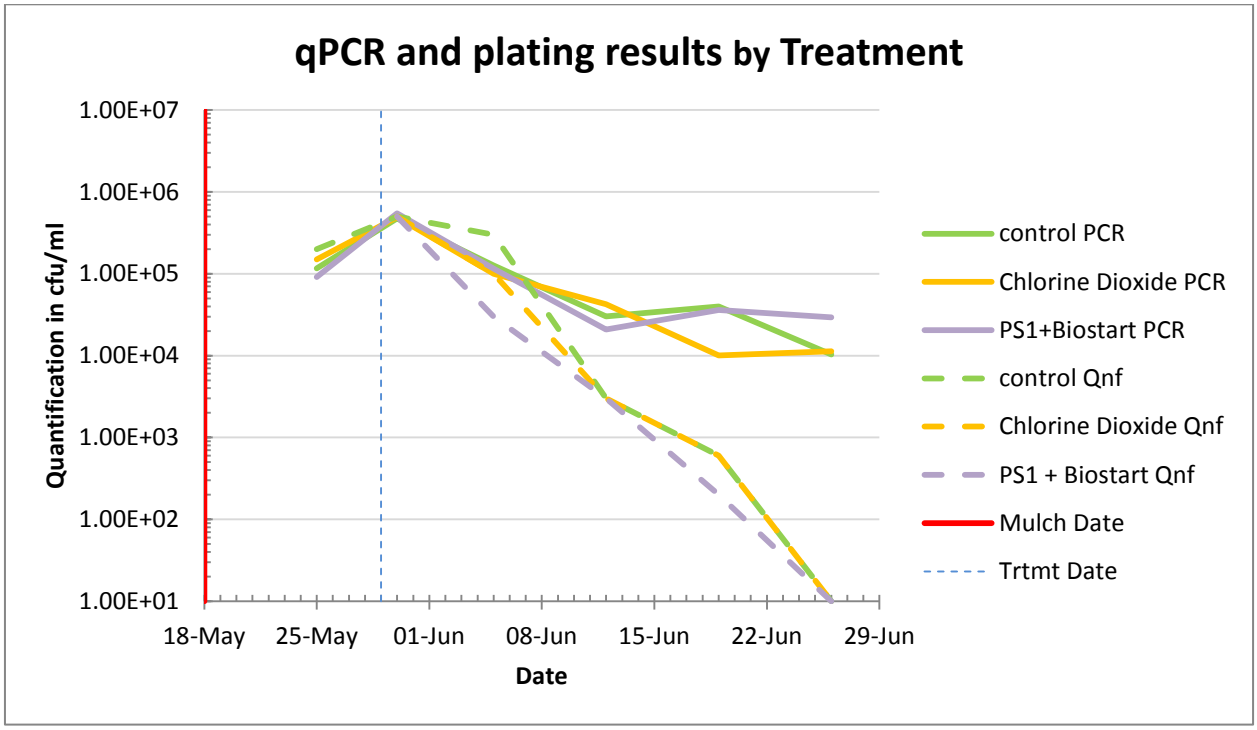


Table 9: Summary of quantifications in cfu/mL post treatment v/s no treatment on mulch samples

Treatment applied	sample date	Control	Chlorine Dioxide	Ps1 + biostart
pre trtment	25-May-12	200,000		
29-May-12	30-May-12	500,000	500,000	500,000
29-May-12	5-Jun-12	300,000	100,000	30,000
29-May-12	12-Jun-12	3,000	3,000	3,000
29-May-12	19-Jun-12	600	600	200
29-May-12	26-Jun-12	10	10	10

We again convert the qPCR data to cfu/mL and plot both estimates on the same graph on a log scale to maintain visibility over a large number of decades (Figure 13).

Figure 13. qPCR and plating results for $ClO_2$and PS1+Biostart. Cq values converted to cfu/mL

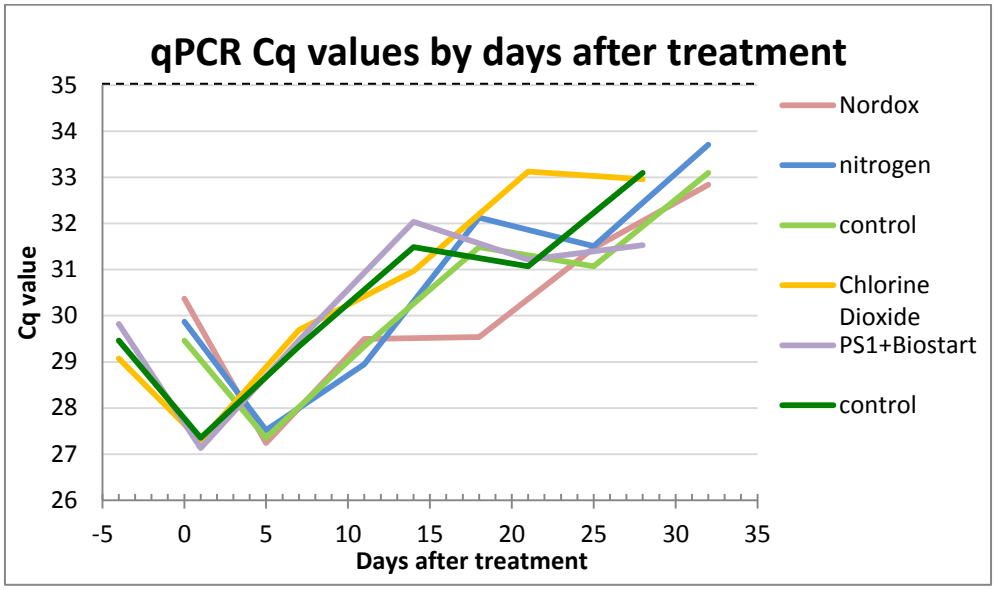


Again we see remarkably good agreement at the first 2 samplings and again we see the qPCR estimates declining much more slowly than those from plating and enumeration. Invoking the same argument as we used above it would appear that the number of live Psa drops rapidly to undetectable levels by 5 weeks after sampling. In this case both treatments seem to hasten the degradation of Psa in the first week after the maximum levels are reached. From the on there is little to choose between treatments.

Controlling variate: Treatment or Mulching

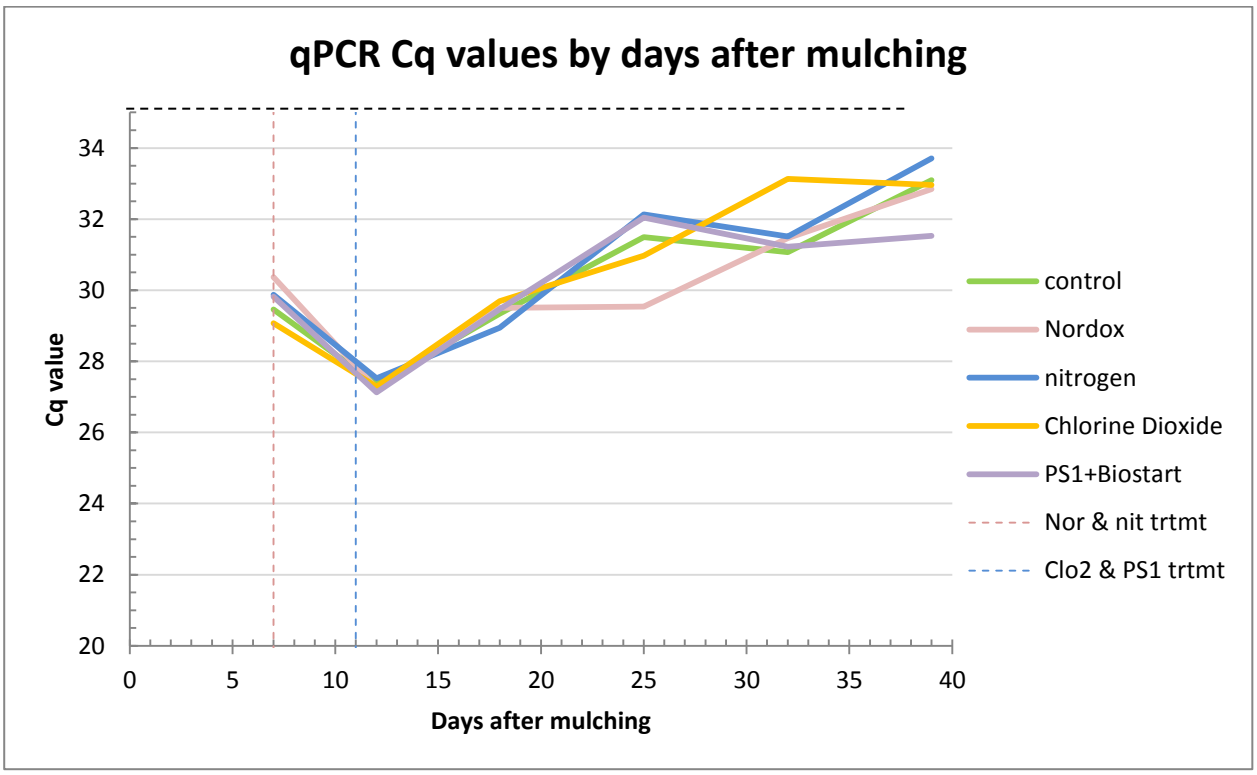
The fact that we applied treatments at different times allows us the option of comparing results aligned by days after treatment or days after mulching (corresponding with date in this case). Plotting against both options allows us to evaluate the likely controlling variable (if such exists) indicated by the degree of collapse of the lines. Figure 14 shows the data aligned by days after treatment and clearly the data does not collapse (we have 2 distinct curves) indicating that the applied treatments are not controlling the growth and decay of Psa.

Figure 14. Data from trial 1 A and B plotted against days after treatment.



If we show the same data plotted against time from mulching (Figure 15) we find a remarkably good collapse indicating that either weather or time of mulching is controlling bacterial populations. In these weather conditions, the bacterial numbers peaked around 12 days after mulching (within the accuracy of our weekly samples) and then declined steadily.

Figure 15. Data from Trial 1 A and B aligned to cays after mulching



The enumeration data, along with the weather data through the trial period is shown in Figure 16 At a simple observational level there appears to be no obvious link between weather and the Psa levels. Average temperature is relatively flat through the period (≈10°C) and rainfall occurs intermittently. We will compare this with the later compost trial which took place approx. 1 month later.

Figure 16. Enumeration data from trial 1 (right hand axis) together with rainfall, solar radiation, and average temperature (left hand axis).

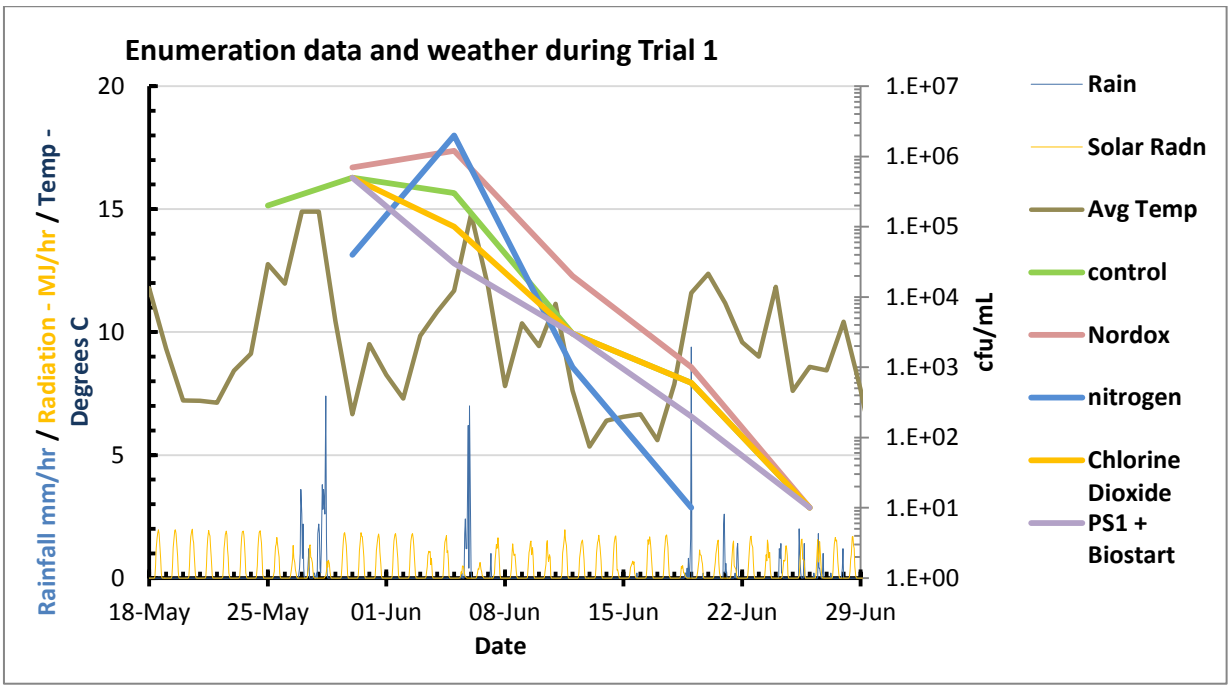
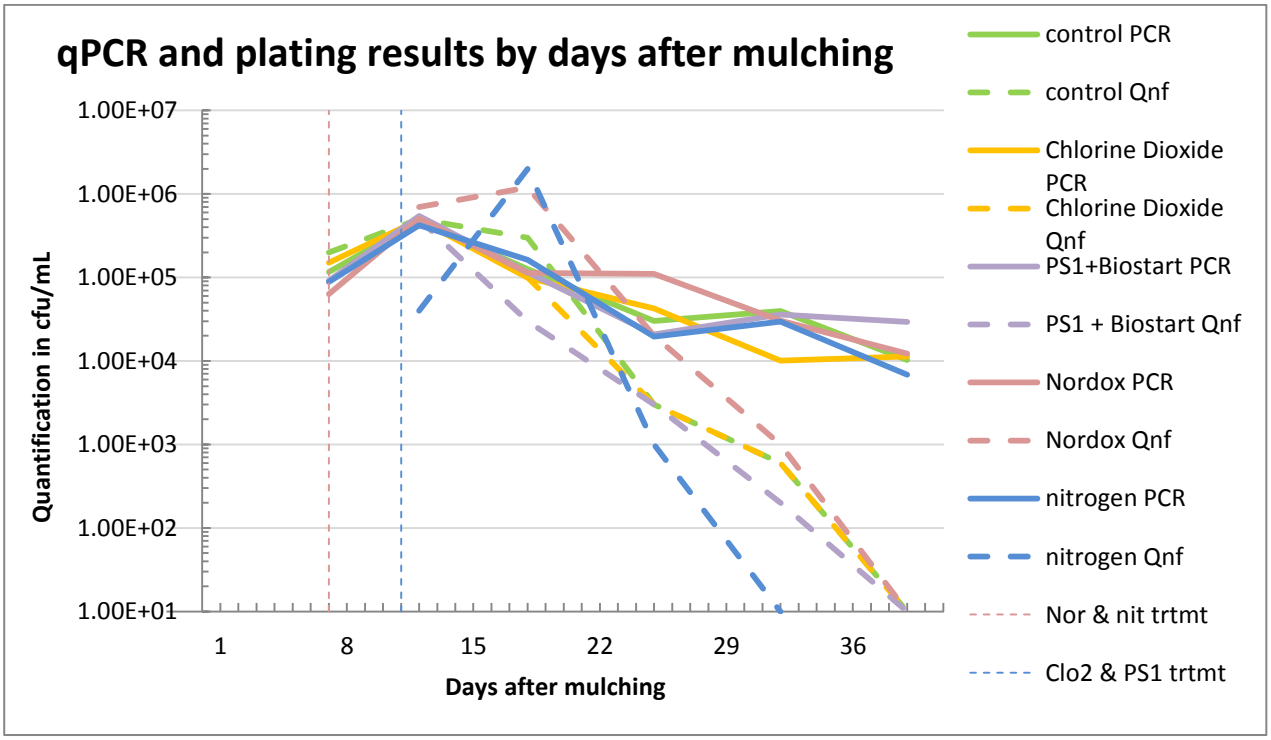


Figure 17 shows the combined data from both parts of the trial and including qPCR data and plating enumerations. We note again that while the qPCR data reached its maxim 12 days after mulching some of the plating enumeration data shows live populations rising for another week. In the longer term the nitrogen treatment appears to drop fastest with the others all following about a week later. If these data are at all accurate Psa dropped to very low levels in the mulch especially when we remember that values of 10cfu/mL are arbitrarily imposed when the plates showed zero growth i.e. the lower limit of resolution.

Figure 17. qPCR and plate data from Trial 1 A and B combined against days after mulching.



Field Trial 2: Raking, mulching, Nordox

Two types of treated samples were collected for this trial comparing leafy and grassy samples. The trial started after both mulching (25th May) and Nordox (27th May) had been applied and therefore a control sample was not available for comparison.

Two soil samples were taken on the first sampling date. Both came back negative.

Table 9: Summary of soil sampling results Field trial 2

Soil sample	PCR results (Cq)	Growth of Psa	PCR results(Cq)	Growth of Psa
Sampling date	Soil sample 1	Soil sample 1	Soil sample 2	Soil sample 2
1-Jun-12	ND	No growth	ND	No growth

ND = not determined i.e. no Psa found

We have done little work looking for Psa-V in soil however we tried this measurement since the ground was so bare after raking.

We then took weekly samples from the two surface "types" for the following 5 weeks. The surface categories represent the fact that some of the orchard (which had been cropped last season) had little grass and consequently the only cover after raking was a few leaves and a little other debris. Other areas had retained more grass (presumably less canopy shading during the season) and consisted of a grass sward with some leaves and other debris.

Table 10 shows the results from the weekly qPCR determinations which are graphed in Figure 18.

Table 10: Summary of results Field trial 2

	PCR results (Cq)	Growth of Psa	PCR results(Cq)	Growth of Psa
Sampling date	Leafy mulch	Leafy mulch	Grassy mulch	Grassy mulch
1-Jun-12	29.2	Growth	30.81	Growth
8-Jun-12	34.32	Growth	35.9	No growth
15-Jun-12	32.2	No growth	35.2	No growth
22-Jun-12	ND(38)	No growth	ND(38)	No growth
5-Jul-12	30.1*	No growth	ND	No growth

*** Outlier not included in graph; ND = not determined and assigned a value of 38 for plotting**

The quantification results showed leafy mulch had a higher load of bacteria (lower Cq) compared with the grassy mulch. N***o*** viable Psa-V colonies were detected after week 2 from either sample and no reliable Cq value after week 3. A Cq value was detected on the 5th July (leafy mulch; table 10) which seems to be an outlier given the weight of other evidence after week 2. This point is shown on figure 18 as a hollow diamond. It might have been associated with a particular piece of debris or it may simply highlight the fact that Psa ***may*** be found erratically in a contaminated orchard environment under almost any circumstances.

Enumeration from the plated samples is given in table 11 and graphed in Figure 19. These show an abrupt population drop over the first 3 samplings followed by zero results for the last two samples. If we undertake the same approach as we undertook for the first trial we can compare the qPCR and enumeration results (Figure 20). Again we get reasonable agreement between qPCR and plate enumeration for the first 2 samples with followed by a faster drop in the enumeration results than in the qPCR (as we saw in trial 1) which have suggested may be due to the faster drop of a live bacterial population.

Figure 18. qPCR data for each sample type from Trial 2. The points where a Cq was not determined are indicated by the dotted lines (heading up to the *assigned* maximum of 38) and the outlier which appeared at the final sampling is shown by the hollow blue diamond.

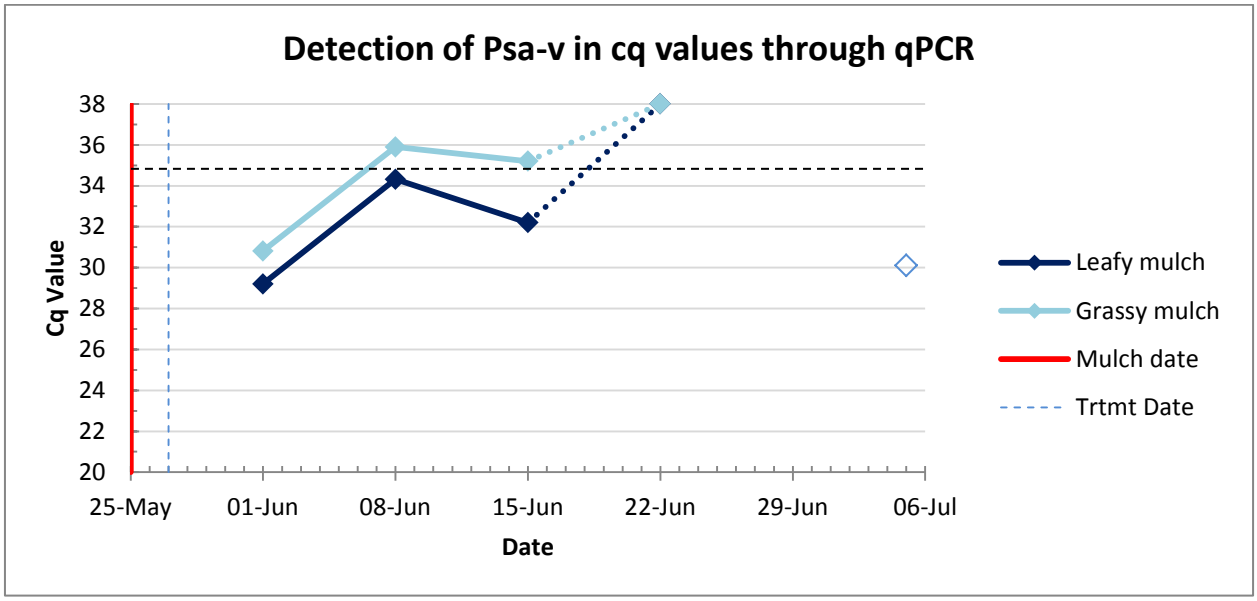


Table 11: Summary of quantification results (cfu/mL) from field trial 2

Sampling date	Leafy mulch	Grassy mulch
1-Jun-12	400,000	100,000
8-Jun-12	6,000	12,000
15-Jun-12	100	100
22-Jun-12	10	10
5-Jul-12	0	0

Figure 19. Quantification of Psa (cfu/mL) from plating. The penultimate samples are shown at 10 cfu/mL (the detection threshold) as their plate value=0. Final zero values not shown.

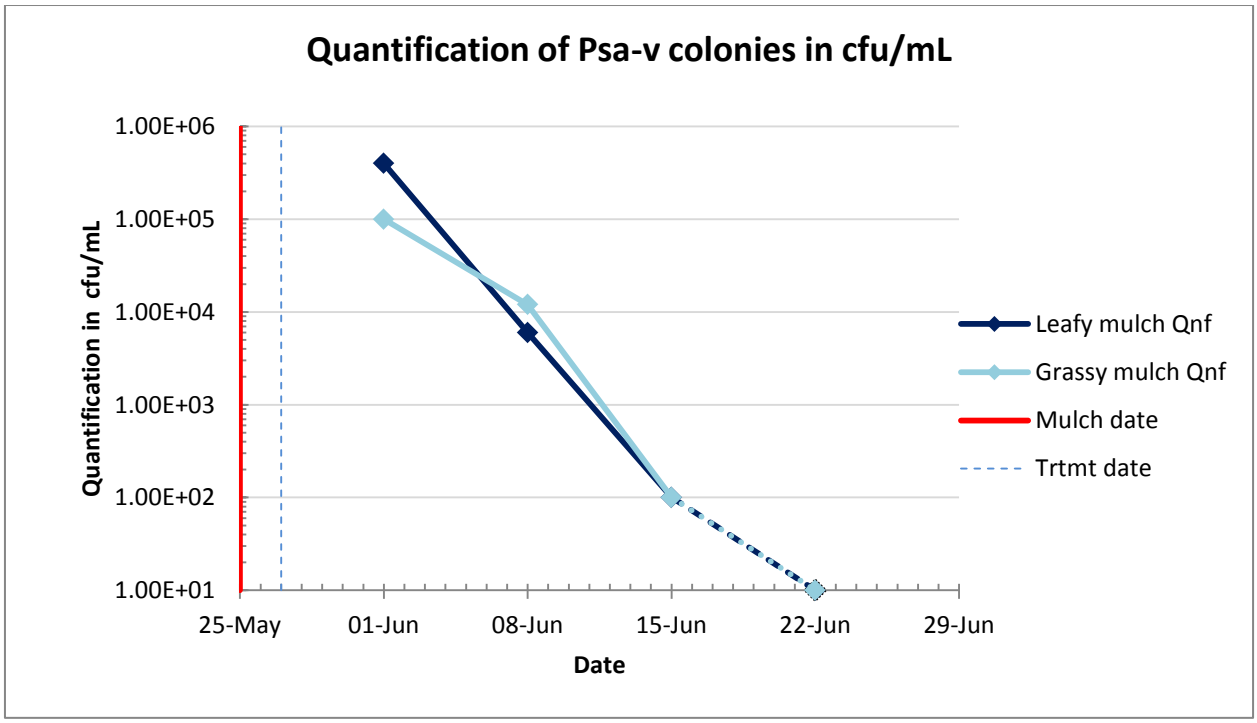
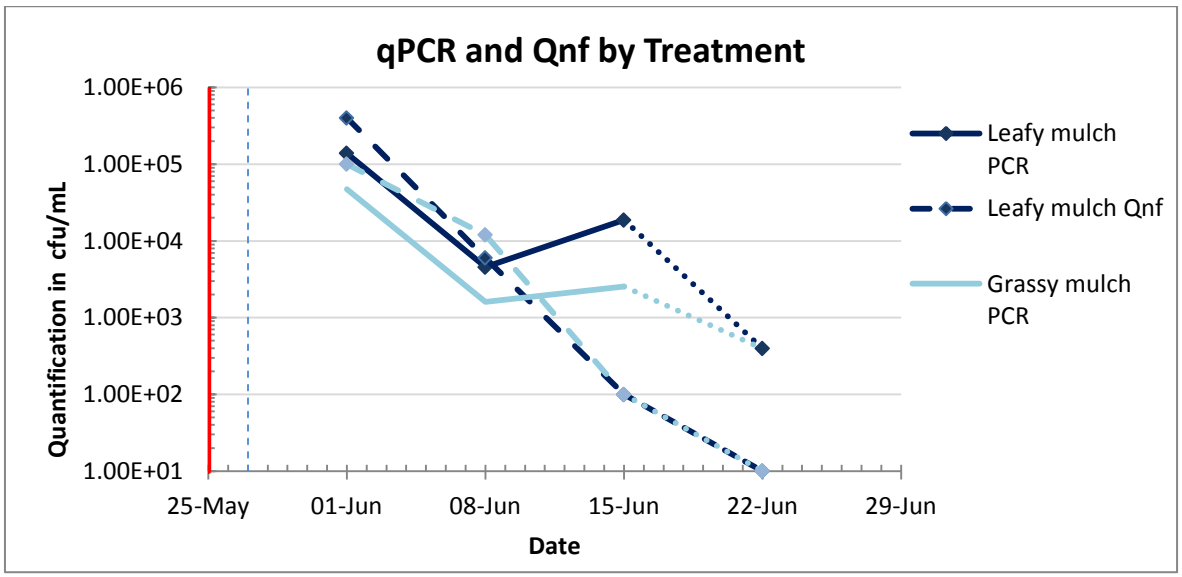


Figure 20. qPCR and plate enumeration for treatments in Trial 2. Undetermined values assigned arbitrary values indicated by dotted approach lines.



Field Trial 3: Mulch + compost

This trial was undertaken several weeks after the first trial at another orchard. Photo 5 shows a single compost plot (band across the row) and a view of the surface. Ten tonnes/Ha of compost corresponds to 1 kg/m^2. As in trial 1 the mulching (11th July) and compost treatments were applied immediately after canopy removal. Table 12 and Figure 21 show the data from this trial.

Photo 5. Newly applied compost plot (10 t/Ha) onto the mulched surface

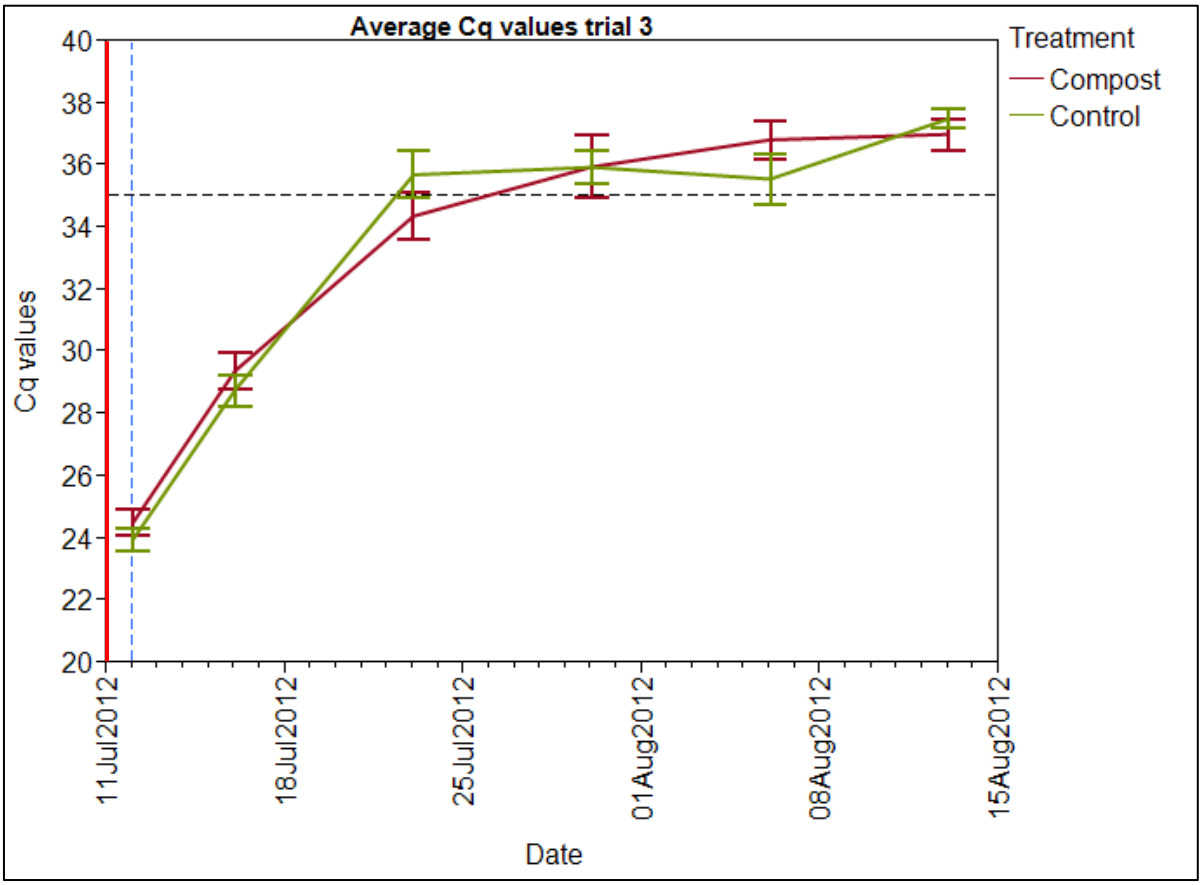
Table 12: Summary of qPCR results (Cq values) from field trial 3 showing all replicates

Trtments	Date	compost replicate						control replicate					
Applied	Sampled	R1	R2	R2b	R3	R4	R5	C1	C2	C2b	C3	C4	C5
Pre-Trtmt	12-Jul-12	22.59	25.11	24.69	25.22	24.94	24.65	22.87	23.11	23.96	24.07	25.30	24.6
12-Jul-12	16-Jul-12	30.7	28.47	27.21	30.62	30.59	28.88	27.69	27.23	30.44	29.48	29.50	28.4
12-Jul-12	23-Jul-12	34.14	33.23	33.02	33.91	34.05	38	32.67	37.98	36.2	35.23	35.14	37.1
12-Jul-12	30-Jul-12	32.02	34.93	34.9	38	38	38	36.22	35.21	36.65	34.39	35.31	38
12-Jul-12	6-Aug-12	38	37.7	37.13	36.12	34.14	38	36.86	38	34.45	32.93	36.92	34.3
12-Jul-12	13-Aug-12	38	38	37.04	38	36.09	35.03	36.9	38	38	38	36.24	38

Red values were "undetermined" i.e. no Psa detected. For the purposes of averaging they have been assigned 38 which is just above the highest "determined" value = 37.98

Again there was good agreement between replicates however in this trial there were a significant number of non-determined Cq values i.e. no evidence of Psa in these samples. This raises the issue of how to treat these in relation to averaging for treatment effects. While Cq=35 is our nominal detection threshold (for our pre-compost calibration") numerous determinations were greater than that – the maximum Cq in this trial being 37.98. We have decided to assign a value of 38 to the non-determined samples i.e. an integer corresponding with the maximum determined value from this trial. While the resulting effects are small, Figures 21 and 22 reflect these decisions. Using the *compost* calibration the value of Cq = 38 corresponds to 14 cfu/mL which is shown in figure 22 by the horizontal green dashed line.

Figure 21. qPCR results for Trial 3: mulch and mulch + compost



This qPCR data only shows a couple of marginal treatment differences between treatments (figure 21), overall there was little effect of adding compost on the rate of Psa decay from the qPCR data. The lack of a "growth" phase is certainly different from previous trial results however since it appears in the control it would appear to be associated with the difference in initial infection or the weather at the time.

As with the earlier trials we also plated and enumerated samples from these plots (using the selective media) with the results shown in Table 13. They are combined with the qPCR data using the calibration for mulch + compost in Figure (22).

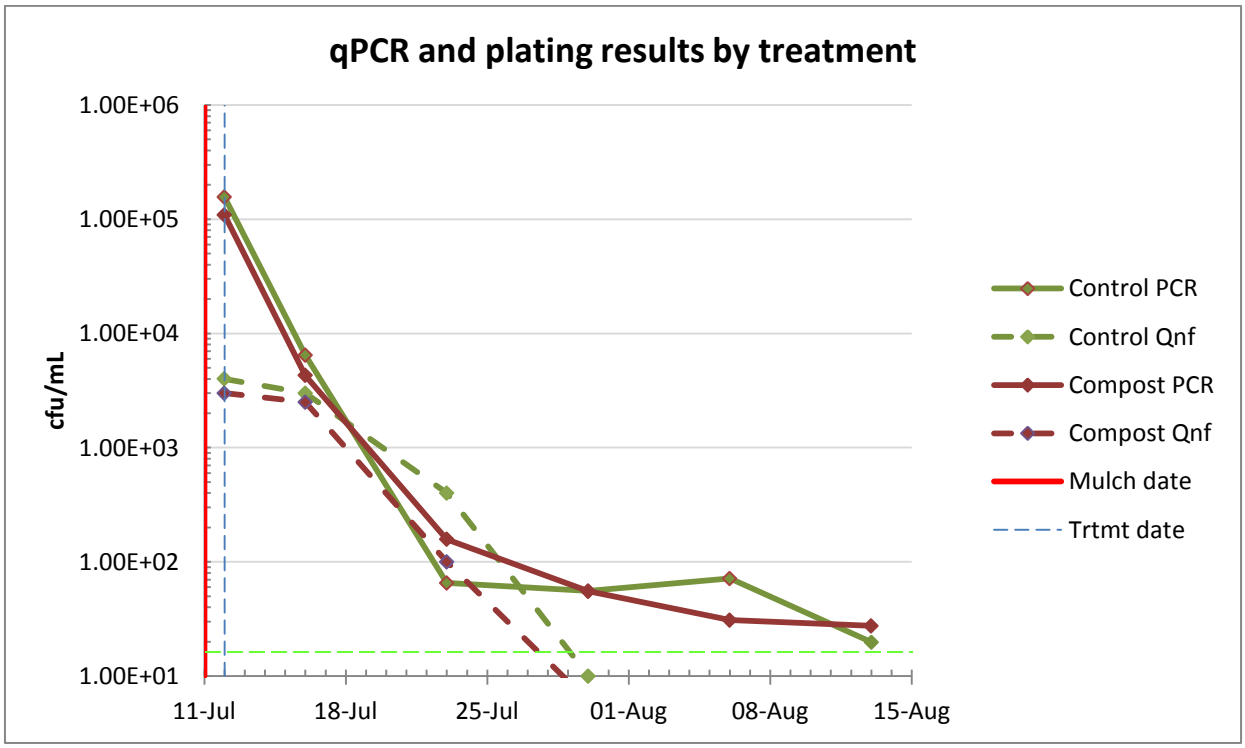
Table 13: Summary of quantification results in cfu/mL compost treatment Field trial 3

Date	Control	Compost
12-Jul-12	4,000	3,000
16-Jul-12	3,000	2,500
23-Jul-12	400	100
30-Jul-12	10	6
6-Aug-12	0	0
13-Aug-12	0	0

These results are broadly similar to the early trials i.e. a rapid decline in numbers, however they are ***qualitatively*** and ***quantitatively*** somewhat different – maximum numbers from this trial (including the control) were lower (4 x 10^3 cfu/mL compared with starting values in the other trials of 10^5-10^6 cfu/mL i.e. 100 times lower), there was no evidence of an increase in numbers followed by a decline and there was more difference between the curves from qPCR and plating enumeration.

While we can postulate possible causes for reductions in bacterial populations (less infected orchard, different temperatures, different weather conditions etc) explanations of this ilk do not explain the discrepancy between the qPCR and plating methodologies at the start of the trial which have hitherto shown closer agreement.

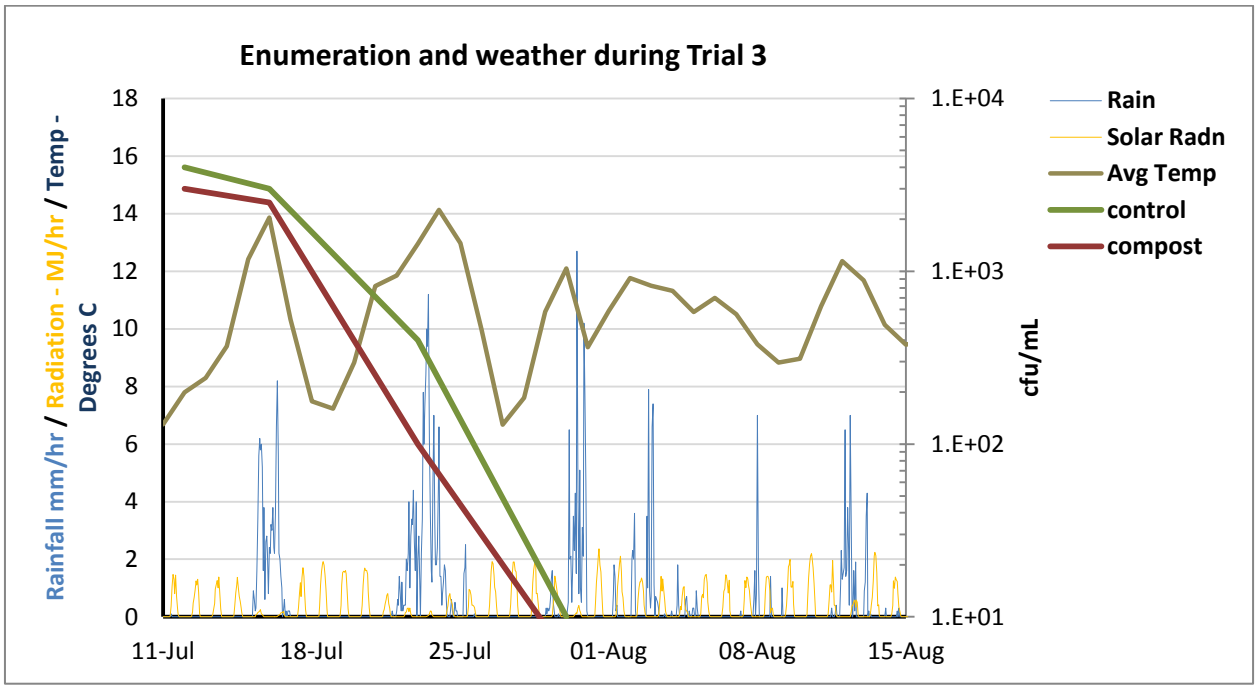
Figure 22. qPCr results transformed to cfu/mL using the earlier calibration along with plate enumeration results for trial 3. The green dashed line shows the minimum possible value for qPCR results corresponding to Cq=38 (see text)



The rate of decline in Psa-V levels in the ***enumerated*** population is about the same as for the previous trials – it reaches 10cfu/mL sooner only because it started from a lower level. A comparison of weather data (Figure 23 and Figure 16) shows rainfall and temperatures to be similar through both trials i.e. weather is not an obvious reason for the differences noted in this trial. Despite the minimal differences between the qPCR data it appears that compost may be causing the differences we have noted in this trial and which are

particularly apparent in the plating enumeration results. It is unfortunate that compost was not included in Trial 1 as that would have eliminated initial level of infection and weather as possible reasons for the differences we have noted in the compost trial.

Figure 23. Trial 3 Enumeration data together with some weather variates. The trial continued for the extent of the graph, enumeration was 0 for those times.



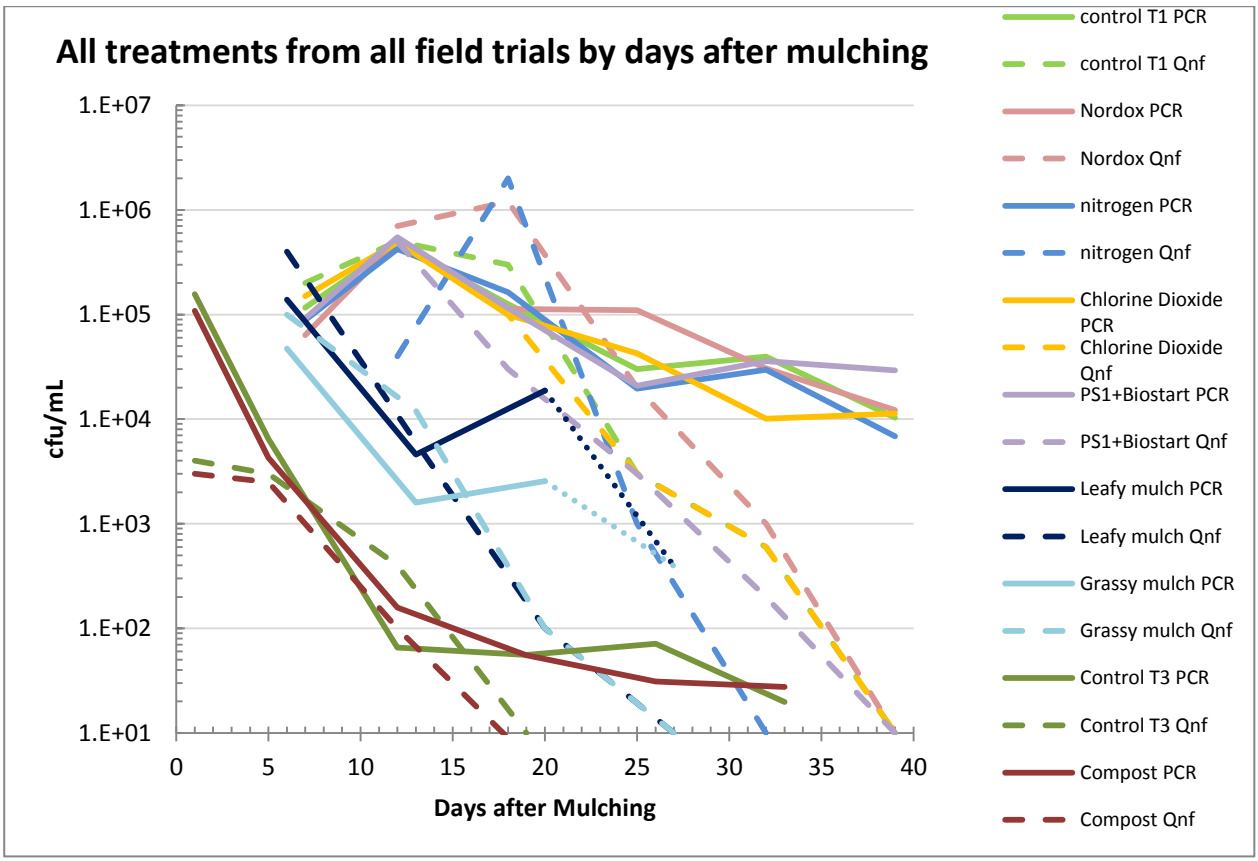
Combined data

If we show all data against "days after mulching" we can combine it all on a single (busy) graph (Figure 24).

We see that the general rate of population decline from enumeration (dashed lines) is comparable from all trials at a rate of ≈ 5 days per decade. The nitrogen treatment in trial 1 is the steepest. The early arrival of the compost trial (treatment and control) was due to very low initial enumeration whereas the initial data from the other 2 sites was comparable at around 1 x 10^5 cfu/mL.

The rate of decline of the PCR data is also roughly consistent from all trials (although there is little evidence from trial 2) at a much slower rate of ≈35-40 days per decade. We note that the lower values seen in trial 3 were measureable because we were using a different calibration from that used in the first two trials where no compost was involved.

Figure 24. Psa-V data from all trials from enumerations and from qPCR after conversion to cfu/mL plotted against days after mulching.



The effect of temperature and relative humidity on survival of Psa-V in mulch under laboratory conditions.

Samples of mulch from Trial 1 were kept under two different temperature and humidity regimes for 35 days in the laboratory.

1. Wet/humid by spraying water every second day into a plastic container maintained in incubator. Conditions stayed very close to 100%RH and 26°C.
2. In the incubator at 26°C without added water. RH varied from 25-45% (Figure 25).

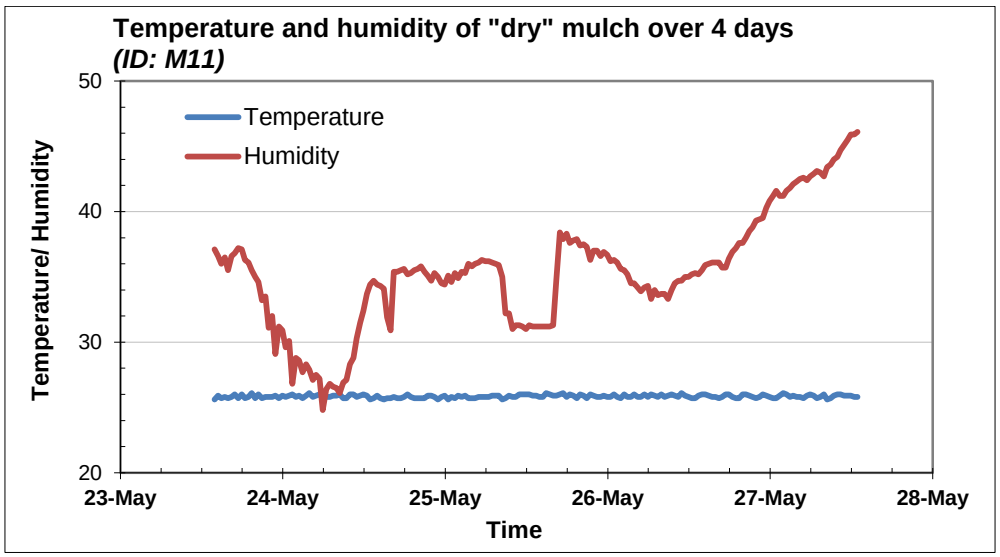
All treatments were tested periodically for Psa using both qPCR and culturing and the results are shown in Table 14 and Figure 26.

Table 14: Summary of effects of temperature and relative humidity on infected mulch.

Days after Trt	Date	Treatments	Trt ID	Cultures	Colony confirmed	Cq from qPCR	PCR result
0	23-May-12	dry incubator	M11	G	confirm	24.17	Detected
0	23-May-12	wet incubator	M12	G	confirm	24.17	Detected
2	25-May-12	dry incubator	M11	G	confirm	25.44	Detected
2	25-May-12	wet incubator	M12	G	confirm	26.41	Detected
5	28-May-12	dry incubator	M11	G	confirm	23.11	Detected
5	28-May-12	wet incubator	M12	G	confirm	29.54	Detected
8	31-May-12	dry incubator	M11	G	confirm	25.58	Detected
8	31-May-12	wet incubator	M12	G	confirm	33.43	Detected
13	5-Jun-12	dry incubator	M11	G	confirm	25.43	Detected
13	5-Jun-12	wet incubator	M12	G	confirm	33.28	Detected
21	12-Jun-12	dry incubator	M11	G	confirm	25.22	Detected
21	12-Jun-12	wet incubator	M12	NG	N/A	35	ND
28	19-Jun-12	dry incubator	M11	G	confirm	25.34	Detected
28	19-Jun-12	wet incubator	M12	NG	N/A	35.94	ND
35	26-Jun-12	dry incubator	M11	G	confirm	26.40	Detected
35	26-Jun-12	wet incubator	M12	NG	N/A	35.54	ND

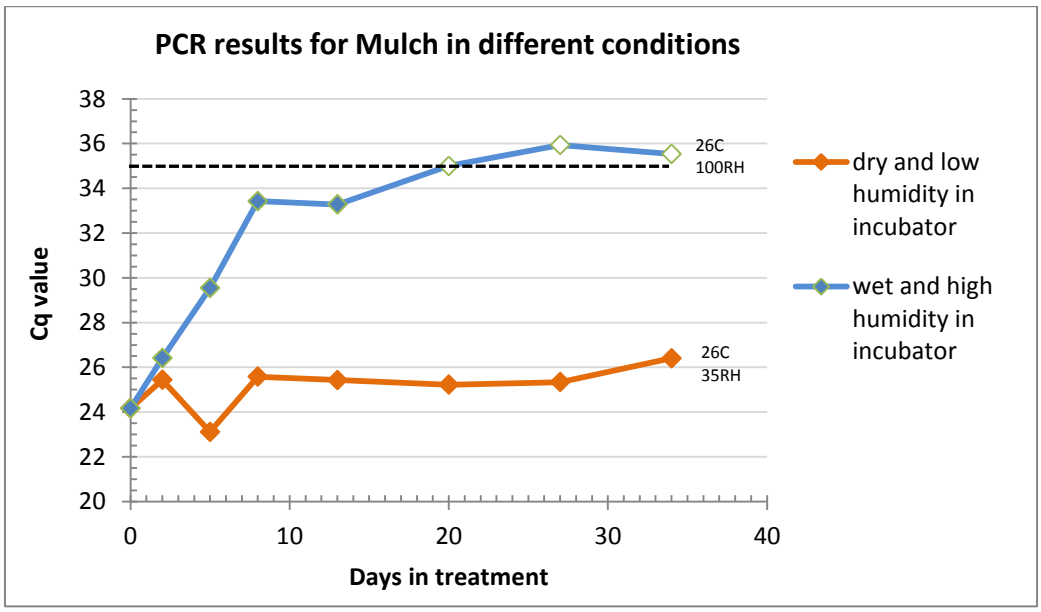
Key: *G= Growth; NG- No growth; 35=Artificial value for ND=Not detected*

Figure 25. Temperature and RH within the "dry" mulch treatment – 4 day typical record.



The trends in Figure 26 seem quite robust. No population decline was seen in the low (30-40% RH) humidity incubator bag after the first 2 days, whereas the decline continued for 20-30 days in the saturated atmosphere bag in the same incubator. Given both bags were at the same temperature the effect of RH was dramatic. The rapid rate of decline of Psa-V is much faster than we observed in any field trial. We might postulate that under these conditions, in the absence of sunlight, the other bacteria in the mulch rapidly destroyed the Psa-V – remembering that qPCR detects Psa-V DNA not just the presence of live bacteria. If this is not the case we struggle to understand the rapid loss of Psa-V in the presence of what we expected to be a significant food source (and bearing in mind that the high population was maintained in the low RH treatment.

Figure 26. Psa survival on mulch at different relative humidities. The hollow blue points indicate there was no growth on the plate.



Conclusions and suggestions for further work

1. In field conditions Psa-V declined on mulch at a rate of ≈1 decade /5 days.
    a. This was not much affected by treatments (Nordox, ClO_2, urea and Biostart+PS1) although the nitrogen treatment (and perhaps the PS1+Biostart) seemed to hasten this decline.
    b. The similarity of decline for treatments such as copper and chlorine dioxide was unexpected – chlorine dioxide should give a short term and immediate knockdown while the other should be persistent.
    c. Populations initially increased for about 12 days before the decline which we attribute to the sudden exposure of substrate. We expect the weather to affect both the level and duration of this increase:
        i. It will affect the rate of decay of the mulch (substrate)
        ii. It should also affect growing/survival conditions for Psa-V.
    d. At the levels of inoculum initially found in the first trial (10^5 – 10^6 cfu/mL) Psa was detectable in the field for 7-8 weeks.
    e. The pattern of growth and decline seemed to be determined by the time after mulching – not the time after treatment application or weather events.
        i. If we had a treatment which was more dramatic this may change.
    f. The significant and continuing PsaV population drop we found here consistently is quite different from previous long term monitoring within orchards where Psa could be found for months (15 weeks) on undisturbed litter beneath vines (Horner et al 2011). It points to the efficacy of mulching litter and so greatly depleting the food source and living environment of Psa.
    g. The compost treatment followed similar trends but with significant differences:
        i. level of inoculum was initially much lower
        ii. We did not see a period of increase prior to decline with the compost treatment.

iii. Agreement between plating and PCR quantification was worse – a soil DNA extraction kit might help eliminate the problem of PCR inhibitors present in soil (and compost) to facilitate the determination of a new PCR threshold in the future.

iv. plating and enumeration was not possible for determination of the threshold detection limit since the selective media was not available at that time.

Because it was undertaken as a separate trial it is not possible to be definitive about the causes of the first 2 differences however it may be that the compost was inhibiting Psa growth.

2. Dead Psa-V was found all over the mulcher 18 days after its final use. Clearly extensive disinfecting should be undertaken if mulching is used.
3. The selective substrate from Otakaro Pathways made much of this work possible – when it was unavailable we could not follow Psa-V populations on plates because of the forest of other micro fauna.
4. The lab work provided convincing calibrations for qPCR and overall the level of agreement between qPCR and plating (and enumeration) was pleasing – especially given the challenging nature of the problem.
    a. The change in the calibration associated with compost was significant.
5. As with all explorations of this type there are a number of technical things we would do differently both in the field (multiple treatments on one site) and in the lab (more controls and tighter procedures over temperature and RH monitoring, sample mixing, treatment application to inhomogeneous material etc)
6. The differences associated with the compost application are intriguing and deserve further study.
    a. It may be worthwhile to isolate and identify the compost components which cause antagonism to Psa-V
    b. A closer look at survival of Psa in compost may be worthwhile in itself and also provide a basis for examining its survival in soil. Quantification using selective media (rather than qPCR) in conjunction with a soil kit would be valuable
    c. The effect of timing (season/weather) on mulching would be valuable if the industry is facing further vine removal (e.g. H16A in new areas).
7. The lab experiments on growth and of Psa-V on mulch and compost raise several interesting questions and further work using the tools we have developed here may give greater insights as to the possible role of compost in Psa control/management.
    a. The extremely rapid loss of Psa-V on mulch under humid laboratory conditions was particularly intriguing.
8. In the field we have not examined issues such as
    a. The effect of weather
    b. Different treatment concentrations or application rates.
    c. The differences associated with the compost application

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