final report

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Survival of Mycobacterium paratuberculosis

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EXECUTIVE SUMMARY

Reducing the survival of *Mycobacterium paratuberculosis* (Mptb) outside the host animal has long been attributed to environmental factors such as elevated temperature, pH, ultraviolet light and dryness although without direct scientific evidence of the contribution on these factors. The experiment reported here evaluates these four factors; ultraviolet radiation, soil temperature, pH, moisture plus organic matter, for their effect on the survival of Mptb from soil as measured by proportional recovery using the Whitlock double incubation and BACTEC culture method.

In this study, soil moisture and soil temperature were the most significant environmental factors affecting the survival or death of Mptb. Ultraviolet radiation appeared to have no effect and different soil types and variable sensitivity of culture obscured the effect of pH.

Results for different environmental factors were evaluated on the low organic soils where the recovery of organisms in culture was not affected by soil type. Sand of acid pH and low organic matter had no loss in analytical sensitivity compared to the Mptb contaminated faeces with which it was inoculated. Fewer organisms were recovered from other soil types mixed with the same faeces. Clay soil of acid pH and high organic matter had a detection limit of 10^3 colony-forming units per gram. Tray trial results from the two low organic soils showed distinct effects between treatments, however, differences were inconsistent in high organic soils due to the lower analytical sensitivity and low total number of Mptb isolations.

The results were statistically analysed by logistic regression and a model developed which provides predicted recovery estimates for each combination of treatments. Results are presented in terms of the predicted mean recovery with standard errors of the predicted means. The conditions applied were within the range of environmental exposure and results of the treatments are directly applicable to field situations. The rate of death of organisms may have been accelerated in high temperature treatments because the temperature was maintained constantly rather than with diurnal variation.

Soil dryness and high temperature resulted in shorter survival times for Mptb in low organic soil. After 8 weeks, there was approximately 100% survival in wet, low temperature (10°C) acid sand, survival in the same soil with cyclic moisture was reduced to 74% and in dry soils to 32% of initially detectable organisms. At higher temperature (30°C) there was an 81%, 37% and 10% survival for wet, cyclic and dry treatments respectively. The alkali loam low organic soil demonstrated a lower culture sensitivity but the same survival trends as for the acid sand for moisture and temperature were observed.

Alkaline soil pH indicated a weak influence with shorter survival in wet and dry soil at both high and low temperature. There was no effect by UV light at the levels used, which may have been too low in intensity or exposure to ultra violet light may not be effective in killing Mptb in soil due to low penetration of the soil.

From these results it should be concluded that dry soil, high soil temperature and possibly alkaline pH are significant in reducing survival of Mptb in soil and should be used to best effect when implementing control procedures on properties.

In contrast wet soil at low temperature due to protection from sunlight and possibly with acidic pH are conditions where Mptb is likely to survive for longer periods.

This study provides only semi-quantitative estimates of the death of Mptb under different environmental conditions. Further work quantifying the log reduction death of Mptb influenced by soil temperature and soil moisture in acidic low organic soil is recommended to assess the level of risk associated with restocking properties undertaking Johne's disease control.

Recommendations

Further work is recommended to quantify the log reduction rate of death of Mptb under critical moisture and temperature conditions to predict appropriate de-stocking intervals. The markedly different effect of moisture and soil temperature on the death of Mptb provides an opportunity to design de-stocking strategies over different land systems.

It is recommended that studies of death rates of Mptb should be conducted in low organic sandy soil due to the high sensitivity of culture in this soil compared with soils containing clay and organic matter.

Further work to elucidate the factors involved in the loss of culture sensitivity in clay and high organic soil types is needed. This will determine if Mptb culture procedures for soil need to be made more sensitive and improve the interpretation of results based on soil type. The low sensitivity of culturing Mptb from loam and high organic soils may mean contaminated areas may go undetected by current culture techniques.

A detailed case controlled study across dairy herds looking for association of herd JD prevalence with soil, environmental and management factors would help determine the influences of moisture and temperature plus local and regional differences and management practices.

SOIL TRAY TRIAL *MYCOBACTERIUM PARATUBERCULOSIS* BOVINE ISOLATE SURVIVAL EXPERIMENT

1.0 Introduction

Johne's disease (JD) is a chronic inflammatory bowel disease that can afflict most ruminant species and is caused by infection of the ileo-caecal region by *Mycobacterium paratuberculosis* (Mptb). The disease manifests in two stages, the first subclinical stage is essentially one of bacterial colonisation through the intestinal tissue and mesenteric lymph nodes. The afflicted animal will intermittently shed organisms in their faeces during the later stage of subclinical infection which can be detected by faecal culture. The animal is seen to 'breakdown' in the clinical stage with chronic scouring and weight loss through malabsorption of nutrients, which will eventually lead to the animal's death. Young animals are most susceptible to infection via the faecal-oral route through exposure to contaminated faeces on teats, in milk and water supply, and on pasture.

Transmission of the organism via this route requires a survival period outside the host where the organism will be subject to desiccation, temperature fluctuations, UV irradiation and soil environmental factors. Mptb is not believed to multiply outside the host animal, however, it will survive for extended periods of time given the right environmental conditions. Answers from current and future research will indicate ways of manipulating the external conditions to provide a self limiting Mptb environment.

Survival of Mptb outside the host animal is an area of importance having implications for Johne's disease control and herd management strategies. De-stocking of sheep properties and dairy herd management strategies are issues which depend on robust and up to date information provided by research. The soil tray trial is a component of the national JD investigation strategy that sets out to determine what environmental factors influence the survival time of Mptb outside the host and elucidate the interaction of environmental factors involved in its survival.

The aim of the experiment in this report was to determine under controlled conditions the influences of five natural environmental parameters, UV exposure, moisture, temperature, soil pH and soil organic matter, that may attenuate or prolong the survival of Mptb.

Issues Associated with Soil Experiments using Mptb

Selection of Soil Type and Characteristics

Soil pH is a product of organic and inorganic factors and soil usually exhibits a strong buffering capacity, which was demonstrated in a previous experiment where adjustments to pH were attempted. The pH began to alter over the course of the experiment, illustrating the unstable nature of soil pH when chemical modifications are made. It is commonly noted in farming situations that after the application of lime, the

resulting change in soil pH is not stabilised until after 1 to 2 years as the soil chemistry is gradually altered. In this study it was decided to use natural soils that were selected on their intrinsic factors and to incorporate the soil type variation into the interpretation of the experimental model. This produced a more complex model but provided more stability in the soil chemistry and more accurately reflected the field situation.

Sterilisation of Soil

The requirement for sterilising soil by a suitable method was investigated after indications that soil sterilisation may solve problems of contamination of cultures by saprophytic Mycobacteria. However, soil sterilised by autoclave and allowed to remain damp resulted in germination of fungal spores leading to a mass of hyphae throughout the soil. Autoclaving and microwave oven drying was also unsuccessful in destroying all spores as fungal hyphae was still found to grow in cold wet soil treatments in a preliminary trial. A previous experiment with the ovine Mptb strain had problems with contamination by Pseudomonas spp in BACTEC cultures. This presented the possibility that sterilising soil may encourage over-growth by rapidly growing organisms due to a lack of competitive inhibition exerted within an active community of soil micro-organisms.

Following preliminary trials with a variety of sterilisation treatments and untreated soil, the untreated soils produced minimal contamination from fungi or bacteria in radiometric culture (RMC) which had been prepared in accordance with the double incubation Whitlock method. For this reason no soil sterilisation was undertaken before commencement of the experiment with the soil trials using bovine strains of Mptb.

Faecal Inoculum

Sampling soil spiked with Mptb can be prone to error introduced at the inoculation stage. Loading soil with faecal material containing Mptb requires an even distribution of the bacterial media within the soil. Incomplete dispersal of the organisms throughout the soil will result in large sampling variation and therefore errors in recovery estimates. Soil type may also have an effect as clay and aggregated soils may prevent an even distribution by adsorbing the inoculum. Clay particles are up to 100 times smaller than sand and as such will require more thorough mixing.
Faecal material used for seeding should contain large numbers of Mptb organisms, at least 10^4-10^5 organisms/gm, to ensure a reduced sampling variation and to increase the probability of detection in the later stages of the trial when there is an even further reduction in bacterial numbers. The final concentration of organism in the soil should be in the order of $5x10^3$ colony forming units (CFU)/gm to achieve these aims.

Experimental Conditions

The experimental environmental conditions were achieved within narrow margins, well within the range of acceptable variation. A low relative humidity was required to achieve a high evaporation rate and was produced using refrigerated air-conditioning and room heaters running simultaneously. Combining wet, dry and a cyclic moisture treatments and high and low temperatures together in the same environment space

required a controlled atmosphere with substantial infrastructure of heating and cooling systems and water delivery equipment.

Recovery versus Survival

It has been assumed that the degree of recovery of organisms is indicative of their survival given defined environmental conditions. In strict terms however, survival relates to the metabolically active status of the organism which may or may not be recovered in culture. Therefore the number of organisms recovered from soil in culture is a proportion of the total number of surviving organisms, and has been assumed to be 100%.

2.0 Materials and Methods

Culture Sensitivity for High and Low Organic Soils

The limits of detection of Mptb from soils of high and low organic matter (but equivalent pH) was investigated by comparisons with the same faecal material used to inoculate the tray trial. Ten grams of faeces was mixed thoroughly with 90 gms of each high and low organic soil and processed through the double incubation procedure. The final pellet underwent serial dilution to 10^{-6}. Each serial dilution was replicated five times for each soil type. Two grams of faeces was cultured separately and underwent serial dilution to 10^{-6} with 5 repeats.

Tray Trial Experimental Design

Five factors ie. soil pH, moisture content, organic matter content, temperature, and ultra violet light exposure, were investigated due to their suspected influence on Mptb survival in soil. The experiment was a completely randomised design with 48 unique treatments encompassing implied replication due to the balanced design.

Five factor variables:

Soil pH	- 2 levels: Acid = <6.5, Alkali = >7.2
Soil organic content	- 2 levels: Low = 4%, High = >6%
Soil temperature	- 2 levels: warm (30°C ±6) & cold (~11°C ±1)
Ultra violet light	- 2 levels: high (250 mW/cm^2) & low (10 mW/cm^2)
Soil moisture	- 3 levels: wet, cyclic & dry

2 x 2 x 2 x 2 x 3 = 48 treatments

Sampling of treatments involved randomly taking approximately 15 grams of soil from throughout the tray at each sampling and processing 6 grams as 3 x 2 gm repeats.

Experimental Equipment and Parameters

The experiment was conducted in a single room under controlled environmental conditions of low relative humidity (~33%) and moderate room temperature (~25°C). This was to enable favourable conditions for evaporation for the cyclic moisture treatments and to prevent condensation on the cold treatments. An air-conditioner was installed to dehumidify the air and fan heaters kept room temperature elevated. A large oscillating fan was used to keep room air circulating (Figure 1).

Soil Tray Construction and Temperature Regulation

Polythene trays 31cm x 13cm were mounted in the tops of expanded polystyrene vegetable boxes (Figure 2). The high temperature treatment was approximately 30°C (±6) and was achieved by using plant nursery heating mats under the soil trays to provide elevated soil temperature. The dry and cyclic moisture treatments used a non-regulated mat (Thermofilm TP020) which provided +5°C above ambient temperature.

The wet treatments required a thermostat controlled heating mat (Thermofilm TPS030) to compensate for evaporative cooling effects.
Cold temperature treatment was approximately 11^{0}C (±1^{0}C) and was achieved by using a refrigerated recirculated water system. Cooling the soils was effected through a gravity fed recirculated water system which syphoned chilled water through the cold treatment boxes and down to a sump. The water was returned by pump back to the header tank through a refrigerated chiller system. Through the use of insulating foam boxes and water conduits, stable and constant temperatures for both hot and cold treatments were achieved.

Moisture Treatments

Moisture treatments were dry, cyclic or wet. As all soils were air dried prior to faecal loading, the dry treatments received no added moisture other than the moisture in the faecal load.
Wet-high temperature treatments were vulnerable to rapid drying and required a very low but constant supply of water. Distilled water was delivered using elevated intra-venous drip bags attached to a 25 cm length of 0.28 mm (inside diameter) polyethylene capillary tube with a blunt 26 gauge needle inserted at one end to connect the supply tube. A capillary length of around 25 cm long was needed to establish a constant flow rate of 0.06 ml per minute.
High temperature cyclic treatments were wetted with 400 ml of distilled water on a weekly basis. This allowed a very consistent wet-dry cycle in which the soil was dry completely for around two days prior to rewatering. Cold cyclic treatments dried more slowly and therefore were wetted with 150 mls of distilled water using a spray bottle, which was sufficient to wet but not saturate the soils. Frequency of rewatering was around 2-3 weeks allowing for 3 days to be completely dry before rewatering.

Ultra Violet Light

Ultra violet light was measured as the amount of UV-A using a UV-A light meter (International Light Radiometer/Photometer, Model IL 1400A). There were two levels of ultra violet light designated UV and no UV which were designed to provide a difference between two UV which should produce a cumulative effect with time. Fluorescent UV lamps of type NEC FL20BL_24 were mounted 34 cm above the UV treated soils providing UV levels of approximately 250 milli-Watts per cm ^{2}of UV-A radiation. UV light was blocked from no UV treatments using cardboard sheets. UV levels over the No-UV treatments were approximately 10 mW/ cm ^{2}.

Sampling and Culture Technique

Soils were sampled according to a prearranged schedule. Sample collection occurred immediately following faecal loading (Day 1) and at weeks 1, 2, 3, 4, 6, 8. Processing the samples for Day 1 began the following day after being stored at 4°C overnight. All other samples were stored at –80°C immediately after sampling. Sampling consisted of pooling random samples from throughout the soil tray totalling approximately 12-15 grams into sterile specimen jars. The remaining soil was mixed and the surface smoothed over. Culture processing for Mptb was performed on Day 1, Weeks 2, 4, 6

and 8 collections. The samples were briefly thawed at room temperature before each being weighed into 3 x 2 gm lots in 8.5 ml of 0.9% sterile saline in polythene centrifuge tubes. The samples were shaken for 30 minutes followed by a settling period for another 30 minutes. Five millilitres of the saline/soil suspension was decanted into 20 mls of 0.95% Cetylpyridinium chloride (HPC) and 1.85% brain heart infusion medium (BHI) and allowed to incubate at 37°C for 20 hrs. The tubes were centrifuged at 10°C for 30 minutes at 900g. The supernatant was discarded and the pellet resuspended with a 1 ml mixture of Vancomycin 100 µg, Amphotericin 50 µg, Nalidixic acid 100 µg (VAN) and 1.85%BHI combination and allowed to incubate for a further 36 hrs at 37°C. Egg yolk supplement was prepared as a mixture of 50% egg yolk, 10% PANTA Plus (Becton Dickinson), 5% Mycobactin J (working solution 50 µg/ml) and 35% sterile deionised water. Two millilitres of the egg yolk supplement was added to each BACTEC 12B radiometric culture vial and inoculated with 0.2 ml of the VAN/BHI pellet suspension and incubated at 37°C for 16 weeks. The incubations were read the day following inoculation then weekly. Positive growth in BACTEC's was sub-cultured onto Herrolds egg yolk media and/or BACTEC 12B media. A culture was considered positive with Mptb only when confirmed by IS900 PCR.

A total of 240 soil samples collected from the trays over weeks 0, 2, 4, 6 and 8 were inoculated into 720 BACTEC bottles, resulting in 210 sub-cultures and IS900 PCR tests. All BACTEC cultures that tested IS900 positive also took less than 8 weeks BACTEC incubation to have positive growth indices. The majority of IS900 negative BACTEC cultures (64%) did not have positive BACTEC growth indices until after 8 weeks incubation. Contamination rates were for Day 1 = 0.7%, Week 2 = 27%, Week 4 = 6.3%, Week 6 = 5.6%, Week 8 = 3.5%. Week 2 had an unusually high contamination rate in which 86% of the contaminants were slow growing organisms which did not produce a growth index in BACTECs until 9 weeks or greater incubation.

Soils

Four naturally occurring soils were sourced and tested by the State Chemistry Laboratory (SCL) for their intrinsic combination of pH and organic material. Soils were classified as acidic with a pH<6.5, alkali pH>7.2, high organic matter >6% and low in organic matter < 4%. Chemical analysis was performed by the SCL. Electrical conductivity and pH analysis was done using 5:1 water:soil (and $CaCL_2$:soil for pH) methods, available Fe, Zn, Cu and Mn were measured using DPTA and total elements were measured by acid extraction and atomic absorption analysis. The chemical profile of the selected soils is shown in Table 1, a complete chemical analysis shown in Appendix A. Using naturally occurring soils adds the complexity of soil type to the model but allows for actual soil factors in the comparisons. Preparation of the soils consisted only of being sieved and air dried (but not sterilised), and weighed out into 600 gm lots in paper bags in which they were stored until used.

Soil Type	Low Organic Acid	Low Organic Alkali	High Organic Acid	High Organic Alkali
Soil Description	Langwarrin sand	Werribee red brown earth	Dalmore clay	Monbulk clay loam
pH (H_20)	5.8	7.4	5	7.7
pH ($CaCl_2$)	4.8	7.3	4.6	7
Organic Carbon (% $^{w}/_{w}$)	1.9	1.2	4.6	3.6
Organic Matter (% $^{w}/_{w}$)	3.6	2.3	8.7	6.8
Total Nitrogen (% $^{w}/_{w}$)	0.08	0.1	0.4	0.31
Fe (available mg/kg)	160	21	170	46

Table 1: Profile of the soils used in the Mptb tray trial.

Faecal Load and Soil Inoculation

Faecal samples were collected from animals showing clinical signs of JD. Samples were examined by Ziehl-Neelson stain for acid-fast organisms and the final selection of the faeces to be used based on these results.

The number of colony forming units per gram (CFU/gm) present in the faecal material used for the soil trial was estimated using the double incubation and Most Probable Number technique (Australian Standard 1766, 1975) to be in the order of $2.4x10^6$ CFU/gm. To ensure an even distribution throughout each soil tray and that an adequate number of organisms were detectable throughout the trial, a large faecal inoculum was used. As the detection sensitivity in high organic soils was less than low organic soils, and the death rate of Mptb when exposed to the experimental factors was unknown, a dilution factor of 20:1 soil to faeces was used giving the final concentration approximately 1.2×10^5 CFU's/gm of soil. Thirty grams of faeces was blended into 600 grams of soil using a Kenwood Chef food mixer for 5 minutes and were further mixed by hand for another 3 minutes to ensure complete distribution. Each 630 gm lot was then placed in its respective tray and the soil surface smoothed. Samples were taken immediately and stored a 4°C over night and processed for culture the following day (Day 1).

Statistical Analysis

Each sample was replicated 3 times in the culturing phase and the proportion of positive growths (p) to total cultures (n) ie. $^{p}/n$, for each tray and treatment was used for the initial data analysis. Data was prepared using Microsoft EXCEL© and proportional logistic regression modelling was performed using the computer statistics package GENSTAT.
The number of positive growth subsamples for each time of sampling was initially analysed as a 2 x 2 x 2 x 2 x 3 factorial assuming no interactions were higher than second

order using binomial errors with possible overdispersion and a logit link function (McCullagh and Nelder 1983).

It was assumed that the highest order of interactions was 2 so that overdispersion could be estimated. This is a standard approach in designed experiments with many treatment combinations (Cochran and Cox 1957). Binomial type errors were used as this is appropriate when sampling the number of positives from a fixed number of cultures (3) in each experimental tray, at a given time. Overdispersion was considered to allow for possible plot effects caused by positional or particular tray factors. The logit link implies a logistic regression formulation.

In week 4, all the dry trays had 0 (out of 3) positive cultures. Thus, it was impossible to estimate the fitted values for dry treatments on the logistic scale. Thus formal analysis at week 4 did not include dry trays. In week 6 soils from dry trays were not cultured and hence the analysis for week 6 only included cyclic and wet trays. In week 8 only soil from low organic soils was cultured due to the lower sensitivity and higher variability in high organic soils, and hence analysis was restricted to low organic trays.

At day 1 the temperature, UV and moisture treatments had not been differentially applied. Thus the analysis at day 1 was a 2 x 2 pH by organic matter factorial with 12 replicates. Since the results for weeks 4 and 6 were very similar, and the estimates of some treatment effects on the logistic scale were imprecise due to many trays having 0 positive readings when analysed as a single week, a combined analysis of the total number of positive cultures (out of 6) for weeks 4 and 6 combined was also performed.

In most analyses the mean residual deviance was less than 1 and in no analysis was the mean residual deviance substantially above 1. Thus it was considered safe and appropriate to assume that errors were standard binomial without overdispersion, hence we know that all information is in the predicted means. Further analyses showed that, at each time, all or nearly all of the effects of 2 factor interactions could be explained by 3 specific 2 factor interactions. These were the pH by moisture, the temperature by organic matter and the organic matter by pH interactions. Also none of these 3 interactions could be reasonably dropped from the model nor could any of these 3 interactions be reasonably substituted by other 2 factor interactions.

Thus, the final model used for each time was

$$\log[p/(1-p)] = \text{main UV effect}$$
$$+ \text{ main temperature effect}$$
$$+ \text{ main pH effect}$$
$$+ \text{ main organic matter effect}$$
$$+ \text{ main moisture effect}$$
$$+ \text{ organic matter by pH interaction}$$
$$+ \text{ temperature by organic matter interaction}$$
$$+ \text{ pH by moisture interaction}$$

where p = the population proportion of cultures which are positive.
The day 1 model only includes pH and organic matter.

Since there is only an additive effect of UV in the model, and no interactions of the model included UV, odds ratios of the effect of UV on the proportion of positive cultures were calculated. Approximate confidence intervals of these ratios were calculated using asymptotic normal theory of the UV effect on the logistic scale, and then backtransforming.

Predicted means from the model, after adjusting for UV effects, were calculated on the logistic scale for each combination of temperature, pH, organic matter and moisture. Confidence intervals for each combination were calculated using asymptotic normal theory. Predicted proportions and approximate confidence intervals were then obtained by backtransformation.

In this report predicted means are presented for weeks 4 and 6 combined, rather than for weeks 4 and 6 separately, to avoid problems with the predicted means on the logistic scale having unacceptably poor precision. This was caused by many plots, at either week 4 or week 6 individually, having 0 positive cultures.

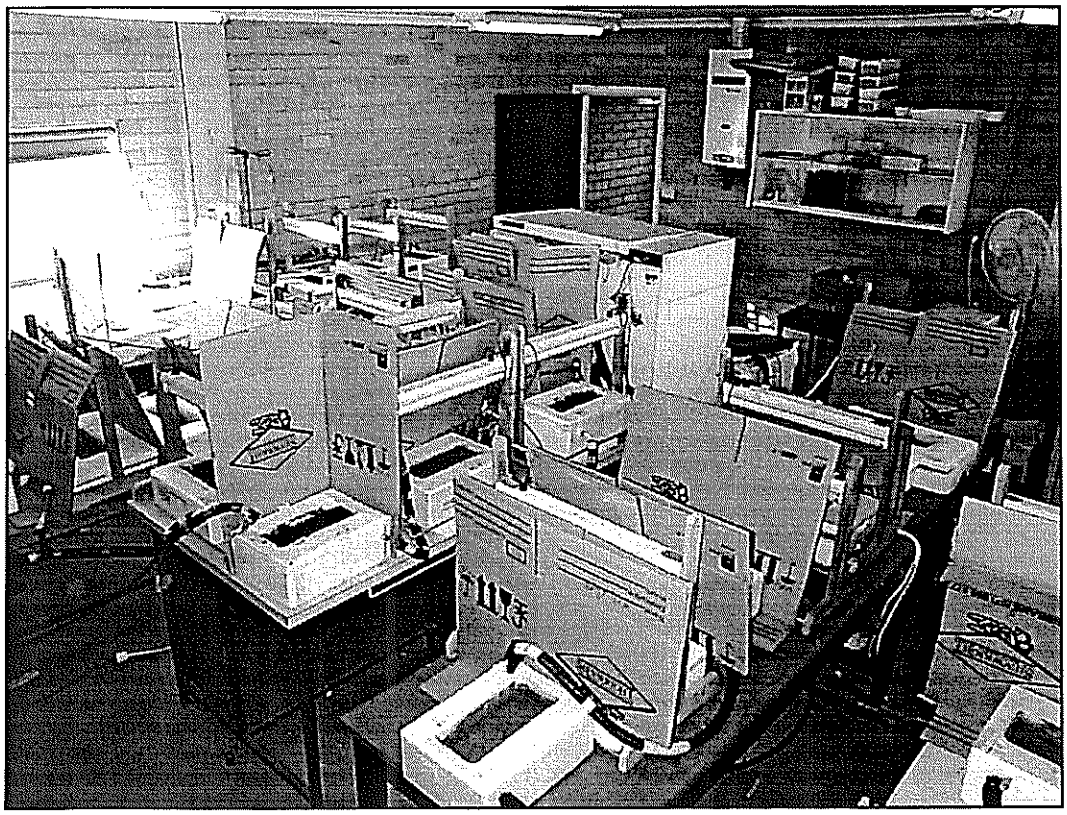

Figure 1: Soil tray trial experiment room.

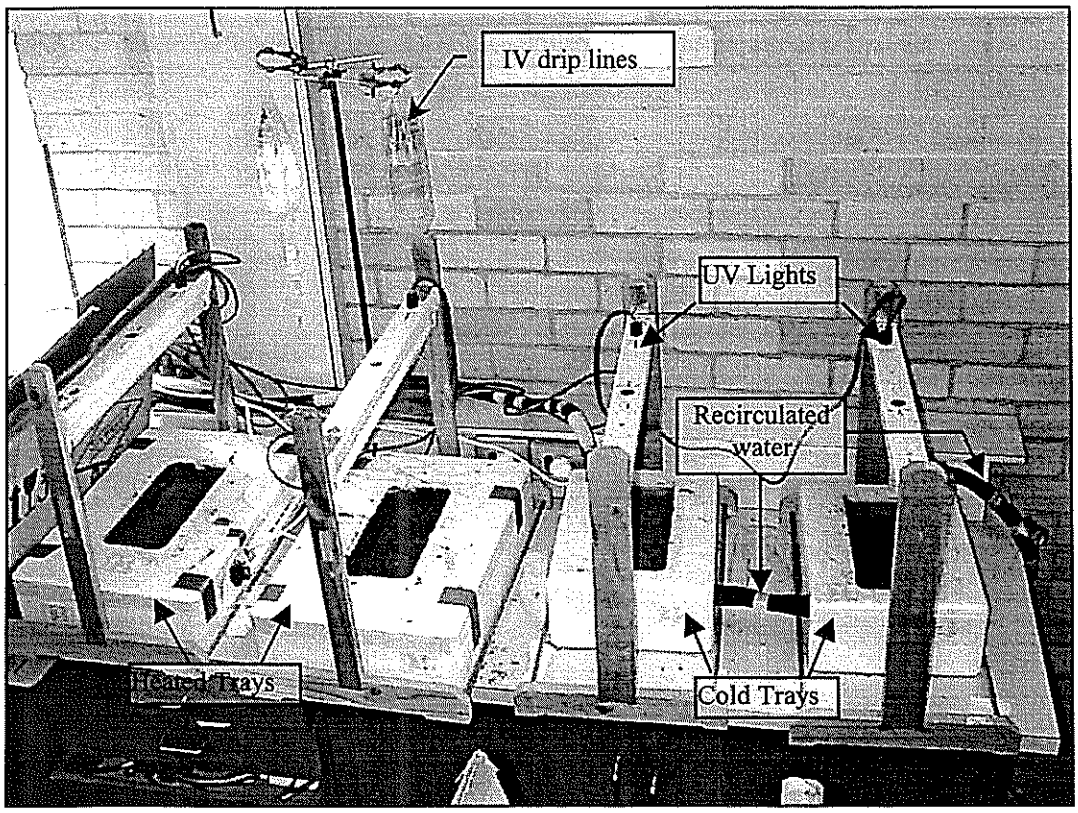

Figure 2: Example of hot and cold treatment trays with drip lines to the hot wet treatments.

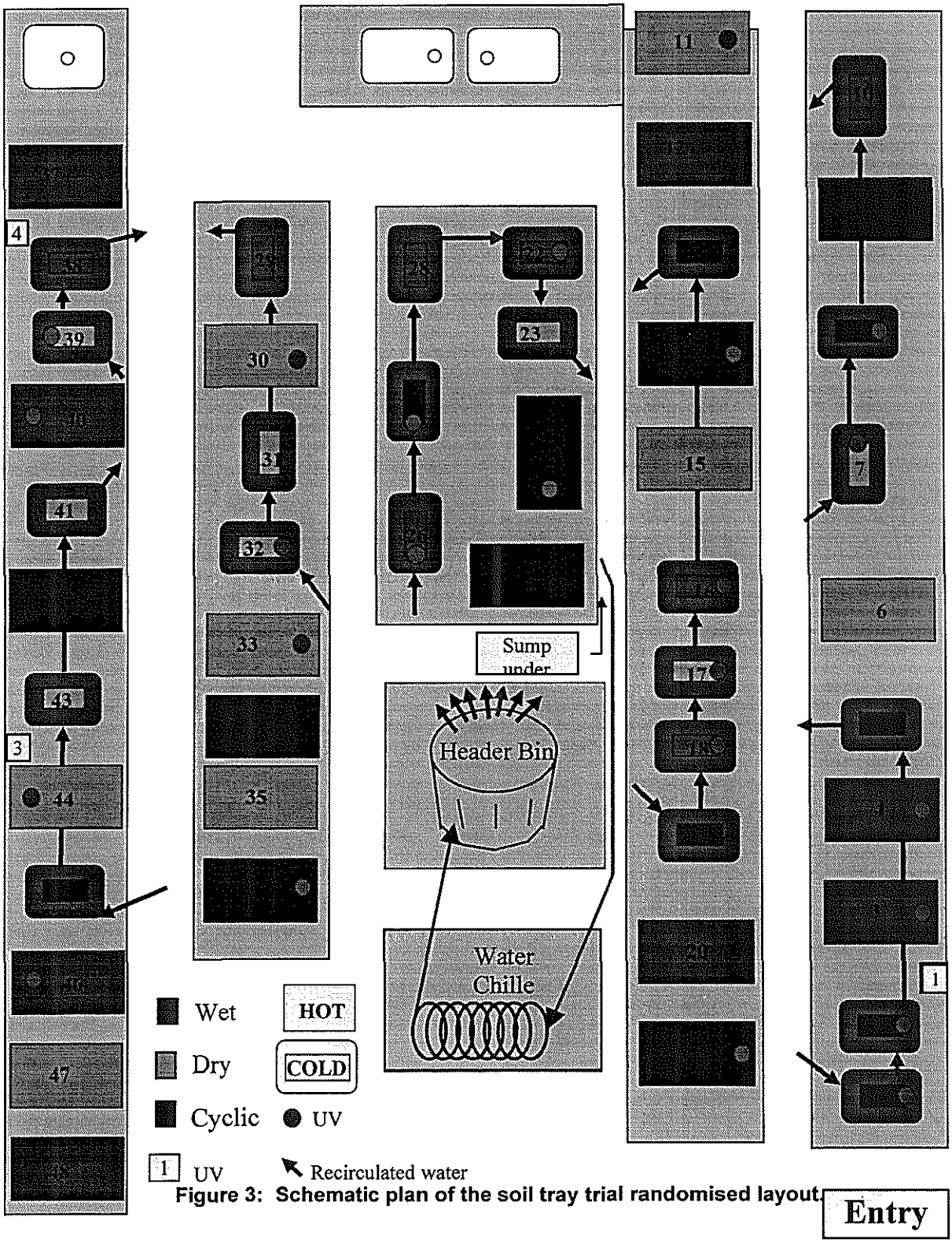

Figure 3: Schematic plan of the soil tray trial randomised layout

Tray	Ultra Violet Light	Temperature	Moisture	Organic Matter	pH
1	Yes	Low	Cyclic	High	Acid
2	Yes	Low	Cyclic	Low	Alkali
3	Yes	High	Wet	High	Alkali
4	Yes	High	Wet	Low	Alkali
5	No	Low	Cyclic	High	Alkali
6	No	High	Dry	Low	Alkali
7	Yes	Low	Dry	High	Alkali
8	Yes	Low	Cyclic	Low	Acid
9	No	High	Cyclic	High	Acid
10	No	Low	Wet	Low	Alkali
11	Yes	High	Dry	Low	Acid
12	No	High	Wet	Low	Alkali
13	No	Low	Cyclic	Low	Alkali
14	Yes	High	Cyclic	Low	Alkali
15	No	High	Dry	Low	Acid
16	Yes	Low	Wet	Low	Acid
17	Yes	Low	Dry	Low	Acid
18	Yes	Low	Wet	High	Acid
19	No	Low	Cyclic	High	Acid
20	No	High	Wet	High	Acid
21	Yes	High	Cyclic	High	Acid
22	Yes	Low	Wet	Low	Alkali
23	No	Low	Dry	Low	Acid
24	Yes	High	Cyclic	High	Alkali
25	No	High	Cyclic	Low	Alkali
26	Yes	Low	Wet	High	Alkali
27	Yes	Low	Cyclic	High	Alkali
28	No	Low	Wet	Low	Acid
29	No	Low	Wet	High	Acid
30	Yes	High	Dry	Low	Alkali
31	No	Low	Dry	High	Alkali
32	Yes	Low	Dry	Low	Alkali
33	Yes	High	Dry	High	Acid
34	No	High	Cyclic	Low	Acid
35	No	High	Dry	High	Alkali
36	Yes	High	Cyclic	Low	Acid
37	No	High	Wet	Low	Acid
38	No	Low	Wet	High	Alkali
39	Yes	Low	Dry	High	Acid
40	Yes	High	Wet	High	Acid
41	No	Low	Dry	Low	Alkali
42	No	High	Cyclic	High	Alkali
43	No	Low	Dry	High	Acid
44	Yes	High	Dry	High	Alkali
45	No	Low	Cyclic	Low	Acid
46	Yes	High	Wet	Low	Acid
47	No	High	Dry	High	Acid
48	No	High	Wet	High	Alkali

Table 2: The randomised layout of the tray trial 5 factors. The location of the trays can be seen in figure 3.

3.0 Results

Experimental Conditions

While all environmental factors were maintained within acceptable ranges small drifts were noted. Analysis of pH by moisture treatment showed that alkali soil pH remained constant over the period of the experiment (Figure 4). Acidic soil pH increased only marginally, the largest increase was in the wet-acid soil by 0.5 pH units from 5.6 to 6.1 (Figure 4). Overall, pH varied in 3 of the soil types by 0.31 to 0.53 (Figure 5). There was no correlation of pH drift with soil type. Small changes were also seen in organic matter and nitrogen content, a result of microbial metabolism of carbon and nitrogen (Figures 5 and 6).

The high temperature trays were the most susceptible to temperature variation but on average were 30^{0}C (24 to 36^{0}C). The variation being due to outside temperatures prior to installation of airconditioning. The average temperature of the cold trays was very stable at 11^{0}C (9 to 13 ^{0}C) (Figure 3) with condensation on the cold dry treatments only a minimal problem on days of high humidity when the edges of some dry treatments became moist, but rectified with airconditioning. Maintaining a heated and constantly wet soil in low relative humidity environment (33%) proved difficult but was accomplished with only 4 of the 8 hot wet trays partially drying out on average 3 times each. Comparison between the hot wet trays which did not suffer any dry periods (n=4), and those that did (n=4), indicated that there was no difference in recovery from these trays or any influence on the final results. The frequency of inspecting the trays ensured that the duration of any dry period was only a matter of hours. Ultra violet light remained constant throughout the experiment providing on average 250 mW/cm^2.

Faecal Material used for Soil Inoculation

Faeces were obtained from a post-calving dairy cow exhibiting clinical JD signs. Examination of the faecal material by Ziehl-Neelson stain indicated a large number of acid fast organisms present. The number of CFU in the faecal material was estimated by MPN calculations to be in the order of 2.4 x 10^6 (Table 3) and the organism was confirmed as Mptb by IS900 PCR.

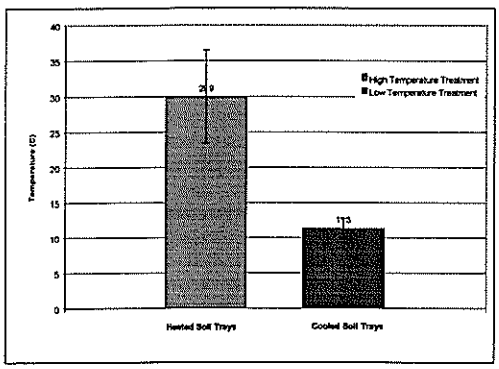

Figure 3: Temperature variations for the Hot and Cold treatments

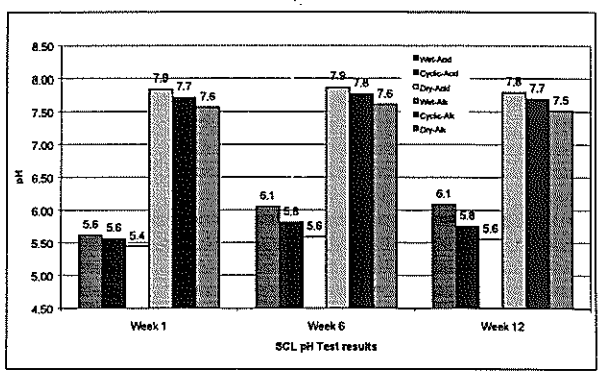

Figure 4: Mean soil pH for each moisture treatment over 12 weeks of the experiment

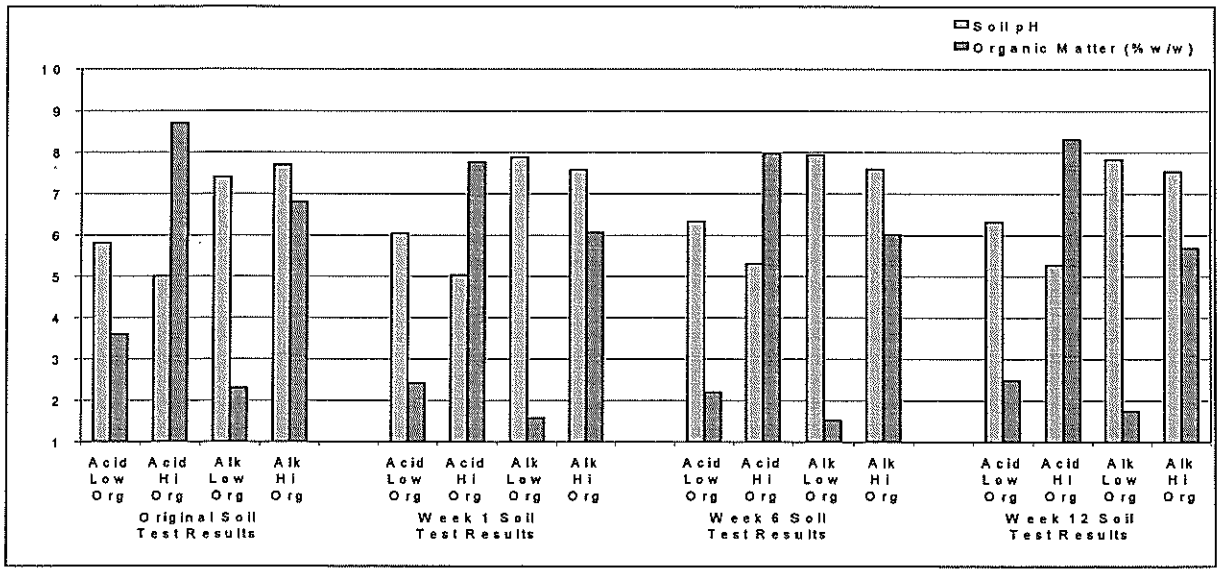

Figure 5: Soil pH & Organic Matter, by soil type test results over 12 Weeks. Y scale is read as pH or % w/w for organic matter.

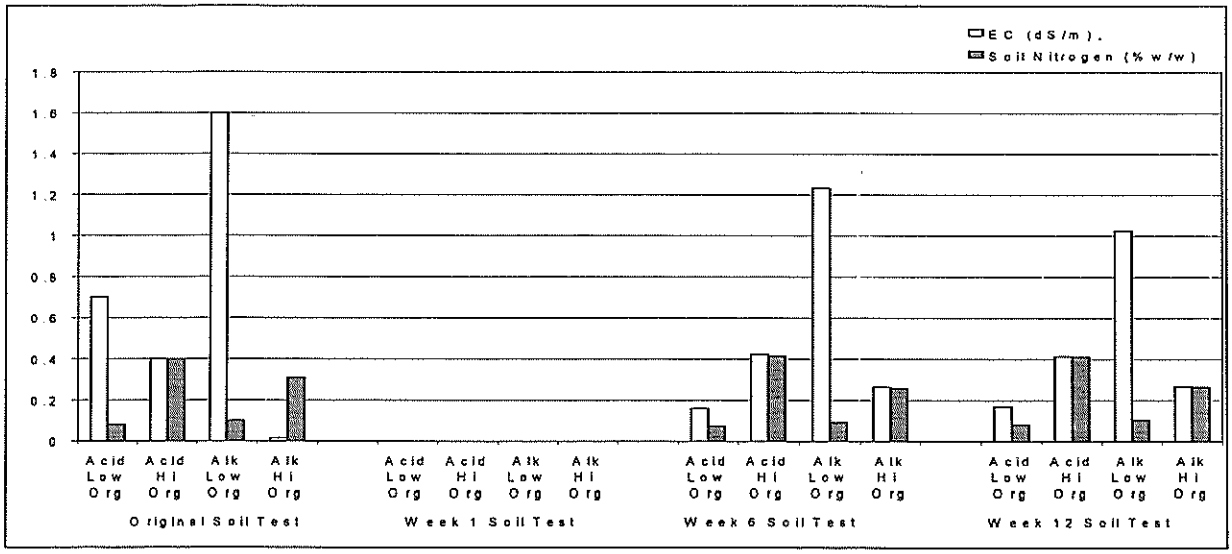

Figure 6: Soil electrical conductivity and total nitrogen, SCL test results over 12 weeks. Y scale is read as dS/m for electrical conductivity or %w/w for soil nitrogen.

Soil Sensitivities for High and Low Organic Acidic Soils

After mixing infected faeces with soils Mptb was reisolated and concentrations estimated by MPN (Table 3). A difference in recovery was seen between high and low organic soils. The results indicate a high sensitivity for recovery from low organic soil of approximately 100% while recovery from high organic soil had a 2.5 log reduction in detection of the original inoculum. The soil types were classed as sand and clay for the low and high organic soils respectively.

Substrate	Recovery CFU/gm
Original Faeces	2.4×10^6
Low Organic Soil (Langwarrin sand)	2.7×10^6
High Organic Soil (Dalmore clay)	7×10^3

Table 3: Most probable number calculations for the recovery of organisms from the original faeces allowing for dilution when mixed with two soils of different composition.

Tray Trial Results

Summary

Overall the most significant factors contributing to the recovery of Mptb from soil were high moisture, low temperature, low organic matter and acidic soil pH. Recoveries decreased over time in dry and cyclic moisture treatments but remained relatively high in wet treatments, especially the cold temperature treatment. UV did not show any effect at the intensity used. Interactions were found between pH by moisture, temperature by organic matter and pH by organic matter although none of the interactions were additive. This means these factors were interacting differently at different levels, eg. the pH effect demonstrated in low organic soils was not present in high organic soils. The actual role of organic matter and clay in decreasing recovery of the organism was not determined.

Logistic Modelling

The logistic statistical model generated from the recovery data was used to generate predicted means and standard errors for the four treatments, from which the major effects and interactions were analysed. The logistic regression model, being based on the raw data, reflects the raw data results (Appendices B and C), but it also smooths data and provides balanced predictions for each of the treatments for similar situations. As UV was not found to contribute any effect, the UV and No-UV tray recovery results

were pooled so that for each treatment of organic matter, temperature, pH and moisture n = 6 cultures.

The model predicted that at Day 1 there would be 78% recovery of the original inoculum in acid sand soil and 47% recovery in alkali loam soil. Organic soils were predicted to have poor recoveries at 11% and 6% of inoculum for acid and alkali soils respectively.

Dry soil was predicted to give the shortest survival time for Mptb in soil, especially in high temperature soil (Table 4, Figures 7 and 8). In the two dry low organic soils, there was 0% survival in less than 2 weeks in high temperature alkali soil and only a 10% survival in high temperature acid soil at 8 weeks. Cyclic moisture treatment increased survival over the dry treatments (Table 4, Figures 7 and 8). Wet low organic acid soil at low temperature significantly maintained the highest survival of organisms to the extent that there was no decline in the eight weeks of the experiment and only a relatively small decrease (19%) in the high temperature soil. At alkaline pH wet soil similarly maintained a higher survival than cyclic moisture which was higher than dry. These results demonstrate a gradient effect of Mptb survivability related to soil moisture. The effect is consistent between the 3 moisture levels when comparing respective pH and temperature combinations in low organic soil.

High temperature was consistent in decreasing survival of Mptb in low organic soils at all weeks and both pH levels. Survival at high temperature in wet acid low organic soil was reduced by 19% of detectable organisms, while there was 100% survival at low temperature. Survival in cyclic moisture acid soils was reduced by 26% and 66% for low and high temperatures respectively, and by 68% and 90% in dry soil (Table 4, Figures 9 and 10). The temperature effect was equivocal in high organic soils, illustrating that the lower analytical sensitivity in high organic soil may preclude the detection of this effect.

There was no apparent pH effect in cyclic wet soils or in high organic soils. Alkaline soil made a minor contribution to the decline of Mptb relative to acid soils, mostly at high and also in low temperature wet soil (Figures 11 and 12). There was a 100% reduction in survival by week 2 in dry alkaline soil at high temperature and a 90% reduction in the acid soil by week 8 relative to their respective day 1 recoveries (Figure 12). The interaction between pH and organic matter illustrates that any pH effect is not easily detected at the high organic level and or that soils with a clay component may confound any effect because of the reduced analytical sensitivity.

Low Organic Acid Soil	Moisture Level		
Temperature	**Wet**	**Cyclic**	**Dry**
Low	100%	74%	32%
High	81%	34%	10%

Table 4: Survival of Mptb after 8 weeks at each moisture and temperature treatment combination on low organic acidic sand as a proportion of Day 1 recoveries.

UV light did not show any influence on the recovery of Mptb as a main effect. A small additive effect was included in the logistic model to determine errors and any UV effects were determined to be of no significance using odds ratios.

Culture Results For Each Soil Type (Raw Data)

Combining the results for all environmental conditions for each soil type indicated that the greatest recovery was from low organic acidic soil (Appendix A) which was also found to have the most sensitive culture results in the preliminary study. Unlike the low organic acidic soil, the other three soils had a clay component.

The highest total recovery was from Day 1 samples where 56% of trays grew Mptb in culture which dropped to 29% by week 4 (not all trays were cultured in week 6 and 8). There were no recoveries from dry soil treatments at week 4 in which recoveries in general were down when compared to the other weeks. Most recoveries were from the low organic soils of both pH, although more consistently from acid than alkali (Table 5). Recoveries from high organic alkaline soil trays were varied and inconsistent.

Soil Types		Total Recovery	Recovery by Organic Matter	Acid Soil	Alkali Soil
Day 1	High Organic Soil Low Organic Soil	56%	25% 88%	33% 100%	16% 75%
Week 2	High Organic Soil Low Organic Soil	54%	50% 50%	41% 75%	66% 25%
Week 4	High Organic Soil Low Organic Soil	29%	16% 41%	16% 50%	16% 25%
Week 6	High Organic Soil Low Organic Soil	37%*	25%* 50%*	0%* 75%*	50%* 25%*
Week 8	High Organic Soil Low Organic Soil	41%+	+ 37.5%	+ 75%	+ 8%

* = Dry treatments not cultured. + = High organic treatments not cultured.

Table 5: Proportion of soil trays with positive cultures for Mptb.

The soil sensitivity experiment demonstrated an approximate 10^3 times decrease in culture sensitivity from high organic loam soil compared to the original faeces or low organic sand. This effect was again evident in the Day 1 recoveries where only 25% of high organic soil trays (8% of cultures) recovered Mptb organisms as compared to 87.5% of low organic treatment trays (63% of cultures) and this was generally consistent throughout the experiment. The reduced sensitivity of culture for high organic soils maybe the reason for the variation in recovery, consequently results from low organic trays were considered to be of higher quality and have been used to analyse and interpret results.

Intrinsic Soil Factors

The soils were of four different types (Table 1) and with correspondingly different structures. The acid and alkali low organic soils were classed as sand (Langwarrin sand) and loam (Werribee red brown earth) respectively. Langwarrin sand was hydrophobic until properly wetted and maintained no structure when wet or dry. Werribee red brown earth was a moderately structured clay loam with some pedal characteristics. The acid and alkali high organic soils were a clay (Dalmore clay) and fine sandy clay loam (Monbulk clay loam) respectively. Dalmore clay is highly structured self-mulching clay with shrinking characteristics and maintained good structure when wet and dry, whereas the Monbulk clay loam was a dispersive soil with little structure.

The amount of available iron was higher in acidic soils (160-170 mg/kg) than the alkaline soils (21 and 46 mg/kg), and therefore also correlates to the pH and degree of organism recovery from soils. The total Fe content was highest in the high organic soils of either pH (17000 and 27000 mg/kg) compared to low organic soil (4500 and 11000 mg/kg) but was not related to available Fe content. Soil acidity was loosely correlated with lower total soil concentrations of elements Mn, Ca, B, Mo and available Cu. The Langwarrin sand from which the most recoveries were obtained, was deficient in many soil elements such as exchangeable P and Ca, and total Ca and S. It also had the lowest exchangeable cations of the four soils and the lowest NO_3 concentration (Appendix C).

Effect of Moisture Conditions

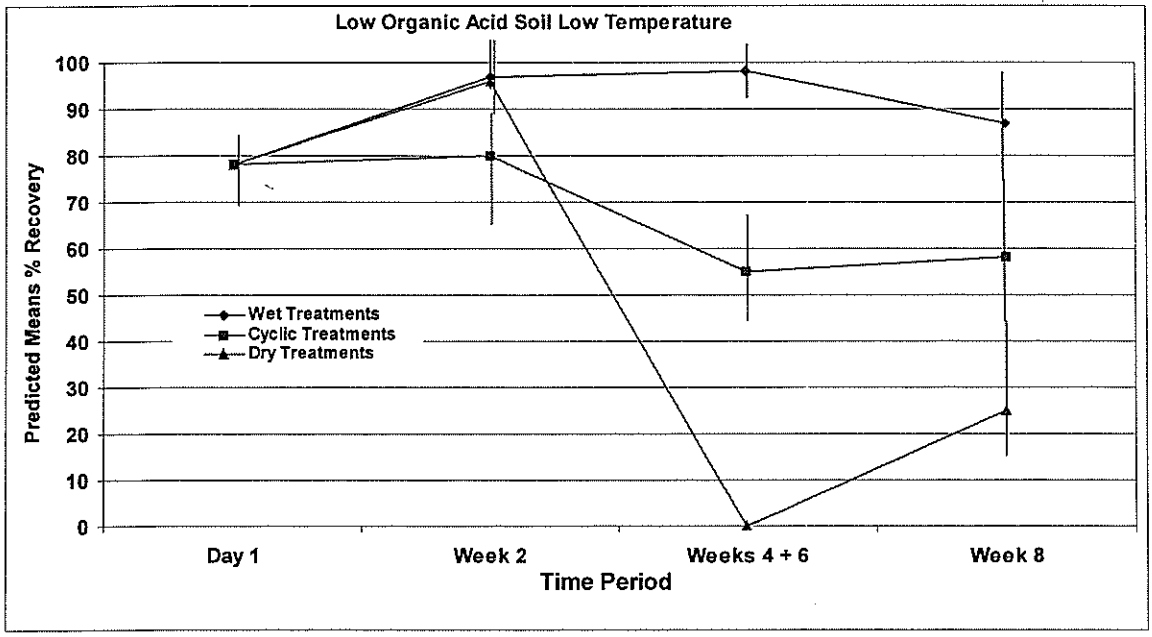

Figure 7: Comparison of Mptb survival (predicted mean ± SE mean.) at low temperature (10°C) for each moisture treatment (wet, cyclic, dry).

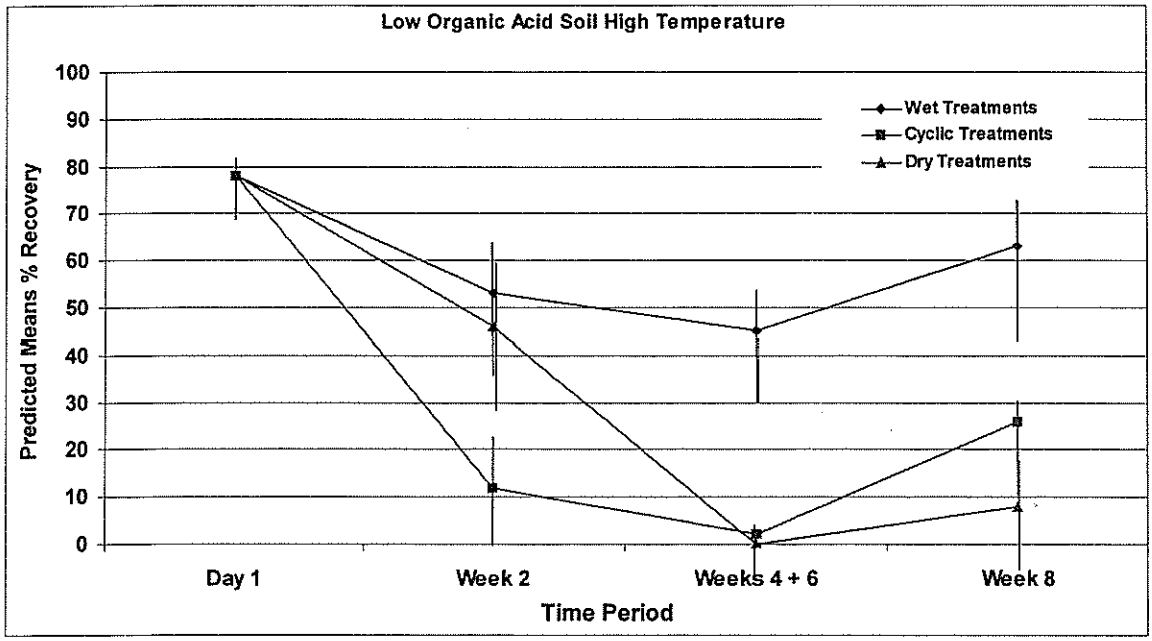

Figure 8: Comparison of Mptb survival (predicted mean ± SE mean.) at high temperature (30°C) for each moisture treatment (wet, cyclic, dry)

Effect of Temperature

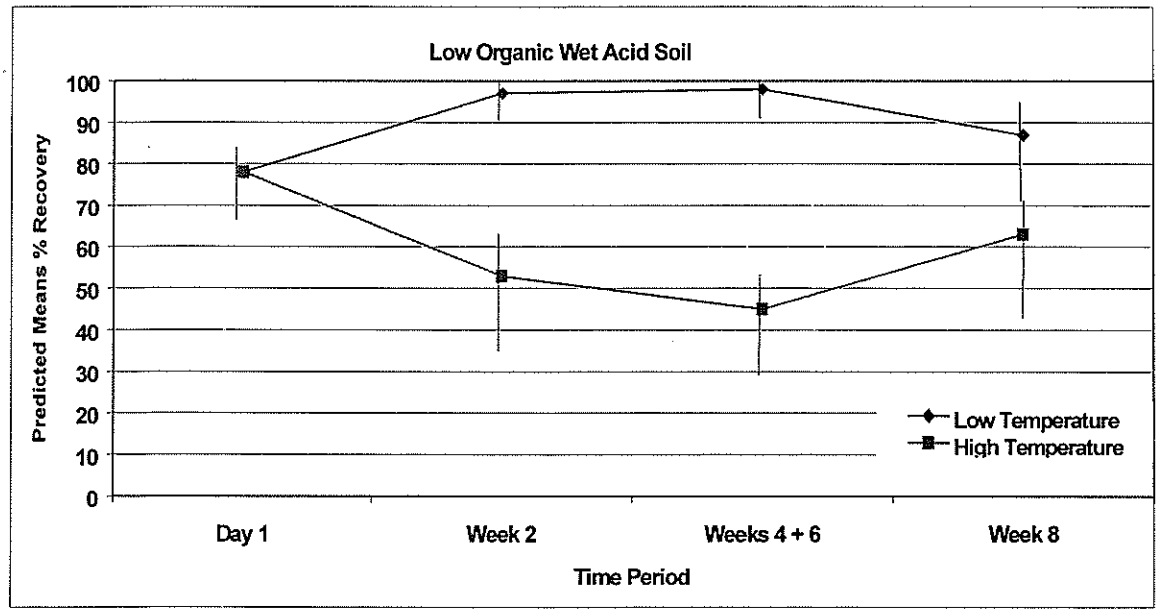

Figure 9: Comparisons of Mptb survival (predicted mean ± SE mean) in wet conditions for low and high temperatures.

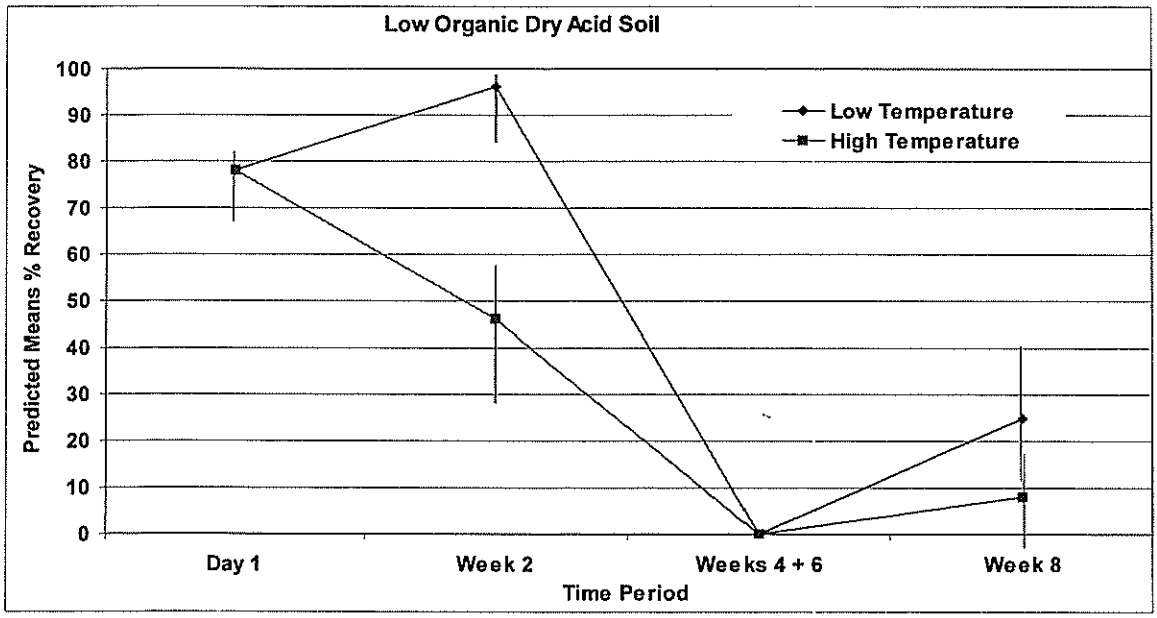

Figure 10: Comparisons of Mptb survival (predicted mean ± SE mean) in dry conditions for low and high temperatures.

Effect of Soil pH

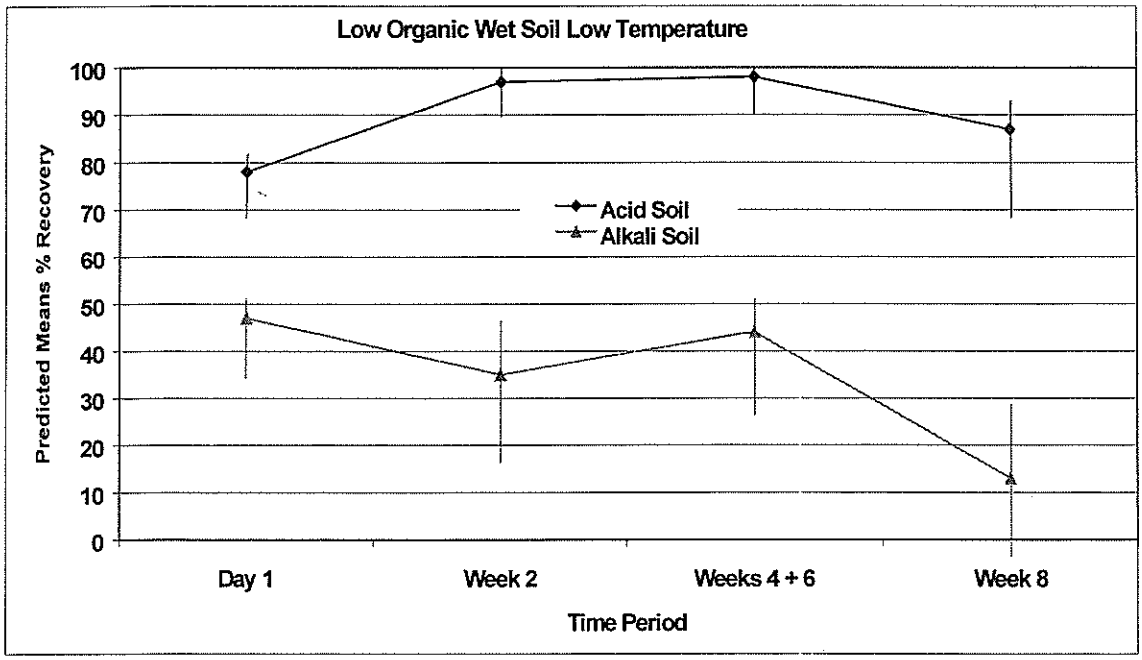

Figure 11: Comparison of Mptb survival (predicted mean ± SE mean) soil pH in wet soil at low temperature for acidic and alkaline soils.

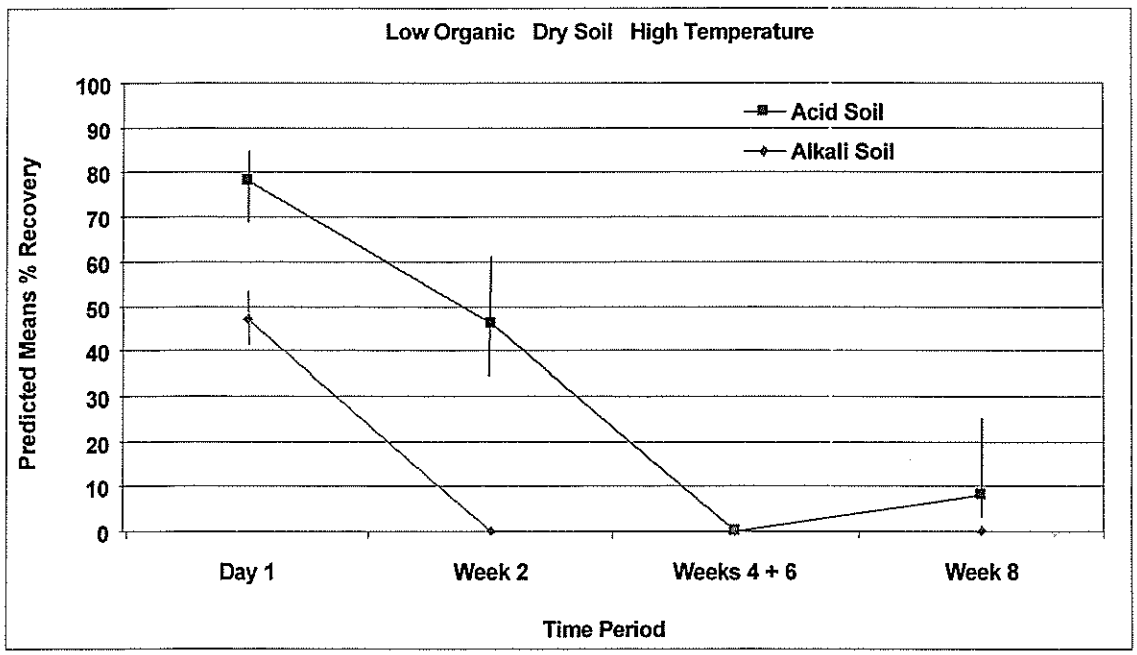

Figure 12: Comparison of Mptb survival (predicted mean ± SE mean) soil pH in dry soil at high temperature for acidic and alkaline soils.

4.0 Discussion

Moisture had the greatest influence on survival of the organism. Dry soil showed the largest reduction in survival over the 8-week period. This was independent of soil pH and temperature and present for both high and low temperature treatments and acid and alkaline soil pH. There was a progressive increase in survival as moisture content increased. Highest survival was observed with the continuously wet treatments followed by cyclically wet treatments and then constantly dry treatments. Few organisms were cultured from trays after week 2 in the dry treatments while the wet treatments showed very little reduction over the 8 week period. Soil bacterial activity is greatest when the soil is just below water holding capacity (van Overbeek and van Elsas, 1997), as water activity drops bacterial metabolism is restricted by lack of substrate availability, then under further water stress dehydration prevents bacterial activity. As Mptb is not believed to be metabolically active outside the host, lack of nutrients may be of no importance but dehydration could be the major survival factor. This may be the explanation why a gradient of survival was established with the three moisture treatments. The results demonstrate that moisture is critical for the survival of the organism and as such any control programme should take this factor into account in destocking strategies.

Soil temperature also had a significant effect on survival of the Mptb. Low temperature greatly favoured the survival of the organism compared with the higher temperature. Apart from the temperature effect on desiccation, survival in hot wet conditions was less than cold wet conditions. At higher temperatures overall soil biota activity will be greater increasing competitive and scavenging organisms. These findings are consistent with E.coli experiments which have demonstrated a 1.5 $\log_{10}$ increase in survival at 10°C compared with survival at 26°C, and 4 $\log_{10}$ reduction of survival at 37°C demonstrating the importance of low temperature for survival (Prescott *et al,* 1999).

The physical conditions applied to the individual trays were consistent with the range of conditions encountered in field situations (P Meele, Personal Communication). Greatest extremes in conditions occur on the surface and due to the heterogeneous variation across farms conditions are difficult to measure and predict. In NE Victoria typical soil temperatures (measured at a depth of 5cm) are; summer 18-30°C, autumn 15-25°C, winter 5-10°C and spring 10-20°C. The presence of stubble reduces soil temperature 5-10°C. In these tray trials although the high temperature conditions were similar to maximum summer soil temperatures, these were maintained constantly rather than cooling to 20°C at night. Soils, which are fully saturated with water may occur adjacent to water courses or springs and periods of 4 months total dryness of soil are typical in northern Victoria with only periodic rainfall events.

When comparing factors in combination there was a marked difference between those factors which prolonged survival, and those that rapidly reduced recovery in low organic soils. The combination of factors which gave the largest reduction in survival was dry, high temperature in alkaline soil. There were no viable organisms detected after two weeks in this treatment combination. This is in contrast to the very favourable conditions (wet, acid, low temperature) which did not demonstrate any loss of recovery from initial loading to week 8.

The soil sensitivity experiment demonstrated an approximate 100% recovery of organisms from low organic soil and a 3 log reduction in recovery from soil with high organic matter. This reduction has been consistent throughout each stage of the tray trial experiment. The effect may be due to a biological suppression on the organism restricting its growth during recovery or a mechanical separation in processing specimens for culture. During the saline step of culture preparation, the organism may bind to large particulate organic matter by a mechanism such as electrostatic or hydrophobic forces and settle out, reducing the numbers of organisms transferred for incubation. Whether a direct interaction exists between bacteria and clay particles is not clear, however clay minerals will demonstrate retention of organisms in perfusion and leaching experiments and increased release when sonication, surfactants and other disruption techniques are used (Stotsky, 1997). Direct binding of organisms to clay minerals is thought unlikely because generally most micro-organisms and clays have similar electrokinetic net negative charges, which would result in repulsion (Stotsky, 1997), although a double positively charged intermediary layer may couple the organisms to the clay concentrating them at the clay particle surface (Prosser, 1998). Clay minerals can exert an indirect influence on micro-organism survival, possibly by modifying the physicochemical environment such as the pH, water potential, nutritional status or activity of toxicants (Stotsky, 1997).

Soil organic matter (SOM) is generally defined by Australian laboratories as all organic materials in soil regardless of origin or state of decomposition (Baldock, J.A.and Skjemstad, J.O, 1999). It is measured as soil organic carbon (SOC) from which SOM is calculated and both results are usually reported. SOM is largely plant material but a significant portion is made up from cell walls of microorganisms. During degradation, organic matter is first broken down to its constituent components before being rebuilt into complex organic acids that constitutes humus. A major component of the breakdown materials are cyclic aromatics as a result of lignin decomposition. Fungi are primarily responsible for lignin degradation, releasing lignases acting on lignin to produce weakly acid cyclic alcohols such as phenol's that may cause injury to Mptb.

The reduced recovery that high organic soils have on recovery of Mptb in culture occurs nearly immediately as the soil sensitivity experiment indicated. The high and low organic soils were sampled and processed for culture two hours after inoculation yet resulted in a difference of 10^3 in recovery. This effect was again demonstrated with the Day 1 soils where there was a delay before processing of about 20 hours, and then throughout the following weeks. The difference in recovery was reasonably consistent. The rapidness of the effect suggests a binding rather than an antimicrobial effect. The culture sensitivity findings in this study were similar to the results from soils used by Elizabeth Macarthur Agricultural Institute in NSW where a sensitivity of detection of 10^3 was observed in studies using low organic loam. Loam soils typically contain a clay component.

High organic soils will support higher numbers and more diverse ecology of micro-organisms, which in the context of competition between species could put Mptb at a disadvantage. Anti-microbials produced by bacteria and especially fungi as competitive mechanisms may injure Mptb and contribute to their death rate. Resource competition from soil organisms may limit resource availability to Mptb, especially iron (Fe). Richards (1989) considers that competition between siderophores and iron deprivation could be highly significant factors in

attenuating the survival of Mptb in soil, culture and animals. However the question remains whether any of the biological processes could act as quickly as was observed.

Availability of soil Fe may be a possible factor in limiting Mptb survival in soil. Availability of Fe increases with soil acidity where soluble Fe^{2+} species predominate assisted by sub-oxic (reducing) conditions. As pH increases available Fe in the soil solution hydrolyses and becomes oxidised through several redox states until insoluble Fe^{3+} hydroxides form, when at pH 7.4 - 8.5 availability of inorganic Fe is at a minimum. As available Fe is a function of pH, it has not been demonstrated whether it is the pH or available Fe which is the significant factor, it remains to be seen whether Mptb will survive as well in acid soil with very low available Fe. The normal range for available Fe is 30 – 550 mg/kg, which corresponds to the soils used in this trial as the alkaline soils where at 21 and 46 for pH 7.4 (7.3 $CaCl_2$) and 7.7 (7.0 $CaCl_2$) respectively. The availability of iron favouring survival is at odds with the quiescent state that the organism is purported to be in when outside the body. The lack of metabolic activity would suggest that the requirement for inorganic element such as Fe, Cu etc would be very low.

Although the minor difference between survival of Mptb in soils of varying pH in this tray trial do not suggest that soil pH is a major factor affecting death rate of Mptb, the epidemiological relationship between JD prevalence and soil pH has been reported in several publications. Kopecky (1977) found JD associated with acidic soil regions of Wisconsin and mentions of regional evidence in England, France and the Netherlands where JD was found to be absent in cattle herds living on calcareous alkaline soils, but prevalent in neighbouring acidic regions. Johnson-Ifearulundu and Kaneene (1997) cautiously accept the anecdotal evidence and limited scientific data but consider the other confounding factors such as available iron, calcium, other bacteria and fungi, soil type and moisture need to be elucidated. In their two most recent reports of their analysis, Johnson-Ifearulundu and Kaneene (1998, 1999) found an association between properties that applied lime to pastures and lower JD herd prevalence. In the second paper they also found using regression modelling, an increase of soil pH by 0.1 and available Fe by 10 ppm was associated with an increase in JD herd prevalence of 5% and 4% respectively. However in these papers a history of Johne's disease and other farm management practices were also significantly associated with the prevalence of Johne's disease and included spreading of manure on pasture for calves and cleaning of calf pens.

Ultra violet light did not produce any change in the pattern of recoveries despite the expectation that an effect would be observed. The lack of response could be explained by 2 possible reasons. The impact of UV light on micro-organisms is a function of exposure and intensity and at 10% of summer daylight UV, the UV light treatment may not have been sufficiently intense to cause significant bacterial injury. Also as the organisms were evenly distributed through 1.5 cm of soil and even though the soil was mixed weekly, the organisms were most probably shielded from direct UV light due to its poor ability to penetrate soil. This probably closely mirrors what would be expected in the field, where the organisms would be shielded to a large degree from the effects of UV light. It must be remembered that UV light intensity and exposure due to sunlight will be very closely correlated with soil temperature and desiccation which may play a larger part than UV light alone. In experiments on air dried organisms, Webb (1963) found low levels of moisture had protective property against the damaging effects of ultra violet light. There is also a possibility that a UV intensity threshold

exists above which Mptb are affected followed by a cumulative effect with time may mean that the threshold was not reached hence the organisms may not have been affected.

Implications

- The results in general support current thinking and strategies for on-farm control, although the death of viable Mptb in hot dry conditions was more rapid than expected and provides an opportunity to reduce de-stocking intervals if death rates can be quantified.
- Moist cool environments provide the best conditions for survival of the organism and thus susceptible stock should be excluded from such areas for longer periods.
- The markedly different effect of moisture and soil temperature on the death of Mptb provides an opportunity to review de-stocking strategies to manage the risk of Johne's disease infection.
- Quantitative information of death rates of Mptb in soil in response to the major factors influencing survival (moisture and temperature) are required to re-assess de-stocking strategies
- The conditions used in this experiment closely resemble field conditions although further work to quantify death rates should include diurnal variation in high temperature series.
- This study demonstrated a weak relationship between survival of the organism in soil and soil pH. Application of lime to contaminated areas has been recommended in the past and may be beneficial despite a lack of strong evidence in this trial.
- Although UV light showed no significant effect in field situations, UV light from natural sunlight will be closely correlated with drying and elevated temperature of the pasture and soil. Areas that experience high UV radiation are also likely to have higher ambient soil temperature and higher levels of evaporation.
- This study has shown that the presence of clay and organic matter in soil significantly influences the recovery of Mptb in culture. Variable culture performance and results from different soil types means that soil composition needs to be taken in account when assessing the potential presence of viable organisms in soil.
- An understanding of the mechanism affecting culture performance would assist the development of modified procedures to improve culture sensitivity from soils containing clay or organic matter.
- Quantification of death rates of Mptb in soil will be more reliable if conducted in low organic non-clay containing soils where culture sensitivity is optimum.

Proposed Further Work

- **Determine death rates for different moisture and temperature conditions in a tray trial**

Aim: Quantify the death rates of Mptb under different moisture and temperature conditions using sandy low organic soils.

Outcome: Provision of information on which to base recommendations for de-stocking procedures. This will provide more flexibility and allow customised de-stocking strategies for different land systems and different classes of livestock through a risk management approach.

- **Effect of organic matter and clay soils**

Aim: Elucidate the mechanism which reduces the sensitivity of culture in clay and high organic soils. Develop other techniques for increasing sensitivity of culture for these soil types.

Outcome: Clarification of whether failure to culture Mptb is due to lack of viability of Mptb in clay and high organic soils or if Mptb is lost during the culture procedure. Better understanding of the factors involved will also lead to improved culture procedures, improved interpretation of results from soils of different types and may lead to determining the infective doses of Mptb in different soil types.

- **Epidemiological case controlled study**

Aim: Evaluate risk factors including soil type and chemistry, environment and management practices for their association with herd prevalence of Johne's disease using an epidemiological case controlled study.

Outcome: Identification of significant risk factors such as those found in this study plus other soil and management factors for persistence and spread or decrease of infection will help determine local and regional influences and focus control measures on strategies which are most effective.

- **Effect of pH and Fe on Mptb Survival**

Aim: Evaluate the effect of pH and available Fe in soils of similar type.

Outcome: Quantify the effect of pH and Fe to determine if ameliorating soil with lime is a viable treatment for reducing the risk of infection.

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APPENDIX A:

Culture Results (Raw Data)

A1: DAY 1 SOIL TRAY TRIAL RECOVERIES BY SOIL TYPE

Soil Type	pH	Organic	No. of Trays Positive N=12	% of Trays Positive	No. of Culture Positive Bactecs N=36	% Culture Positive Bactecs	% Positive Cultures Organic N=72	% Positive Cultures pH N=72
Sand	Acid	Low	12	100	28	77%	63%	
Loam	Alkali	Low	9	75	17	44%		
Clay	Acid	High	4	33	4	11%	8%	44%
Clay Loam	Alkali	High	2	16.7	2	5%		26%
Total			27/48		47/144	33%		

A2: DAY 1 SOIL TRAY TRIAL RECOVERIES

UV	Temp	Organic	pH	Dry	Cyclic	Wet	*Total*
Yes	Low	Low	Acid	3	2	2	78%
No	Low	Low	Acid	1	3	3	78%
Yes	High	Low	Acid	1	3	2	67%
No	High	Low	Acid	3	3	2	89%
Yes	Low	High	Acid	0	0	1	11%
No	Low	High	Acid	0	0	0	0%
Yes	High	High	Acid	0	1	1	22%
No	High	High	Acid	1	0	0	11%
Yes	Low	Low	Alkali	3	0	2	56%
No	Low	Low	Alkali	2	2	2	67%
Yes	High	Low	Alkali	1	3	0	44%
No	High	Low	Alkali	1	1	0	22%
Yes	Low	High	Alkali	0	0	0	0%
No	Low	High	Alkali	0	0	1	11%
Yes	High	High	Alkali	0	1	0	11%
No	High	High	Alkali	0	0	0	0%
			Total	33.3%	39.6%	33.3%	35.4%

A3: WEEK 2 SOIL TRAY TRIAL RECOVERIES

UV	Temp	Organic	pH	Dry	Cyclic	Wet	*Total*
Yes	Low	Low	Acid	3	2	3	89%
No	Low	Low	Acid	3	3	2	89%
Yes	High	Low	Acid	2	0	3	56%
No	High	Low	Acid	0	0	2	33%
Yes	Low	High	Acid	0	0	0	0%
No	Low	High	Acid	2	0	1	33%
Yes	High	High	Acid	1	1	0	22%
No	High	High	Acid	0	0	1	11%
Yes	Low	Low	Alkali	0	2	2	44%
No	Low	Low	Alkali	0	2	0	22%
Yes	High	Low	Alkali	0	0	0	0/9
No	High	Low	Alkali	0	0	0	0/9
Yes	Low	High	Alkali	0	2	2	56%
No	Low	High	Alkali	1	2	1	33%
Yes	High	High	Alkali	0	1	1	22%
No	High	High	Alkali	0	0	0	11%
			Total	25.0%	31.3%	37.5%	37%

A4: WEEK 4 SOIL TRAY TRIAL RECOVERIES

UV	Temp	Organic	pH	Dry	Cyclic	Wet	*Total*
Yes	Low	Low	Acid	0	1	3	44%
No	Low	Low	Acid	0	3	3	67%
Yes	High	Low	Acid	0	0	1	11%
No	High	Low	Acid	0	0	1	11%
Yes	Low	High	Acid	0	0	0	0%
No	Low	High	Acid	0	0	1	11%
Yes	High	High	Acid	0	0	0	0%
No	High	High	Acid	0	0	1	11%
Yes	Low	Low	Alkali	0	2	2	44%
No	Low	Low	Alkali	0	1	2	33%
Yes	High	Low	Alkali	0	0	0	0%
No	High	Low	Alkali	0	0	0	0%
Yes	Low	High	Alkali	0	0	0	0%
No	Low	High	Alkali	0	1	0	11%
Yes	High	High	Alkali	0	0	0	0%
No	High	High	Alkali	0	0	1	11%
			Total	0%	17%	31%	16%

A5: WEEK 6 SOIL TRAY TRIAL RECOVERIES

UV	Temp	Organic	pH	Dry	Cyclic	Wet	*Total*
Yes	Low	Low	Acid		1	2	33%
No	Low	Low	Acid		2	3	56%
Yes	High	Low	Acid		0	2	22%
No	High	Low	Acid		0	2	22%
Yes	Low	High	Acid		0	0	0%
No	Low	High	Acid		0	0	0%
Yes	High	High	Acid		0	0	0%
No	High	High	Acid		0	0	0%
Yes	Low	Low	Alkali		0	2	0%
No	Low	Low	Alkali		0	1	11%
Yes	High	Low	Alkali		0	0	0%
No	High	Low	Alkali		0	0	0%
Yes	Low	High	Alkali		0	0	0%
No	Low	High	Alkali		1	0	11%
Yes	High	High	Alkali		1	1	22%
No	High	High	Alkali		1	0	11%
			Total		13%	23%	20%

A6: WEEK 8 SOIL TRAY TRIAL RECOVERIES

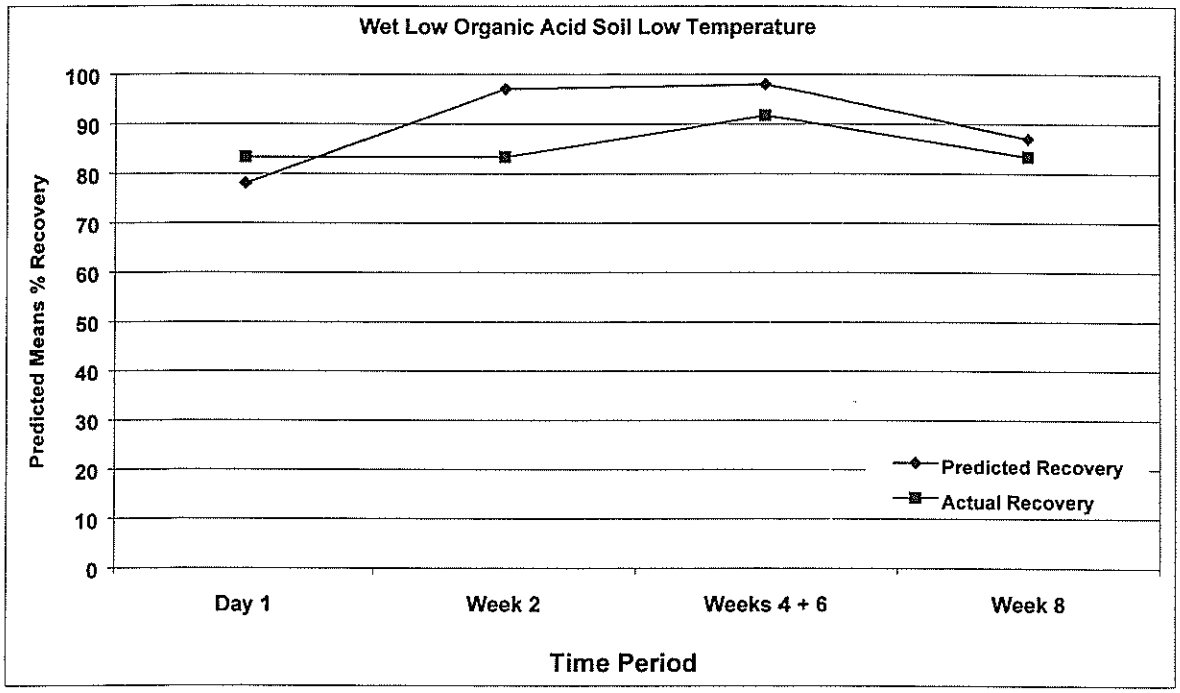
UV	Temp	Organic	pH	Dry	Cyclic	Wet	*Total*
Yes	Low	Low	Acid	2	2	2	67%
No	Low	Low	Acid	0	1	3	44%
Yes	High	Low	Acid	0	0	2	22%
No	High	Low	Acid	0	2	2	44%
Yes	Low	High	Acid				
No	Low	High	Acid				
Yes	High	High	Acid				
No	High	High	Acid				
Yes	Low	Low	Alkali	0	0	0	0%
No	Low	Low	Alkali	0	0	1	11%
Yes	High	Low	Alkali	0	0	0	0%
No	High	Low	Alkali	0	0	0	0%
Yes	Low	High	Alkali				
No	Low	High	Alkali				
Yes	High	High	Alkali				
No	High	High	Alkali				
			Total	4.17%	10.42%	20.83%	12%

APPENDIX B:

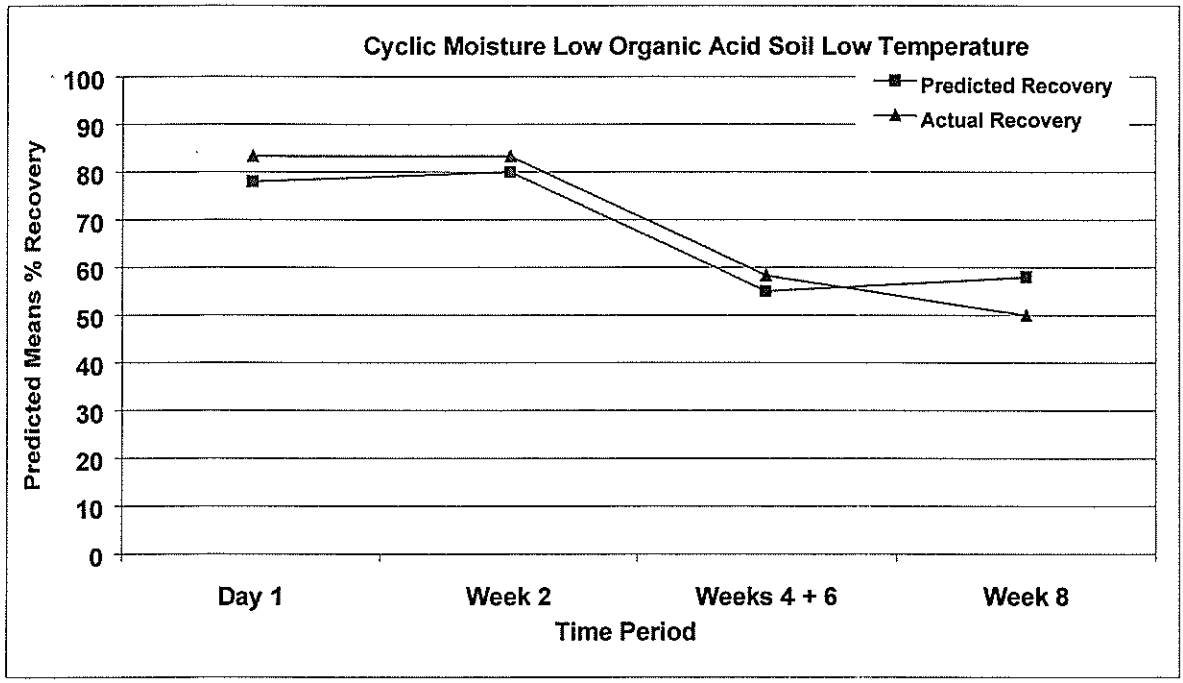
Comparison of Actual versus Predicted Mean Recovery

Actual recovery of Mptb versus results predicted in logistic regression analysis for moisture treatments wet (B1), cyclic (B2) and dry (B3). Predicted results closely follow actual results in all treatments.

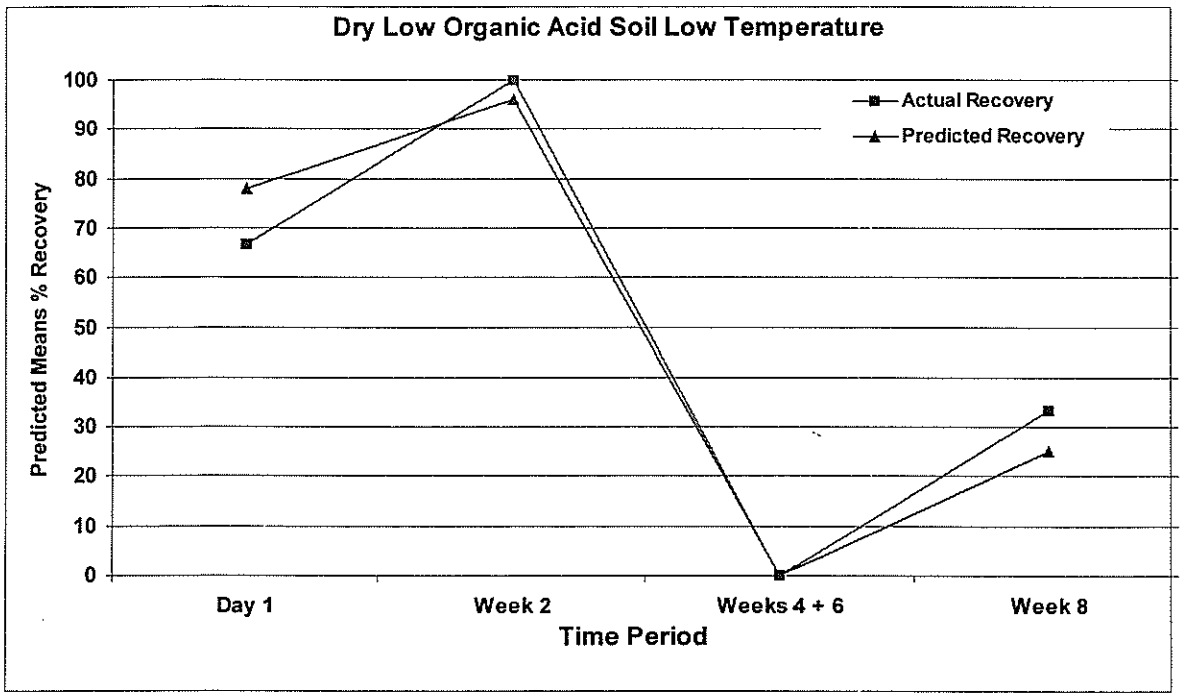
B.1: Actual versus predicted recoveries for wet acid low organic soil at low temperature.



B.2: Actual versus predicted recoveries for cyclic moisture acid low organic soil at low temperature.



B.3: Actual versus predicted recoveries for dry moisture acid low organic soil at low temperature.

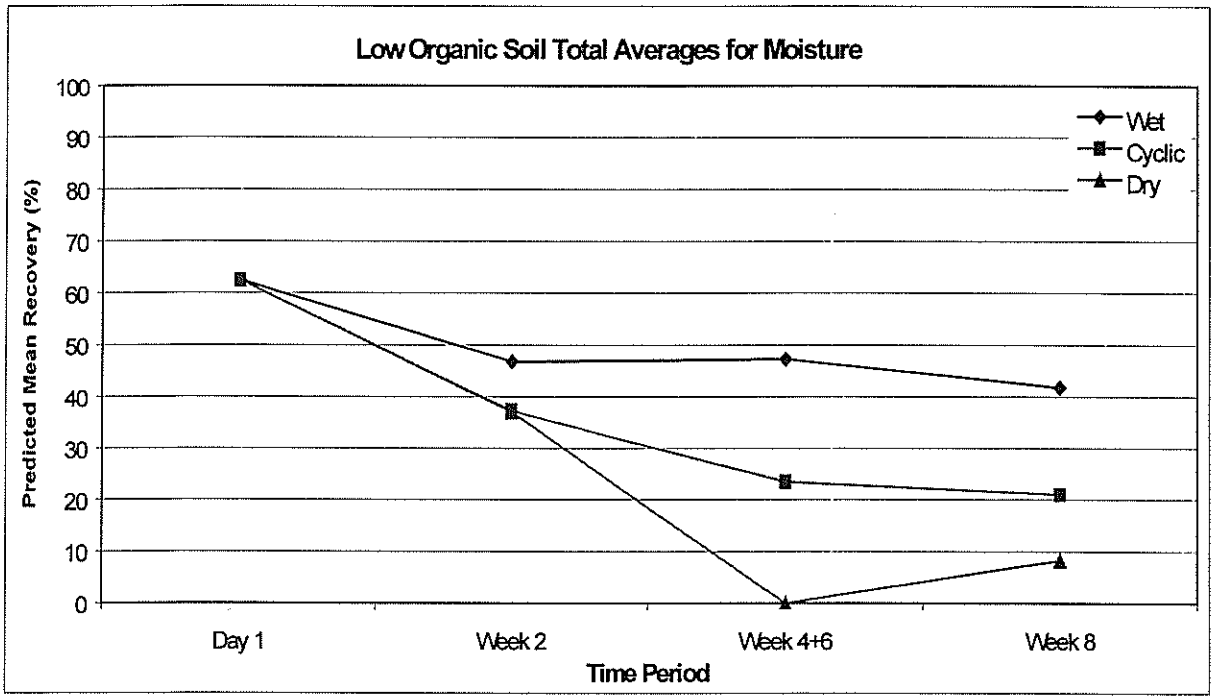


APPENDIX C:

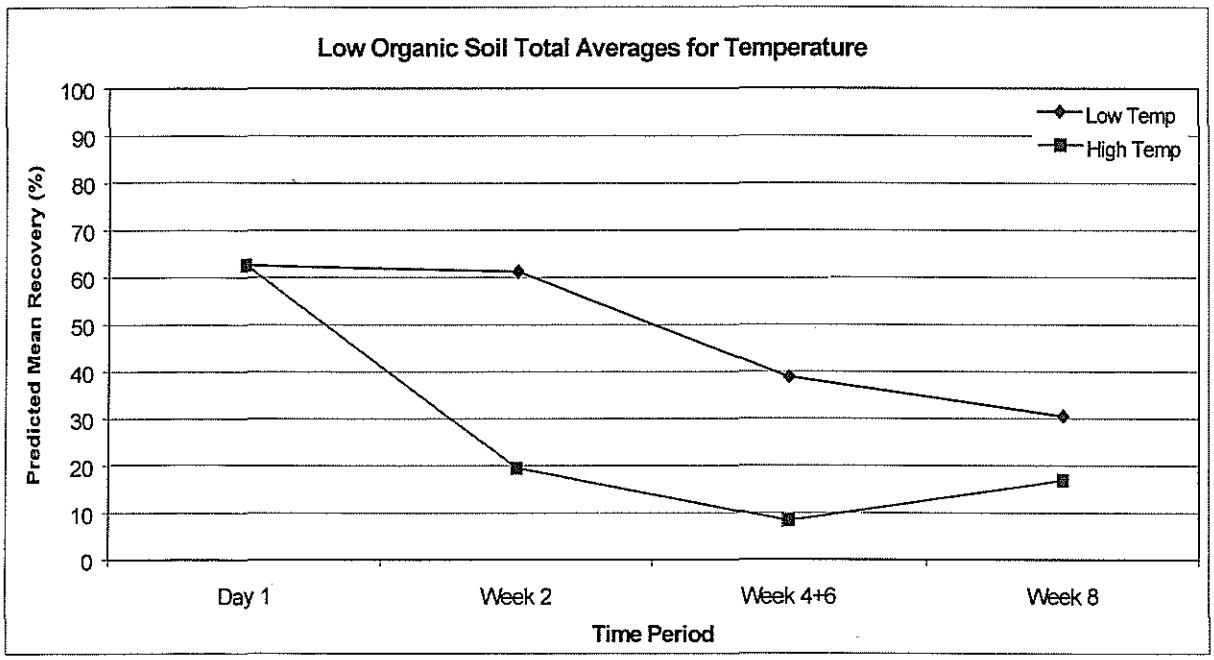
Predicted Mean Recovery Results for Moisture, Temperature and pH using Pooled Data

The graphs in Appendix C are for moisture, temperature and pH as previously presented in the results section however the data has been pooled from all treatments in low organic soils, therefore each data point is a result from 12 Bactec cultures (n=12). These graphs summarise the main outcomes of the results describe earlier.

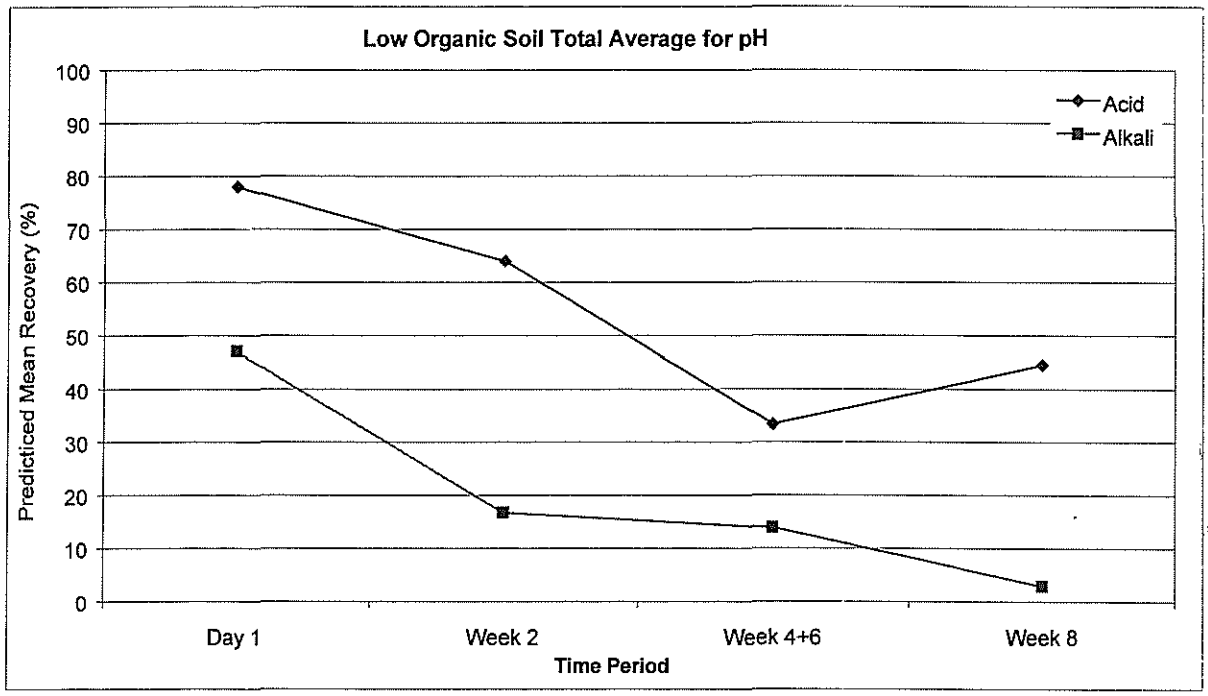
C.1: Recovery results for moisture in low organic acid soil using pooled results for temperature, pH and UV. Data for dry data was not available in the Week 4 means.



C.2: Recovery results for temperature in low organic acid soil using pooled results for moisture, pH and UV. Data for dry data was not available in the Week 4 means.

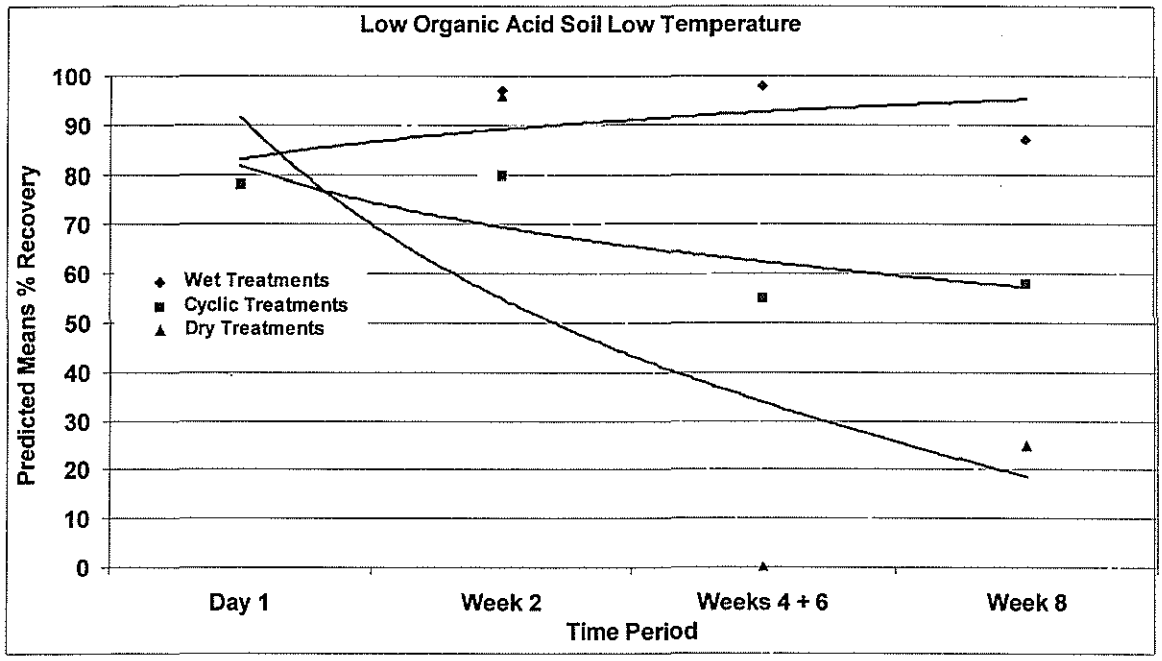


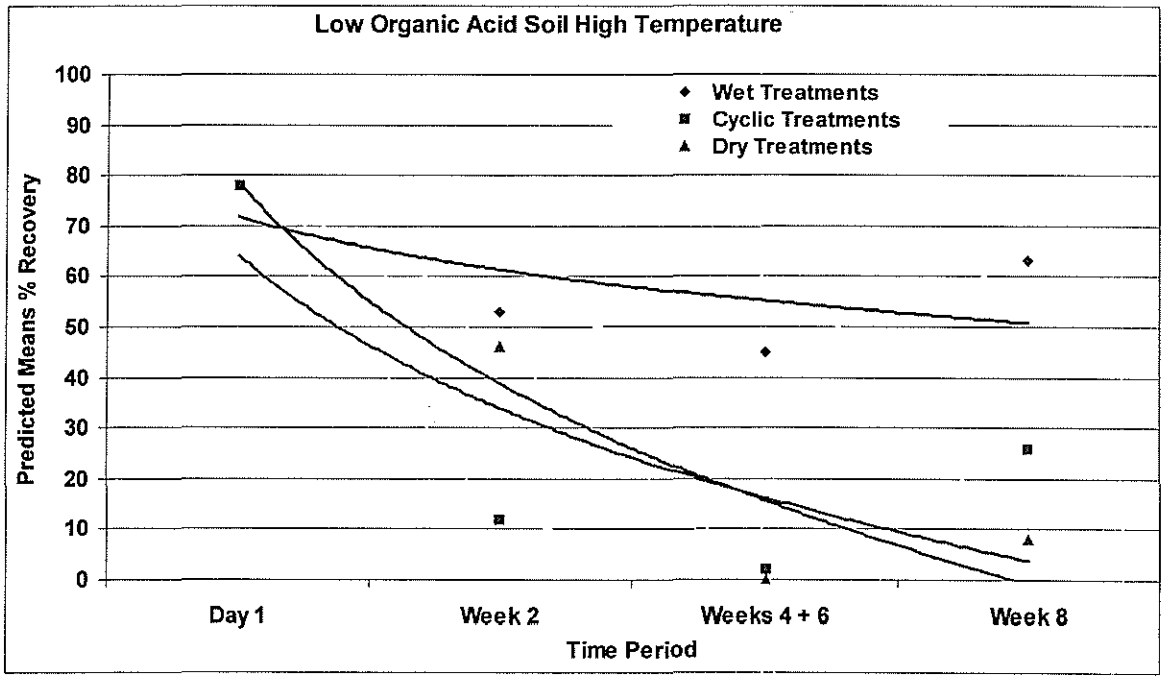
C.3: Recovery results for pH in low organic soil using pooled results for moisture, temperature and UV. Data for dry data was not available in the Week 4 means.

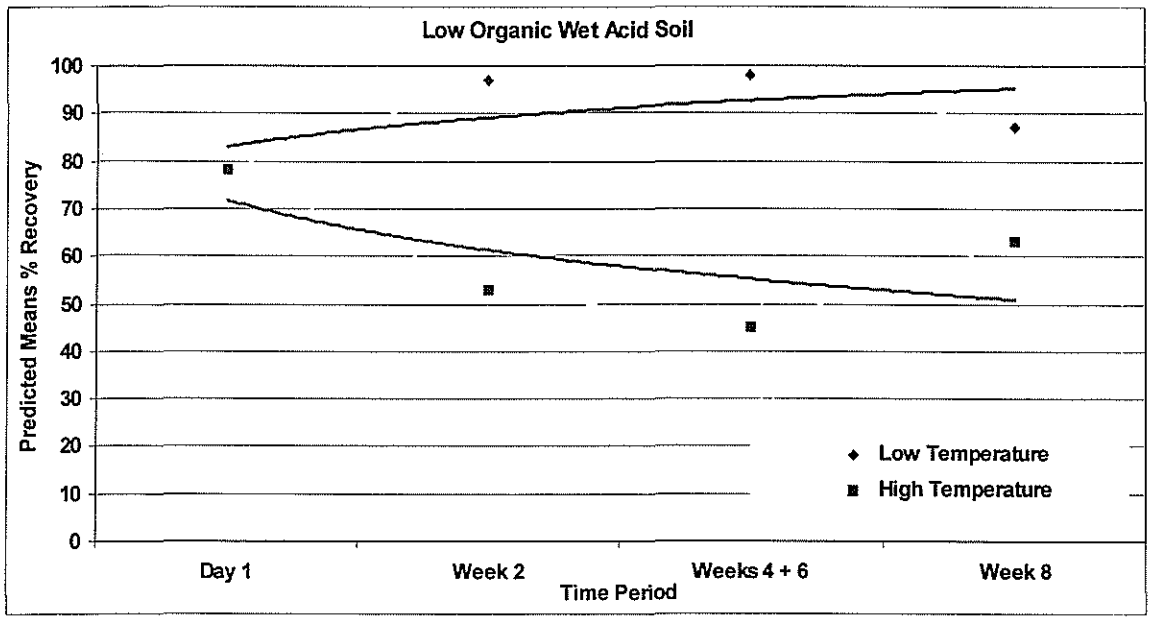


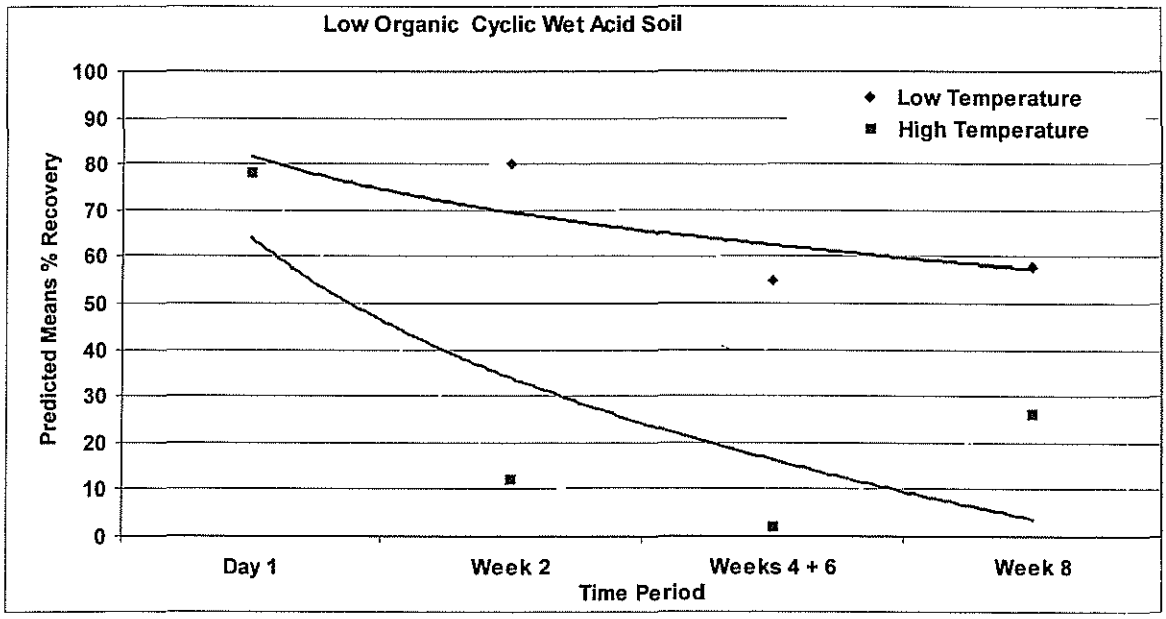
APPENDIX D:

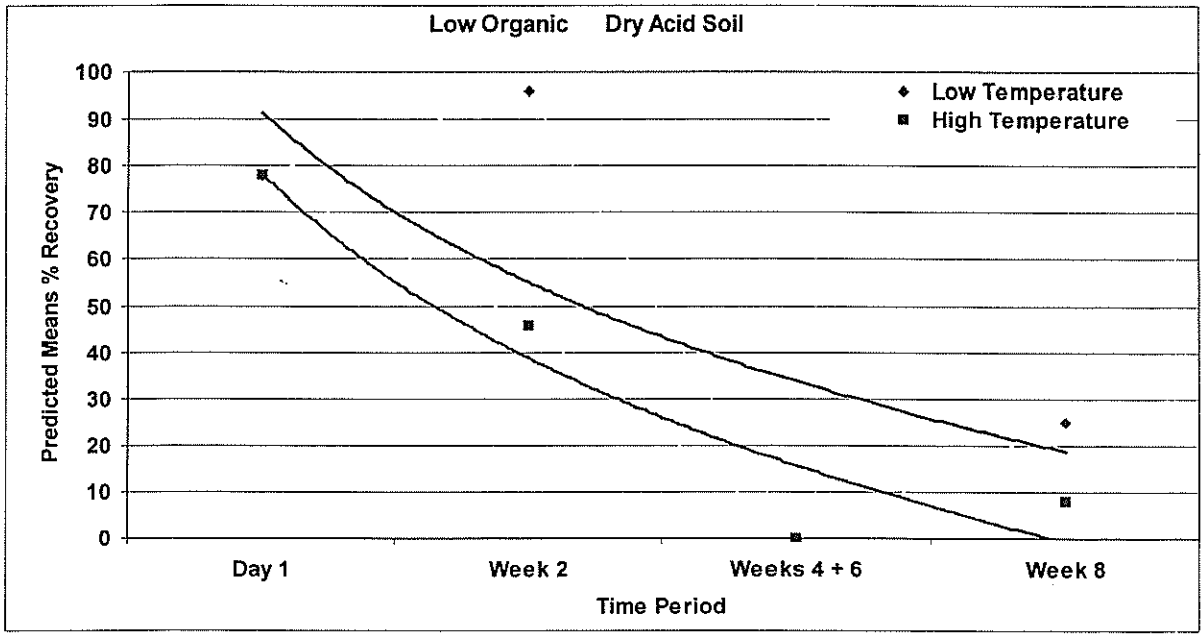
Logistic Regression Data with Logarithmic Regression Line Fitted

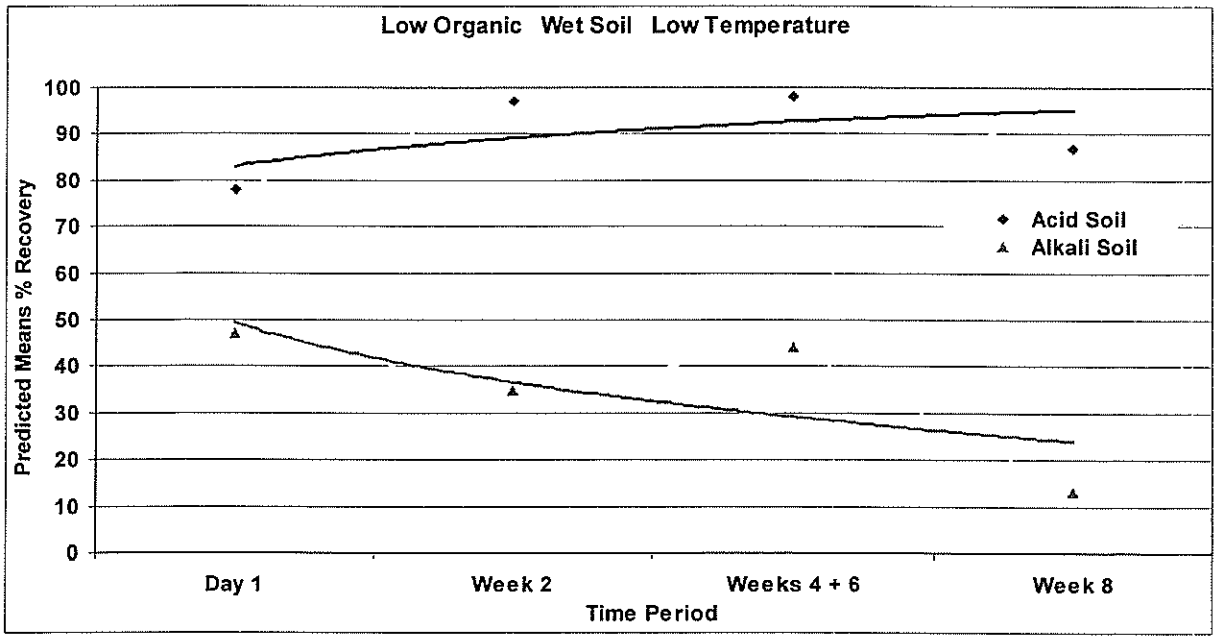

Low Organic Acid Soil Low Temperature
Predicted Means % Recovery
100
90
80
70
60
50
40
30
20
10
0
Wet Treatments
Cyclic Treatments
Dry Treatments
Day 1
Week 2
Weeks 4 + 6
Week 8
Time Period

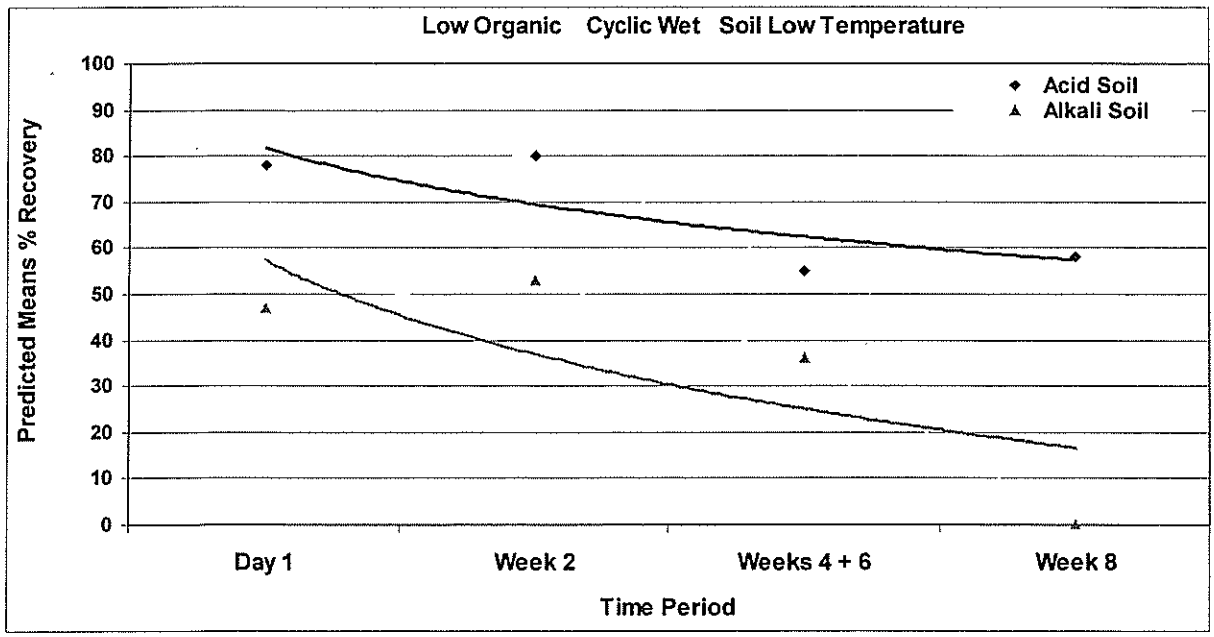

Low Organic Acid Soil High Temperature
Predicted Means % Recovery
100
90
80
70
60
50
40
30
20
10
0
Wet Treatments
Cyclic Treatments
Dry Treatments
Day 1
Week 2
Weeks 4 + 6
Week 8
Time Period

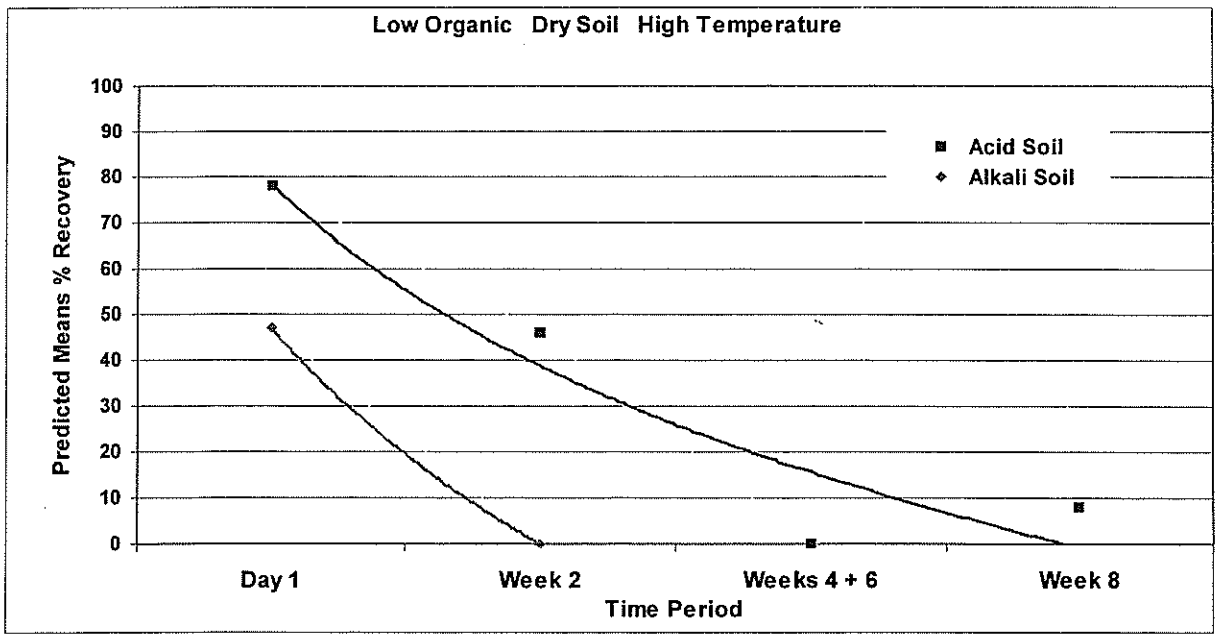

Low Organic Wet Acid Soil
Predicted Means % Recovery
100
90
80
70
60
50
40
30
20
10
0
Day 1
Week 2
Weeks 4 + 6
Week 8
Time Period
Low Temperature
High Temperature


Low Organic Cyclic Wet Acid Soil
Predicted Means % Recovery
100
90
80
70
60
50
40
30
20
10
0
Day 1
Week 2
Weeks 4 + 6
Week 8
Time Period
Low Temperature
High Temperature


Low Organic Dry Acid Soil
Predicted Means % Recovery
100
90
80
70
60
50
40
30
20
10
0
Low Temperature
High Temperature
Day 1
Week 2
Weeks 4 + 6
Week 8
Time Period


Low Organic Wet Soil Low Temperature
Predicted Means % Recovery
100
90
80
70
60
50
40
30
20
10
0
Acid Soil
Alkali Soil
Day 1
Week 2
Weeks 4 + 6
Week 8
Time Period


Low Organic Cyclic Wet Soil Low Temperature
Predicted Means % Recovery
100
90
80
70
60
50
40
30
20
10
0
Acid Soil
Alkali Soil
Day 1
Week 2
Weeks 4 + 6
Week 8
Time Period


Low Organic Dry Soil High Temperature
Predicted Means % Recovery
100
90
80
70
60
50
40
30
20
10
0
Acid Soil
Alkali Soil
Day 1
Week 2
Weeks 4 + 6
Week 8
Time Period

APPENDIX E:

Soil Analyses of Tray Trial Soils

Soil Type	Low Organic Acid	Low Organic Alkali	High Organic Acid	High Organic Alkali
Soil Description	**Langwarrin sand**	**Werribee red brown earth**	**Dalmore clay**	**Monbulk clay loam**
pH (H_20)	5.8	7.4	5	7.7
pH ($CaCl_2$)	4.8	7.3	4.6	7
Organic Carbon (% $^{w}/_{w}$)	1.9	1.2	4.6	3.6
Organic Matter (% $^{w}/_{w}$)	3.6	2.3	8.7	6.8
Total Nitrogen (% $^{w}/_{w}$)	0.08	0.1	0.4	0.31
ASE-CU mg/kg	0.6	3	1	3.8
ASE-FE mg/kg	160	21	170	46
ASE-MN mg/kg	12	3.1	9.3	9.8
ASE-ZNmg/kg	2.1	3.6	4.8	120
Exch.Al mg/kg	<10	<10	48	<10
Exch.Hmeq/100g	3.9	1.1	25	7.3
NH4-N mg/kg	3.2	0.8	40	3.3
NO3-N mg/kg	13	160	180	140
CPC S mg/kg	6	>200	74	28
Colw.P mg/kg	15	330	470	280
Exch. Ca meq/100g	1.7	17	16	16
Exch. Mg meq/100g	1.6	2.5	6.6	3.2
Exch. Na meq/100g	0.2	1.4	0.35	0.27
Exch. K meq/100g	0.3	1.1	4.1	2.6
4 Cat.+ meq/100g	3.8	22	27	22
Ca:Mg	1.1	6.8	2.5	5
Ca %	45	78	60	73
Mg %	43	12	25	15
Na %	6	7	2	2
K %	8	5	16	12
Total P mg/kg	150	1600	1700	1400
Total K mg/kg	400	4000	4000	2100
Total S mg/kg	90	1400	460	300
Total Ca mg/kg	700	6300	3900	5600
Total Zn mg/kg	8	30	48	340

Total Mn mg/kg	57	110	72	280
Total Fe % (w/w)	4500	11000	17000	27000
Total Mg mg/kg	1000	1300	2300	1900
B (CaCl2) mg/kg	0.3	1.6	0.8	1.2
Total Mo mg/kg	<0.6	3.8	<0.6	1.5
Total Co mg/kg	1.8	16	5.6	4
Total Al (%w/w)	0.22	0.7	3.1	1.9

PAPER:

Survival of Mptb in Soil.

Presented to the Australian Sheep Veterinary Association

Survival of Mptb in Soil

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Abstract

Determining environmental factors which influence the survival of Mptb is a priority of the Australian National Johne's disease Program. A collaborative project between agricultural departments in Victoria and NSW is investigating survival of the mycobacteria in several studies. These studies include evaluating techniques to detect viable mycobacteria in soil, describing the persistence of *M. paratuberculosis* on OJD and BJD affected properties, and assessing the relative impact of environmental factors on its death rate. Preliminary findings have shown viable ovine and bovine strains of *M. paratuberculosis* can be recovered from soils on affected properties using a culture technique described in this project. The analytical sensitivity of the technique is being established to enable interpretation of culture negative soils. Information on factors influencing the death rate of *M. paratuberculosis* will be available by December 1998.

Introduction

Livestock infected with M. paratuberculosis may develop Johne's disease, a chronic and fatal bowel disease, which is endemic in dairy cattle and occurs at a low prevalence in sheep flocks in southeastern Australia. The bacteria multiply in the bowel wall of infected animals and are passed with their faeces in concentrations of 102-108 per gram of faeces. Pastures contaminated with infected faeces may be a source of infection for susceptible grazing livestock. Overseas studies suggest the mycobacteria may survive in favourable conditions in the environment for months (Larsen, Merkal and Vardaman 1956, Lovell, Levi and Francis 1944). However, there has been no investigation of survival of M. paratuberculosis in Australia where high ultraviolet irradiation and low soil moisture are likely to accelerate the death of the organism. Information on the survival of M. paratuberculosis in the Australian environment will enable livestock industries to efficiently use land whilst minimising the risk of M. paratuberculosis infection. This paper describes a two-year study in NSW and Victoria which is investigating survival of M. paratuberculosis in the environment.

Materials and methods

This project is the first phase of investigations into the environmental survival of M. paratuberculosis. It consists of a composite of studies which began in May 1996 and will finish in December 1998. These include:

- assessment of the soil culture technique in a pilot study;
- description of recovery of M. paratuberculosis from properties with infected livestock;
- persistence of mycobacteria on infected properties;
- determination of the analytical sensitivity of soil culture;

- investigation of factors influencing death rates of M. paratuberculosis in the field and the laboratory.

The persistence of ovine and bovine strains of M. paratuberculosis are being investigated separately as they have some phenotype differences.

Pilot study assessing soil culture

A pilot study was performed in 1996 to determine the efficiency of recovery of M. paratuberculosis from soil using a double incubation preparation technique described by researchers in the United States (Whitlock and Rosenberger 1990) coupled with radiometric culture and PCR to detect the growth of both ovine and bovine strains of M. paratuberculosis. Mycobacterial concentrations were derived from plate counts or estimated from the rate of release of radiolabelled carbon dioxide during the incubation of radiometric cultures (Lambrecht, Carriere and Collins 1988). The variability of mycobacterial concentrations in soil and overgrowth of cultures with saprophytic mycobacteria and fungi were of particular interest for the design of subsequent studies. To assess the variability of mycobacterial concentrations and to describe the dispersal of M. paratuberculosis, 19 soil cores were taken through and around bovine faecal pats. To assess the variability in changes in mycobacterial concentrations over time, four experimental plots at the Victorian Institute of Animal Science (VIAS) were evenly loaded with a diluted suspension of faeces from faecal culture positive cows and sampled on five occasions over a one month period. Core samples were taken from randomly selected cells from a grid at each plot site before and after loading, the following day, and then seven, 14 and 28 days later.

Recovery of mycobacteria on farms

Since May 1996, environmental samples were taken from 8 sheep and 4 cattle properties in Victoria and New South Wales (Table 1). Samples from three OJD properties in Victoria were taken prior to the destocking of infected flocks. The sites selected in Victoria and NSW included sheep camps, exposed hill sides, holding paddocks for sick sheep, and soils from around carcases of sheep. Other environmental samples included water and sludge from dams where dead sheep had been found, and dehydrated faecal pellets behind carcasses. The samples taken from sheep properties in NSW were used to identify sites where mycobacterial survival could be studied. Sites sampled on four dairy properties in Victoria included irrigation channels, effluent drains, calving pads, holding paddocks and calf paddocks.

Survival of mycobacteria on farms

The death rates of ovine and bovine strains of M. paratuberculosis on affected properties are being described in NSW and Victoria respectively. In the NSW study samples were taken from 120 individual sites from five OJD properties between October and December 1997 (Table 1). In Victoria, four sites on a beef and dairy farm which were recently accessed by faecal culture positive cattle will be investigated. The sites will be fenced to prevent further livestock or wildlife access and six pooled soil samples will be submitted

from each site for culture every three months. Information on temperature, rainfall, soil moisture and UVA irradiation will be regularly collected from the sites.

Analytical sensitivity of soil culture

The analytical sensitivity of double incubation and radiometric culture methods for M. paratuberculosis is being determined in soils with low and high organic content, for both bovine and ovine strains of the organism. Each soil type was loaded with either faeces from a faecal smear positive cow with clinical Johne's disease or a pooled faecal sample from 10 faecal culture positive sheep. For each treatment, a dilution series of 10-1 to 10-8 was made by thoroughly mixing 10 g of the loading sample with 90 g of soil. The samples were divided equally and sent for processing at both Elizabeth Macarthur Agricultural Institute and the Victorian Institute of Animal Science. In November 1997, both laboratories cultured a 2 g sample for each dilution, with aliquots of the final pellet being inoculated into five Bactec bottles. The original mycobacterial concentrations were calculated from dilution series using Most Probable Number or the method of Reed and Muench (1938), and the limit of detection of the system was defined as the highest dilution yielding growth.

Plot trial of factors influencing survival

A plot trial to determine environmental factors which influence survival of ovine strains of M. paratuberculosis was started at two sites in central NSW in January 1998. Forty two plots were set up in a block design with seven treatments repeated in triplicate at each location (7*3*2). The treatments comprised:

1. unshaded soil covered with faecal pellets;
2. moist shaded soil covered with pellets;
3. shaded soil covered with a faecal slurry;
4. unshaded soil covered with slurry;
5. moist shaded soil covered with slurry;
6. moist shaded soil with a low lime application covered with slurry;
7. moist shaded soil with a high lime application covered with slurry.

The faeces used to load the sites was collected from seven faecal smear positive sheep. Each plot was 2.25 m2 and was covered with either 3.5 kg of a slurry of faeces and chaff or 1 kg of faecal pellets. The sites were secured by rabbit proof fencing and drains have been used to protect plots from run-off. Plots are being kept moist using domestic polypipe microsprayers and shaded with shadecloth covered frames. A weather station has been set up at each location for the collection of detailed meteorological information and soil temperature and moisture. Soil samples are collected for culture at monthly intervals. Sample collection involves taking soil cores from 20 randomly selected grid cells on each plot and pooling them into two samples for culture. A similar process is followed to generate two pooled faecal samples from the plots loaded with faecal pellets.

Laboratory experiment of factors influencing survival

A laboratory experiment to determine the relative importance of ultraviolet irradiation, soil moisture, organic content, and soil pH on survival of M. paratuberculosis in soil was started in Victoria in January 1998. The apparatus for the trial was developed by March 1998. The experiment involves loading 48 trays of soil with faeces from JD AGID positive sheep. It is a single replicate fully randomised factorial design, with each tray containing a different treatment depending on the combination of:

1. soil temperature (high or low);
2. soil moisture (wet, dry, cyclically wet);
3. pH (high or low);
4. organic content (high or low);
5. UVA irradiation (+/-).

Measurements of the organic content and pH of the soil will be made at the beginning of the trial. Measurements of soil temperature will be taken daily, UVA irradiation will be taken weekly, and moisture will be taken on days 1, 2, 14, 16, 28, 30, 42, 44, and 56 of the experiment. Mycobacterial concentrations in each tray will be estimated from a pooled soil sample taken on days 1, 12, 26 and 52 of the experiment. This information will be used to assess the influence of the factors on the viability of M. paratuberculosis.

Results

Mptb was cultured from 23% (32 of 140) of environmental samples collected from Victorian dairy farms, 13% (16 of 120) samples collected from OJD properties in NSW, and none of the 37 samples collected from OJD properties in Victoria. The culture positive soil samples from cattle properties comprised two samples taken from a calf paddock on a heavily infected dairy farm and 30 samples collected around faecal pats in the pilot study. The culture positive OJD samples were from widely differing sites including an exposed hillside, a sheep camp, a poorly drained area and dam sediment. On the low prevalence OJD farm, none of 10 sites in an area which had been destocked for one year were culture positive whilst one of 10 sites with continuing animal access was culture positive.

Using the Lambrecht model, the concentration of mycobacteria in soils in the pilot project ranged from 101.5 and 103.3 CFU/g. Counts were highly reproducible with coefficients of variation of 15% (when mean soil concentrations were 103.0 CFU/g) from soils taken through and around faeces from a clinical cow. However, the method could not be applied to 65% of culture positive samples which had unsuitable radiometric growth profiles, and it required daily reading 29% of samples which were ultimately found to be overgrown with saprophytic organisms. One-quarter of culture positive samples which could not be quantified using the Lambrecht method were likely to contain less than 100 CFU/g soil based on the slow development of radiometric growth indexes.

Detection of this concentration of mycobacteria is consistent with preliminary results of the sensitivity study. The culture technique detected ovine strains of M. paratuberculosis when present at concentrations in the order of 1-100 cells per g soil. The detection limit in low organic soil (10 cells/g or less) was slightly better than for high organic soils. Calculation of mycobacterial concentrations of ovine and bovine faecal samples used to load the soils were

highly consistent between laboratories. The limit of detection in high organic soils loaded with a bovine strain of M. paratuberculosis is to be repeated due to the low numbers of organisms in the original sample and the high contamination rate of these soils at VIAS.

Several OJD positive cultures from soils taken from the sensitivity study and from farms showed their first evidence of growth after the routine two month week incubation.

Four experimental plots in the pilot study were estimated to be loaded with suspensions of 102.5 - 102.8 CFU of M. paratuberculosis per cm2. The mycobacteria was cultured from all plots immediately after they were loaded. Multiple samples were required to detect plots containing viable M. paratuberculosis. Three plots were culture positive the first day after loading and one week later, and one of these plots was culture positive a fortnight and a month later. During this period, the pasture plots were exposed to an average of 8.2 sunshine hours each day, a total of 25.2 mm rain, and the moisture loss in the soils in the plots was between 14% and 29%.

One-third of the samples collected in the pilot study were overgrown with non-paratuberculous organisms. Soils from the experimental plots accounted for 93% of this contamination. The contamination rate was particularly high in one plot throughout the month and in all plots at the final week of sampling.

Soil samples taken from plots in the NSW field trial were culture positive immediately after seeding.

The remainder of the results of these studies are pending.

Discussion

The culture technique used in this study is effective at detecting the growth of ovine and bovine strains of M. paratuberculosis (Whittington, Marsh Turner et al. 1998).

Sampling to date suggests that soils containing concentrations as low as 1-100 mycobacteria per g of soil can be culture positive. This assessment of analytical sensitivity is necessary to interpret negative results such as the 37 samples collected from Victorian properties with heavily infected OJD flocks. Although culture negative samples are expected to represent soil which does not contain M. paratuberculosis, cultures may be false negatives if mycobacteria are present in numbers below the test sensitivity or present in dormant or non-culturable forms. The suggestion that sensitivity of the technique may be lower in high organic soils needs to be confirmed.

Reductions in mycobacterial concentrations over time will be determined by analysis of dilution series rather than Lambrecht's method in subsequent studies. This is because Lambrecht's method is not applicable when soil concentrations are less than 102 CFU/g or greater than 105 CFU/g. It is therefore costly to quantify M. paratuberculosis as between 9 and 40 cultures must be set up for each soil sample to be analysed.

Overgrowth of soil cultures with fungi and bacteria has been about 5%. The exceptions are high contamination rates in plots in the pilot study and a high contamination rate in the high organic soil treatment of the sensitivity trial. Contamination in the pilot study was probably a result of sporulation and multiplication of micro-organisms following dumping of 85% of the month's rainfall for the month within a week of the final sample collection. Contamination in the sensitivity trial was directly related to manure used to enrich the soil. It is expected that overgrowth of cultures will be a continuing and frustrating component of this study. Actions taken to reduce contamination include autoclaving soils used in the laboratory study prior to loading with M. paratuberculosis.

The observation that 10 soil samples from a NSW property which had been destocked for one year were culture negative is both encouraging and consistent with a report from a property with infected deer in the United States (Stehman, Rossiter, Shin et al. 1996). However, results of on-going studies are necessary to determine whether non-persistence is representative of the normal situation.

The higher number of culture positive soil samples from OJD infected farms in NSW compared to Victoria may reflect higher environmental loads of M. paratuberculosis, local conditions favouring its survival, the greater number of samples taken from OJD properties in NSW, or incubation of radiometric samples for more than eight weeks. Since M. paratuberculosis has been confirmed in soil samples which first showed evidence of growth at 10 weeks of incubation, cultures in subsequent studies will be incubated for a minimum of three months. This contrasts with the standard radiometric incubation period of eight weeks.

Isolation of M. paratuberculosis from soils taken along a fenceline in a calf paddock on a dairy farm was unexpected. None of 45 calves held in this paddock were faecal culture positive at the time of soil collection. Mycobacterium are likely to have spread to this fenceline by the movement of livestock or machinery as the calf paddock was situated beside a laneway near the hayshed and there were 13 faecal culture positive cows in the herd.

Conclusions

This study has demonstrated that viable M. paratuberculosis in soils from affected OJD and BJD properties can be recovered using a technique combining double incubation and centrifugation preparation of samples with radiometric culture and PCR confirmation. The analytical sensitivity of this technique is being established to enable interpretation of culture negative soils. Preliminary results indicate that culture detects between 1-100 cells per g soil. In subsequent studies, mycobacterial concentrations should be based on a Most Probable Number method or 50% end-point and cultures should be incubated for more than 8 weeks. Although contamination rates for soil samples are usually about 5%, very high rates were observed in some soils and following increases in soil moisture and temperature. Information on factors influencing the death rate of M. paratuberculosis will be available by December 1998.

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Property	Prevalence	Date	Location	No. samples [g]	No. culture positive
1	OJD, 34%[a]	Sep 96	East Vic	14	0
2	OJD, 21%[a]	Sep 96	East Vic	16	0
3	OJD, n/a	Sep 96	East Vic	7	0
4	OJD, low[b]	Oct 97	Cent NSW	20	1
5	OJD, high[c]	Dec 97	Cent NSW	25	2
6	OJD, high[c]	Dec 97	Cent NSW	25	4
7	OJD, high[c]	Dec 97	Cent NSW	25	4
8	OJD, high[c]	Dec 97	Cent NSW	25	5
9	BJD, 4%[d]	May 96	North Vic	8	0
		Aug 96	North Vic	8	0
		Sep 96	North Vic	28 (7)	0
10	BJD, 5%[d]	Aug 96[e]	North Vic	19 (1)	15
11	BJD, 9%[d]	Aug 96[e]	West Vic	16 (1)	15
		Aug 96	West Vic	6 (1)	2
		Apr 97	West Vic	28 (1)	0[f]
12	BJD, 3%[d]	May 97	East Vic	25 (1)	0

Table 1 Recovery of *M. paratuberculosis* from environmental samples

a Prevalence based on untested sheep whose OJD status was confirmed at slaughter

b Low prevalence refers to confirmed OJD property with sporadic mortalities

c High prevalence refers to confirmed OJD properties with mortalities exceeding 5%

d Prevalence based on herd EIA and faecal culture test assuming sensitivity of 75%

e Soil samples taken through and around faecal pats of JD infected cows
f Includes samples taken from two previously culture positive sites
g Brackets refer to the number of contaminated samples
n/a Prevalence not applicable as holding yard on research farm