MEAT & LIVESTOCK
AUSTRALIA

On farm

Survival of Johne's Disease in the Environment

National Ovine Johne's Disease Control and evaluation Program

Project numbers OJD.003, TR.055A, TR.055
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Animal Health and Welfare

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Abstract

This report covers three MLA projects (TR.055, TR.055A and OJD.003) undertaken by NSW Agriculture to determine how long the sheep strain of *Mycobacterium paratuberculosis* survives in the environment to validate destocking recommendations for eradication of OJD. Survival of the organism was prolonged but finite. In the shade it lasted for 13 months while in the open in ungrazed pasture it lasted for 7 months. It survived for a shorter period in fully exposed pastures where grazing was simulated but for much longer in water than on pasture. These times were probably underestimates. Liming pasture did not reduce survival and moisture did not increase it. Shade was the most significant factor favouring survival. Further research is necessary to determine the mechanisms of survival, which include dormancy. Decay rates for the organism were determined for short term and long term destocking. These can be used to estimate how much time must be allowed to render pastures safe for control and eradication of OJD, respectively. When estimates of soil ingestion rates by grazing sheep are combined with within-flock OJD prevalence estimates and bacterial shedding rates determined by PFC, it is possible to make property by property recommendations for the purpose of control or eradication of OJD.

Executive summary

This report relates to components of three MLA projects to determine the duration of survival of the sheep strain of *Mycobacterium paratuberculosis* (TR.055, TR.055A and OJD.003) in order to provide greater confidence in recommendations for destocking and decontamination for eradication of OJD being evaluated under the National Ovine Johne's Disease Control and Evaluation Program. A separate report covers work undertaken by Agriculture Victoria under TR.055.

There is very little information on pasture decontamination. The sheep strain of *M. paratuberculosis* that is responsible for almost all cases of OJD in Australia was extremely resistant in the environment. The results were consistent with those from several laboratory experiments conducted overseas using the cattle strain.

Pasture contamination on OJD affected farms can be widespread and its location is generally not predictable. Low-lying areas may become a focus for contamination due to movement of faecal material. Under the conditions studied contamination was not detectable after one complete summer in most sites previously found to be infected.

In plot and box experiments the duration of survival exceeded 12 months in faecal pellets in a shaded location but was much less in unshaded treatments unless vegetation was not grazed. Sunlight, including factors such as UV, visible and infra-red radiation, is a very significant factor influencing survival of the organism. Temperature flux was proposed as the reason shade was so important because UV radiation probably cannot penetrate pellets. Moisture levels and lime application did not appear to influence survival.

Decay rates for the organism were estimated and were found to be inversely proportional to the period of observation. There was a rapid decay phase lasting about 6 weeks during which the vast majority of viable bacteria declined. This was followed by a period of dormancy of variable duration during which the organism could not be cultured but its DNA could still be detected, and sometimes a period of apparent replication during which its numbers increased. Finally there was a slow decline phase lasting many months.

The organism moves from faecal pellets into the surface litter as pellets break down and also enters the soil profile. Thus it can be cultured readily from the surface layers of the soil. The duration of survival in soil was underestimated because the culture method was imperfect and the data on duration of survival needs to be interpreted with caution. Pasture emerging through contaminated faeces becomes contaminated with relatively high concentrations of the organism. The organism was also found to be associated with infective third stage larvae of intestinal nematode parasites that developed in the faeces of sheep with OJD. These larvae may be found on pasture and may contribute to infectivity of pasture.

The organism moves away from infected sites in run-off water and survives for prolonged periods in water. The duration of survival in water is longer than that in soil in the same environment. Based on studies of other bacteria including the related organism *M. avium*, there is potential for interactions between *M. paratuberculosis* and single-celled aquatic animals.

Faecal shedding rates of the organism were determined using sheep with multibacillary OJD and amounted to 10^8 viable organism per gram or more than 10^{10} viable organisms per sheep per day. Sheep in earlier stages of the disease process would shed fewer organisms. Faecal output ranged from about 500 grams per day to over 1000 g per day among sheep.

A means of estimating decontamination intervals for pasture was proposed and three examples were given. Each was based on reasonable assumptions of prevalence of OJD, faecal shedding levels, bacterial decay rates and rates of soil consumption by grazing sheep.

Specific recommendations were made:

1. Decontamination intervals for eradication of OJD need to take account of: a. Decay rates. Conservative estimates of decay rate should be used. b. The level of infection in the flock prior to destocking which can be measured objectively by PFC (prevalence of faecal shedders and level of shedding). c. The presence of environments likely to be conducive to survival and which might be fenced off. d. The amount of soil ingested by sheep, which can be estimated based on type of soil, stocking rate, pasture type, rainfall and other factors. A decontamination interval of 15 months is likely to sufficient in many but not all cases, depending on the assumptions used.
2. Decontamination intervals following transient contamination of land can be shorter than those following long term contamination by an endemically infected flock.
3. Decontamination intervals to reduce the impact of OJD by pasture spelling or management on endemically infected farms can be quite short because of the rapid decline phase. In general, the practices recommended for control of internal parasites will be beneficial for OJD control provided that adult sheep used to prepare pasture for young sheep are not heavy shedders of *M. paratuberculosis.*
4. Simple spreadsheet-based computer models need to be developed to facilitate estimation of decontamination intervals for individual situations using a stepwise approach based on that outlined in this report. Further information may need to be gathered about rates of soil ingestion by grazing sheep in Australia, but much of this information may already exist in the literature. Probabilistic models that can account for incomplete knowledge can be developed using commercial software (eg @risk).
5. Knowledge about age susceptibility of sheep is required because shorter decontamination intervals might be possible if age-resistant sheep were used as restockers.
6. It is important to measure decay rates for contaminated faeces and soil in the winter months because it is possible that season may be less significant than local or micro-environmental shade influences. This would reduce the component of economic hardship imposed by being required to commence destocking at the beginning of summer. This research should be conducted using the pasture box method developed in this study. Greater flexibility should be given as to when decontamination can start in the summer period.
7. In future experiments using pasture boxes it is important that contamination with faeces be undertaken after transport of boxes rather than before transport because of the potentially deleterious effects of pooling of water caused by vibrations during transport. It would also be desirable to protect boxes from heavy rain using removable covers.
8. Specific recommendations have already been made concerning research on the survival of the organism in water and its association with aquatic invertebrates.
9. In vitro studies of the survival of the organism within faecal pellets exposed to measured doses of UV radiation are required to confirm that incident UV radiation does not sterilise organisms within pellets. Similar in vitro studies of the effects of temperature flux also need to be undertaken.
10. Basic research on dormancy and environmental replication of *M. paratuberculosis* is needed to support the NOJDP.
11. The findings of this study need to be discussed widely because it is impossible to consider all relevant technical issues nor to foresee all relevant present and future policy and farm management factors in a single report.

The results of this project will be of immediate benefit to industry. Reliance can be placed on the current recommendations related to a 15 month destocking period for eradication of OJD under most circumstances. Situations where this might not be sufficient include properties with very high prior prevalence of infection and a high proportion of infected sheep in advanced stages of the disease, where there are extensive areas of shaded environment favourable for survival of the organism, or where pasture and geographic factors result in high levels of soil consumption. Contaminated water is also a risk. Industry can also benefit by an end to speculation that shorter destocking periods (particularly 4 months) might be adequate for eradication. Short pasture spelling periods, such as those used for internal parasite control, are, however, ideal to increase the safety of pasture for young sheep on infected properties by significantly reducing contamination levels.

The results of this project will be of benefit to producers, their veterinary advisers, policy makers, disease control regulators and research coordinators, all of whom are faced with difficult issues.

Acknowledgments

This report covers research conducted over a five year period from 1997 to 2001. Over this time a large number of people contributed directly and indirectly to the outcomes.

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Main research report

1.0 Background and industry context

Johne's disease is a chronic enteric infection transmitted insidiously from one generation of animals to the next mainly by faecal contamination. Central to efforts to control and eradicate Johne's disease is better understanding of the biology of the causative organism, *M. paratuberculosis*. By taxonomic definition[1] this bacterium is an obligate pathogen and parasite of animals, which means that its existence is dependent upon the presence of animals. In theory, the organism can be eradicated by removal of all infected animals. However, we cannot disregard the ability of the organism to survive for long periods of time outside the host animal. This is an evolutionary insurance policy for the organism, enabling it to spread in the environment and to withstand a periodic lack of suitable hosts to infect. The spread of the organism in animal faeces was recognised early in the last century and the question of how long pastures remain infective has been asked since 1912[2].

Under the National Ovine Johne's Disease Control and Evaluation Program (NOJDP) research is under way to evaluate the efficacy and practicality of eradication of OJD by whole flock destocking, pasture decontamination and restocking from clean flocks (Trial 1.1). At stake, among other things, is farm income during the destocking period. Although a range of enterprises such as cattle grazing, short term grazing of sheep and cropping are available to producers, options may be limited by geographic factors, market factors, and by the experience and inclination of the producer to change enterprise. Thus the shortest possible destocking period is desired by most parties to enable a return to the usual enterprise.

The destocking interval required to eradicate the organism from the environment is unknown. However, "global wisdom" is such that a lengthy period is required. It is generally suggested that at least 6 months to a year is required to render pastures safe after grazing by infected cattle[3]. This kind of recommendation was made as early as 1938[4], presumably based on anecdotal field experience. However, experimental confirmation of the resistance of the organism came in 1944[4]. Faeces from a cow with JD was placed in an open bowl in an exposed place in London, England, and examined at intervals. The organism survived for about 9 months.

The "conventional wisdom" is that the Australian environment is harsher than that in the northern hemisphere. Our summers are hotter, longer and drier than those in other places. These factors are generally considered to be favourable for elimination of infectious disease agents. It is one explanation for the lack of certain infections that are common in the northern hemisphere. This is the thinking that prevailed in 1995 - 1996 when recommendations were proposed to deal with OJD. Summer was prescribed as best the time to destock because pastures would be exposed to the highest levels of sunlight and it could reasonably be expected that one full summer (4 months) might be sufficient and that two full summers (15 months) would be more than sufficient to rid pastures of the organism. Property disease eradication plans were approved and producers commenced to destock under the 15 month guideline. In Victoria there were additional recommendations to exclude stock from low lying wet areas as these might provide a more favourable environment for the organism and its survival might be longer in such places.

There is very little published data of relevance to the pasture decontamination process proposed under NOJDP. The available data on the survival of *M. paratuberculosis* is summarised in Table I. All of it relates to the cattle strain of the organism and most of it comes from quite contrived situations. Field data is non-existent. Data from the Victorian companion study under project TR.055[5] relates to the cattle strain under laboratory conditions and cannot be applied readily in the field for control of OJD.

In order to provide greater confidence in recommendations for the destocking and decontamination intervals, research was commissioned directly by MLA and later under NOJDP. The results of this research are provided in this report. It relates to components of three projects undertaken by NSW

Agriculture (TR.055, TR.055A and OJD.003). A separate report covers work undertaken by Agriculture Victoria under TR.055[5]. The projects were commissioned in stepwise fashion with strict milestones to ensure the best value in terms of outcomes for industry.

2.0 Project objectives

The following project objectives are taken from the contracts applicable to the work described in this final report.

Project TR.055

1. By September 1998, to determine the survival of *Mycobacterium paratuberculosis* under a representative range of field conditions

Project TR.055A

1. To continue monitoring contaminated sites on farms in NSW to determine the duration of survival of the organism
2. To study in more detail the survival of the organism in sheep pellets and in the immediate environment of sheep pellets

Project OJD.003

1. To show that a full summer (1999-2000) is adequate for decontamination of OJD
2. To correlate survival with solar radiation levels over a full summer
3. To draw inferences upon extension of research to areas in northern, western and southern NSW

Project activities to meet these objectives can be summarised as follows:

Numerous pasture sites and some drainage areas and dams were surveyed on five OJD-affected farms. Sites found to be infected were sampled again after a full summer (Part 2 of this report).

In separate work experimental pasture plots were established and contaminated with infected faeces at the beginning and end of summer in two successive years. Samples were collected for more than 12 months. A similar trial was conducted in polystyrene boxes containing soil and grass (Part 3).

A pilot study on survival of the organism in water was undertaken after discussions with MLA (Part 4).

Methodologies, including the sensitivity of the culture method, were checked and re-evaluated during the study (Parts 1, 5 and 6).

Table I. Reports of the survival of the cattle strain of *M. paratuberculosis* (except reports on pasteurisation and chemical disinfection)

Substrate	Source of bacilli	Estimated starting concentration or number of bacilli*	Temperature/light	Duration of survival	Reference
Survival in dip fluids, slurry, faeces and urine					
Amitraz cattle dip fluid pH 12.4	Bovine faeces	uncertain	22°C	> 2 < 3 wks	60
Anaerobic bovine slurry (mixture of faeces, urine, straw, water)	Cultured bacilli	10^5/g	5° C	>252 < 287 days	6
Anaerobic bovine slurry	Cultured bacilli	10^5/g	15° C	>98 <112 days	6
Anaerobic bovine slurry	Cultured bacilli	10^3 - 10^4/g	35° C	>21 < 28 days	61
Anaerobic bovine slurry	Cultured bacilli	10^4/g	53° C - 55° C	<1 day	61
Bovine faeces and straw	the sample, containing orange pigmented strain	uncertain	not recorded	>5 mths	62
Bovine faeces	Cultured bacilli	uncertain	uncertain	11 mths	63
Bovine faeces (liquid) in open bowl	the sample	uncertain	Ambient -3°C to 23°C exposed	>246 <284 days	4
Bovine faeces in open bowl	the sample	uncertain	Ambient exposed -2°C to 23°C	>208 <236 days	4
Bovine faeces in open bowl	the sample	uncertain	Ambient exposed -2°C to 23°C	<5 mths	4
Caprine faeces in open bowl	the sample	uncertain	Ambient exposed -2°C to 23°C	>67 days	4
Bovine urine	Cultured bacilli	uncertain	uncertain	7 days	63
Bovine urine	Cultured bacilli	10^7/ml	38° C (dark)	<30 days	64
Bovine urine & faeces	Cultured bacilli	10^7/ml	38° C (dark)	<30 days	64
Survival in water					
Tap water pH 7	Cultured bacilli	10^7/ml	38° C (dark)	>17 <19 mths	64
Tap water pH 5 or 8.5	Cultured bacilli	10^7/ml	38° C (dark)	>14 <17 mths	64
Distilled water - sterile pH 6.4 - 6.8 in sealed bottle	Cultured bacilli	10^3 - 10^4/ml	Ambient 9°C - 26°C	>9 <13 mths	4
Tap water - sterile pH 7.1 - 8.0 in sealed bottle	Cultured bacilli	10^3 - 10^4/ml	Ambient 9°C - 26°C	>9 <13 mths	4
Pond water + mud - sterile pH 5.3 - 5.9 in sealed bottle	Cultured bacilli	10^3 - 10^4/ml	Ambient 9°C - 26°C	>9 <13 mths	4
River water in bottle	bovine intestinal scrapings	uncertain	Ambient - Jan to May in London, shade	>113 <141 days	4
River water in open bowl	bovine intestinal scrapings	uncertain	Ambient -7°C to 18°C shade	>135 <163 days	4
River water in open bowl	bovine intestinal scrapings	uncertain	Ambient -7°C to 18°C sun	>163 <218 days	4
Distilled water	Cultured bacilli	10^6/ml	uncertain	455 days & D-value 69 days (strain Dominic); D-value 92 days (strain ATCC 19698, lab adapted)	15
Survival in the laboratory					
Saline	Cultured bacilli	10^7/ml	38° C (dark)	>17 <19 mths	64
Desiccated culture	Cultured bacilli	10^8	38° C (dark)	>17 mths	64
Desiccated culture	Cultured bacilli	10^8	38° C (dark)	>47 mths	64
Desiccated culture	Cultured bacilli	10^9	-14° C for 5 mths then 4° C for 5 mths then 38° C for 8 mths	viable	64
Desiccated culture	Cultured bacilli	10^9	-14° C for 12 mths then 4° C for 5 mths	viable	64
Desiccated culture	Cultured bacilli	10^9	up to 44° C	>65 <100 hrs	64
Bovine faeces	the samples	mod to high	-70°C	>15 wks	7
Bovine faeces	the samples	> ~ 1000/g	-70°C	>15 wks	8
Bovine faeces	the samples	< ~1000/g	-70°C	<15 wks	8

*based on authors' raw data assuming 1 mg wet weight bacilli = 10^6 organisms and 1 mg dry weight bacilli = 10^7 organisms, or colony count; several authors noted counts dropped off very quickly after T=0 but then declined more gradually[4,6,7]

3.0 Detailed general methodology

3.1 Culture of faeces and soil samples

Pooled soil samples and pooled faecal (pellet or faecal slurry/mixtures) were thoroughly mixed prior to sub-samples being taken for culture. In early stages of the project mixing was undertaken by hand using a mortar and pestle and scissors to break up plant material but in most cases an electric blender with metal cutting blades was used to mix samples as described previously[9]. Samples were cultured as described by Whittington et al[10]. Briefly, a double incubation and centrifugation method was used. Faeces or soil, about 2g, was placed in a 15 ml polypropylene tube containing a swab stick, which was used to break up the material in 10-12 ml sterile normal saline. After mixing, the tube was allowed to stand for 30 min at room temperature (RT). 5 ml of the surface fluid was transferred to a fresh tube containing 25 ml 0.9% hexadecylpyridinium chloride (HPC) (Sigma Chemical Co., St Louis, USA) in half-strength brain heart infusion broth (Oxoid, Basingstoke, England) and allowed to stand at 37°C for 24 hours. After centrifugation at 900 x g for 30 min, the pellet was collected and resuspended in 1 ml aqueous solution of vancomycin (100µg/ml), nalidixic acid (100µg/ml) and amphotericin B (50µg/ml) (all Sigma reagents) and incubated for 72 hours at 37°C. For culture, 0.1 ml of the prepared sediment was inoculated into each culture. The radiometric medium consisted of Middlebrook 7H9 medium (BACTEC 12B; Becton Dickinson, Sparks, MD, USA) with 200 µl PANTA PLUS (Becton Dickinson), 1 ml egg yolk, 5 µg Mycobactin J (Allied Monitor Inc., Fayette, MO, USA) and 0.7 ml water. Vials were incubated at 37°C for up to 20 weeks. Growth index (GI) was determined weekly using an automatic ion chamber (BACTEC 460; Johnston Laboratories, Towson, MD, USA).

3.2 Identification of M. paratuberculosis

Identification was achieved by detection of the specific element IS*900* by polymerase chain reaction (PCR) directly from the BACTEC culture medium, with restriction endonuclease analysis (REA) of PCR product to ensure specificity. The rubber stopper-lid of the radiometric culture vial was wetted with 70% ethanol, left for 20 seconds, then dried. The vial was inverted several times to mix the contents and 200 µl of medium was removed using a sterile syringe and needle and transferred to a screw-capped 1.5 ml polypropylene microcentrifuge tube. 500 µl of absolute ethanol was added and the tube was left to stand for 2 minutes before mixing vigorously on a vortex mixer for 5 seconds and centrifuging at 8 x g for 10 minutes at 22°C. Partially flocculated egg yolk accumulated at the base and sides of the tube. The supernatant was transferred to a clean microcentrifuge tube then centrifuged at 18,000 x g for 5 minutes. The resulting pellet was washed twice in 200 µl sterile phosphate buffered saline by resuspension/centrifugation then resuspended in 50 µl sterile distilled water. The tube was placed in a dry-heating block at 100°C for 20 minutes to lyse the mycobacteria. A volume of 5 µl of the lysate was added to each PCR reaction mix. The lysate was then stored at -20°C. All work was conducted in a Class II Biosafety Cabinet using precautions for containment of radioactivity and personnel protection. If PCR was negative, residual inhibitors of PCR were removed by purifying the DNA in the extract. Lysates (45 µl) were thawed and DNA was purified from the entire lysate by binding to silica in a column using 6 M guanidine thiocyanate according to the manufacturer's instructions (Wizard PCR Preps DNA Purification System; Promega Corporation, Madison, USA) with elution of purified DNA in 50 µl of sterile distilled water. A volume of 5 µl of purified DNA solution was added to each PCR reaction mix.

Briefly, a reaction volume of 50 µl containing 5 µL of the DNA sample, 250 ng of each primer (P90 GAAGGGTGTTCGGGGCCGTCGCTTAGG and P91 GCGTTGAGGTCGATCGCCCACGTGAC)[11], 200 µM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 66.8 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1.65 mg/mL bovine serum albumin, 10 mM beta-mercaptoethanol, 2 U Taq polymerase, in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.8) was used. Amplification was undertaken in 200 µL tubes with individual lids. Only one PCR tube was opened at any time throughout the entire PCR procedure. A 96-place thermal cycler (Corbett Research, Sydney,

Australia) was used with the following conditions: one cycle of denaturation at 94°C for 3 min followed by 37 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 15 sec and extension at 72°C for 1 min. A modified hot start procedure was used for all PCRs: samples were placed in the thermal cycler after the block had reached 94°C during the initial denaturation cycle. Products of approximately 413 bp were predicted and evaluated by electrophoresis at 94V for 45 min in 2% agarose gels stained with ethidium bromide using the molecular size marker number VIII (Roche). The following controls were included with each PCR batch: positive control DNA (100 fg/µL *M. paratuberculosis* strain 316V DNA), negative control (sterile water) and a process control (PCR cocktail prepared in a clean location, without DNA sample). The process control tube was not opened until the completion of the PCR and was included to assess the integrity of the PCR reagents.

Samples that gave a positive result in PCR were subjected to REA with *Alw* I or *Mse* I. The restriction endonucleases were used as recommended by the manufacturer (New England Biolabs Incorporated). REA reactions were prepared by adding 4-12 µL of PCR product, 2 U of the appropriate restriction endonuclease, 1.6 µL of buffer (supplied with restriction endonuclease), 1.6 µL of 100 µg/mL bovine serum albumin (for *Mse* I) and made up to 16 µL with sterile purified water. Restriction digests were incubated for 2 hours at the recommended temperatures and were assessed by agarose gel electrophoresis in 2 or 3% gels. An REA result was considered to be positive when complete digestion of the PCR product to a recognised REA pattern, without non-specific bands, was achieved.

3.3 Grid sampling method for soil and faecal material

A 1 x 1 m galvanised steel 2.5 cm square mesh grid with 1600 numbered cells was used. It was placed on the ground and random numbers were used to select the cells from which samples were collected. Unless otherwise stated, two series of ten cores about 1cm in diameter and 2 cm deep, including the surface organic material and up to an additional 2 cm of overlying pasture was sampled from selected cells. When sampling specifically for pellets, random numbers were used to locate a cell, then a pellet was selected from the nearest grid cell that contained a pellet. After removing the pellet, a soil core was taken from the same grid cell (i.e. the soil beneath the pellet). Pellets and soil cores were pooled in separate containers.

3.4 Enumeration of M. paratuberculosis in faecal mixtures

A 50% end point titration method[12] was employed to estimate the number of *viable M. paratuberculosis* present in faecal mixtures used to contaminate plots, boxes and water troughs, as well as in experiments to estimate the sensitivity of culture from soil and the number of viable organisms present in environmental samples at various times after contamination. Unless otherwise stated in descriptions of specific experiments, five replicate cultures of each sample, each of 2 g, were undertaken. The 1 ml suspension in VAN obtained from each replicate was diluted in a 10 fold series from 10^{-1} to 10^{-8} in a final volume of up to 10 ml PBS or PBS and/or with 0.1% v/v Tween 80 (only after 1999). Tubes were placed on a vortex mixer for 30 seconds before preparing the next dilution. An aliquot of 0.1 ml of each dilution was used to inoculate BACTEC media. The range of dilutions used varied between experiments and was based on the estimated numbers of organisms. For example, where large numbers of organisms were expected based on the culture history of the sample, the first dilution cultured was the 10^{-2} dilution. Where very few organisms were expected the undiluted VAN suspension was cultured in addition to the dilutions prepared from it. Cultures were completed as previously described. The 50% end point titre was used to calculate the number of viable *M. paratuberculosis* per gram of starting material using the following formula: count = 50% end point titre x 1/0.1 x 1/wgt where 1/0.1 is the proportion of VAN inoculum used and wgt is the amount of faecal mixture in grams that was cultured. A variation of this method was used in Part 1 of this study and is described there.

3.5 Culture of grass samples

Grass samples were placed in a re-sealable plastic bag and weighed. 250-500 ml of saline with 0.1%v/v Tween 80 was added so that the grass was completely covered. The bag was sealed then placed in another bag. The bag was placed on a rocking platform for 1 hr at room temperature, and turned over every 15 min to ensure thorough washing of the grass. The washing water was collected and centrifuged at 11,000 x g for 20 min. The pellet was then cultured: it was added to a tube containing saline and the remainder of the procedure was identical to that used for culture of faeces.

4.0 Part 1. Sensitivity of culture of *M. paratuberculosis* from soil samples

4.1 Background

Although culture of ovine strains of *M. paratuberculosis* has been developed and applied to spiked environmental samples (soil and pasture) the sensitivity of the method is uncertain[10]. Similarly there is little or no information on the sensitivity of culture of bovine strains of this organism from environmental samples. For this reason an experiment was conducted to determine whether culture of the organism from soil was as effective as it is from faeces. Two questions were asked:
1. How far can faeces be diluted in soil before the culture technique fails to detect organisms?
2. How many organisms are present in the faeces and is the viable count reduced in the presence of soil?

4.2 Materials and methods

Bovine faeces were collected from a cow with BJD in Victoria and kindly supplied by Anne Hope (VIAS). The sample contained clumps of acid fast bacilli (AFB), visualised in a ZN stained smear. Ovine faeces consisted of a pool of samples from 10 sheep, which were thoroughly mixed. These had been stored at -80°C and were held at 4°C after mixing. Each sample was known to be culture positive; 6 contained scant numbers of acid fast bacilli (AFB) while 4 contained clumps of AFB visualised in ZN stained smears.

Viable organisms in the parent faeces were enumerated by culture of a replicated serial dilution of faeces in PBS. Five 2 g aliquots of the faecal sample were prepared by the double incubation centrifugation method. Each of the 5 final VAN pellets was serially diluted in 10ml PBS (final volume) from 10^{-1} to 10^{-9} to create five parallel serial dilution series. One BACTEC vial was inoculated with each dilution. GI occurring at any time up to and including week 12 was attributed to *M. paratuberculosis* and these cultures were graded as positive. The method of Reed and Muench[12] was used to calculate the 50% end point dilution, with the assistance of a simple computer program. The number of organisms was given by the formula count = dilution x 20/2, where 20 = the fraction of the final pellet (50 ul of 1 ml) placed into each culture vial and 2 is the amount (g) of original material cultured.

Two faecal samples were examined in two types of soil, giving four treatments: bovine faeces in moderate organic soil, bovine faeces in high organic soil, ovine faeces in moderate organic soil and ovine faeces in high organic soil. The moderate organic soil was a dry dusty dirt collected from an exposed area in western Sydney (2-10% organic matter; Ca 19 meq/100g; water pH 6.4; Na 0.49 meq/100g). The high organic soil was a rich dark soil collected from a domestic vegetable garden in western Sydney (5-10% organic matter; Ca 22 meq/100g; water pH 7.1; Na 2.5 meq/100g). For each of the treatments, 10 g of faeces was diluted in 90g of soil by mixing in a commercial blender (Waring) for 6 x 5 sec bursts, shaking the vessel between bursts, to produce 100g of a 10^{-1} dilution of faeces in soil. Ten grams of this mixture was then serially diluted in soil to yield separate samples representing 10^{-1} to 10^{-8} dilutions of the original faeces in soil. A 2 g sample was taken from each dilution and prepared by the double incubation centrifugation method. Aliquots of the final VAN pellet of each were inoculated into 5 x BACTEC bottles. Results were reported as the reciprocal of the highest dilution

yielding evidence of growth. In addition an estimate of viable count was made for the parent faeces using the 50% end point titre method[12].

4.3 Results

End point titration counts of the bovine faecal sample cultured in BACTEC medium suggested that there were approximately 800 viable organisms per gram of faeces. When mixed with low organic soil the apparent number of viable organisms was lower, approximately 50 per gram, and a count was not achieved in high organic soil. Viable organisms were not detectable in soil at dilutions higher than 1:10 for low organic soil and < 1:10 for high organic soil.

End point titration counts of the ovine faecal sample suggested that there were approximately 2.2 x 10^5 viable organisms per gram of faeces. This estimate approximates the number that would have been expected based on the appearance of ZN stained smears of the original faecal samples. When mixed with low organic soil the apparent number of viable organisms was lower (2.2 x 10^3 per gram), and lower still when diluted in high organic soil (0.5 x 10^3 per gram). Viable organisms were not detectable in soil at dilutions higher than 1:1000 for low organic soil and 1:10 to 1:100 for high organic soil.

Several of the replicate cultures were confirmed culture positive after the routine 8 week incubation, and the duration of incubation was extended to 12 weeks for this reason. One additional BACTEC culture vial was detected as positive by extending incubation to week 20.

Table 1. Highest cultivable dilution and viable counts of *M. paratuberculosis* in soil

Parameter	Highest cultivable dilution of faeces mixed in soil	Viable count/g in parent faeces after mixing in soil	Viable count/g in parent faeces mixed in PBS
Bovine faeces			0.8 x 10^3
Bovine faeces in low organic soil	10^{-1}	0.5 x 10^2	
Bovine faeces in high organic soil	<10^{-1}	not possible	
Ovine faeces			2.2 x 10^5
Ovine faeces in low organic soil	10^{-3}	2.2 x 10^3	
Ovine faeces in high organic soil	10^{-1} to 10^{-3}	0.5 x 10^3	

4.4 Discussion and conclusions

There was a reduction in the apparent viable count of *M. paratuberculosis* when faeces was mixed with soil. The reduction was of the order of 2 log 10 dilutions (Table 1). In other words a significant percentage (up to 99%) of viable organisms present in faeces were not detectable when faeces was mixed with soil. The source of the loss was not determined but theoretically could include: binding to soil particulates, attachment to the surfaces of containers, destruction during the disinfection process and failure to grow in culture media. However, as the same disinfection protocol and media were used for both faeces and soils the latter considerations do not apply in this experiment. These data confirm that the analytical sensitivity of the culture method is reduced when bovine or ovine faeces are mixed with soil. The data indicate that samples containing very low numbers of viable organisms require greater than 8 weeks incubation to yield significant growth in BACTEC medium, and that 12 weeks is sufficient to detect most samples. It was resolved to incubate BACTEC cultures of soil for 20 weeks in later experiments to maximise the chances of isolating the organism.

Irreversible binding of organisms in the *Mycobacterium avium* complex to soil particles was noted in a previous study and the extent of this was related to the method of preparation of soil for culture, including the nature and concentration of disinfectant and length of time in disinfectant[13]. However, the magnitude of difference between methods was small (less than 1 log). Overall, less than 10% of the organisms present in soil were recovered regardless of the method. Organisms were also lost by binding to laboratory equipment during processing. Correction factors ranging from 24 to 1100 were proposed to convert observed counts to estimated actual counts.

For these reasons any estimates of viable counts or duration of survival of *M. paratuberculosis* in soil are underestimates.

5.0 Part 2. On-farm studies of the survival of the organism

5.1 Aims

To determine the survival of *M. paratuberculosis* under a representative range of field conditions.

To study survival of the organism on farms with Johne's disease to validate information from plot trials and to increase the credibility of recommendations.
(Project scope - from final project draft document dated 26.9.97)

5.2 Methods

5.2.1 Overview

Five ovine Johne's disease affected properties in the Central Tablelands district of NSW that were judged to be heavily contaminated based on evidence of a high prevalence of infection in sheep were selected. Potentially contaminated areas were assessed on a field visit. Twenty sites representing a range of inclination and orientation, shade and moisture were selected for culture. Each site was identified with a conspicuous survey peg and its location described on a map and by reference to landmarks. Pasture sites needed to have evidence of sheep having grazed the area as assessed by faecal contamination. Drainage areas and water bodies did not need to have this sign. A randomised grid sampling method was used to obtain a pooled soil sample from each site. Dung pats and faecal pellets were also sampled. All samples were cultured in BACTEC medium. Culture positive sites were then fenced to exclude stock and a second series of samples was collected for culture at this time, about 5 months after initial sample collection. A third sample was collected about 12 months after the initial sampling.

5.2.2 Methods in detail

Preliminary property visits were undertaken to establish eligibility and owner cooperation. An initial visit was undertaken to properties 1 to 4 on 17-18.11.97 and property 5 on 26.8.97 to conducting a property survey and the first round of sampling. The sampling strategy for each farm was designed to cover a wide range of micro environments on pastures that had been recently grazed (last 7 days) or that were currently being grazed by a mob of known-infected sheep. Factors considered desirable for sampling included: aspect (north vs. south), elevation (high vs low), exposure (sun vs shade) and contamination rate (camp vs grazing area); dam sediment and drainage gullies were also considered important sites.

For sampling, the grid sampling method was used. The grid was placed on the ground with the top left corner (cell 1) adjacent to a white marker peg labelled with the site number, with orientation to magnetic north. Soil cores approximately 2 cm deep were taken from 10 cells according to a list of random numbers generated for each sampling site, pooled into a sterile 50 ml polycarbonate jar labelled with site number, and placed in an esky at ambient temperature. Two sets of 10 cores were taken from each site. A separate 10 ml syringe barrel was used to collect each series of cores from each site. Soil cores included aerial parts of plants, surface litter, soil and plant roots. Basic observations were recorded for each site.

Single samples of dam/trough water (500 ml), dam sediment (up to 50 g), faeces (up to 50 g), and soil/pasture (up to 50 g single site or pooled multiple site) were also collected from each farm.

Sites found to be culture positive on properties 1 to 4 were fenced off (where possible) and resampled about 5 months (April 1998) and again at about 12 months (November - December 1998) after initial sampling. There were also additional samples taken from culture positive sites on property 5 (April

1998, May 1998, November 1998). An area near a dam (site 24) on property 5 was sampled on 01.06.98 together with a new site 24b at the opposite side of the dam. On 03.11.98 sites 24a and 24b were submerged in the dam however, samples were collected using the grid technique from new sites labelled 31 - 37 which were adjacent to the dam.

Pooled samples were mixed thoroughly using a mortar and pestle or electric blender; plant matter was cut with scissors. Water samples were centrifuged at 10,000 g for 20 min and the pellet was collected for culture.

5.3 Results

5.3.1 Primary collections

These were the first collections aimed at identifying infected sites from 5 properties in the central tablelands district of New South Wales. A list of sites and descriptions is provided in Appendix I. Each property was judged to have a moderate to high prevalence of ovine Johne's disease in sheep or sheep/goat flocks. Except for a section of property 5 which was destocked of small ruminants in November 1996, at the time of first sampling in September or November 1997 each property was currently grazed by stock with ovine Johne's disease. The sites were extremely diverse with respect to aspect, pasture composition, moisture content, degree of exposure and degree of faecal contamination.

A total of 148 primary environmental samples were collected. These comprised 106 soil/pasture samples, 5 water samples from dams or troughs, 23 soil/sediment samples from creeks, gully areas or dams, 2 soil/faeces samples from sheep yards, 5 soil/faeces samples from beneath shearing sheds, 2 cattle faeces, 2 rabbit faeces, 2 sheep faeces (one a pool of 50 from a sheep camp) and 1 duck faeces from near a dam.

Of the primary samples collected, 27 were culture positive. If the pool of sheep faeces is excluded, 26 (17.6%) environmental samples were culture positive. Details for the culture positive sites are provided in Tables 2.1 to 2.5. Of the culture positive sites, 22 were soil/pasture, 3 were sediments from areas adjacent to dams, 1 was a sediment from within a dam while one was a pooled faecal sample collected from a sheep camp. Of the 22 soil/pasture samples, 12 were from sites where faeces from sheep were visible on the ground, and several of these sites were heavily contaminated so that faeces were present in the samples. However, many samples were collected without inclusion of faecal pellets. A range of microenvironments were represented by the culture positive soil/pasture samples, including sheep camps, exposed dry areas, hillsides, lightly shaded areas and gully areas. The samples from sheep yards and from beneath shearing sheds were culture negative. Apart from sediment collection areas near dams and watercourses, there did not appear to be any particular feature that could be used to predict infection status of sites.

For many sites there appeared to be very low numbers of organisms present because development of a growth index was slow, e.g. 12 weeks to reach GI >999. However, there was rapid growth (3-4 weeks to peak GI) indicating relatively large numbers of organisms from some samples from low-lying areas.

5.3.2 Secondary collections

Approximately five months after the primary collections, sites that were culture positive were fenced to exclude stock and samples were collected again for culture. A third collection was undertaken approximately 12 months after the primary collection.

With the exception of a single soil/pasture site (Site 12 on property 5), none of the original culture positive sites were culture positive on a second occasion. Site 12 on property 5 was culture positive on two occasions 8 months apart. The second occasion was approximately 4 months after fencing the site to exclude stock, however, the site was on quite a steep slope and contamination by water run-off or movement of sheep pellets from adjacent pasture could not be excluded.

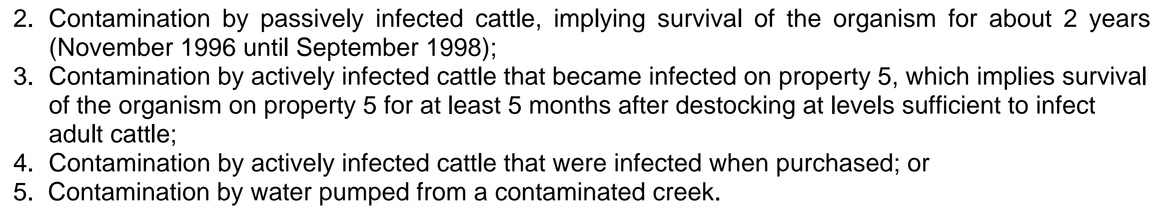
Additional sampling was undertaken on the portion of property 5 that had been destocked for 10 months including one full summer prior to the primary collection, i.e. since November 1996. The culture positive site (site 24) was a low-lying drainage area leading to a dam. The isolate was typed as a sheep strain by IS*1311* PCR-REA. Run-off from the infected part of the farm to this site is not possible because of topography however, site 24 collects the drainage from at least 30 Ha and is at the base of quite a steep hillside so that movement of faecal material onto the site is quite possible during heavy rain. These results prompted more intensive evaluation of the area adjacent to the dam. A site near site 24 (24b) was culture positive in June 1998, about l9 months after destocking sheep and goats. A further 7 sediment samples, a duck faeces and a rabbit faeces collected from this site in November 1998 were culture negative (Table 10).

The apparent prolonged survival of *M. paratuberculosis* at site 24 stimulated an investigation of the Johne's disease status of cattle on property 5. Testing of the entire cattle herd of 135 head by ELISA and individual faecal culture was undertaken in September 1998 (ON98/3873, CM98/1254). There were no serological reactors however, a sheep strain of *M. paratuberculosis* was isolated from the faeces of two cattle with inconclusive culture results from a further 3 head. Blood and faecal samples were collected from these 5 individuals in February 1999 (ON99/0585, CM99/0140). None of the animals reacted in the bovine ELISA and faecal cultures were negative on this second occasion. A trace on the source of the two faecal culture positive cattle was undertaken. Both had been purchased from the Goulburn sales in April 1997 at 8-10 months of age. They originated from two properties in the Goulburn area, one of which adjoins two flocks known to be infected with ovine Johne's disease while the other has no known ovine Johne's disease in the vicinity. Neither property has undergone testing for ovine Johne's disease. The source of the organism in the cattle is uncertain, but could include one of the properties of origin in the Goulburn area, i.e. the cattle were actively infected when they arrived on property 5. Alternatively the cattle could have become infected on property 5, or they may merely have ingested the organism and passively transferred it to site 24. The two culture positive cattle had never grazed on the non-destocked part of property 5, so if infection was acquired actively or passively on property 5 it must be inferred that the organism had survived on pasture between the time of destocking in November 1996 and the time of arrival of the cattle in April 1997, i.e. 5 months. If the dogma that adult cattle are resistant to infection is correct then it would seem more likely that the cattle were infected at source in Goulburn rather than on property 5, but this does not rule out passive excretion of organisms acquired on property 5. It was not possible to examine these cattle at post mortem.

Further advice was provided by the owner of property 5 concerning the area at site 24. The dam was filled by pumping water from an adjacent creek in Summer-Autumn 1998. An ovine Johne's disease affected property is located several kilometres upstream.

Thus the apparent prolonged survival of *M. paratuberculosis* at site 24 could be explained in a number of ways:

1. Survival of the organism at the site since November 1996 when the area was destocked;



2. Contamination by passively infected cattle, implying survival of the organism for about 2 years (November 1996 until September 1998);
3. Contamination by actively infected cattle that became infected on property 5, which implies survival of the organism on property 5 for at least 5 months after destocking at levels sufficient to infect adult cattle;
4. Contamination by actively infected cattle that were infected when purchased; or
5. Contamination by water pumped from a contaminated creek.

5.4 Discussion and conclusions

Approximately 20% of environmental samples collected from 5 properties with ovine Johne's disease were culture positive for *M. paratuberculosis*. The culture positive sites ranged from sheltered sheep camps with heavy faecal contamination and soil/pasture sites on exposed ridges and hillsides through to sediments in low lying water courses/dams. Collectively the results suggested that the organism is likely to be found wherever sheep are present and wherever faecal contamination occurs or can be focussed, for example by gradient and run-off.

Survival of the organism on sites that were culture positive at the first sampling was rarely found at a second sampling about 5 months later and none of these sites were culture positive after 12 months.

Apparent prolonged survival in a low-lying drainage area on one property may have been associated with genuine prolonged survival, contamination from several cattle that were shown to be intermittently excreting a sheep strain of *M. paratuberculosis* or with contamination from a waterway draining an infected property upstream of property 5.

The results do not enable predictions to be made regarding where the organism might be found other than to speculate that contaminated sites are those where faecal contamination has occurred or where faecal material may be focussed by topography. Several low-lying areas tended to have larger numbers of organisms than other areas, evidenced by more rapid growth of the organism. A more intensive survey would be required to confirm these observations.

Although sheep yards and the substrate below shearing sheds were not culture positive, insufficient sites were examined on too few occasions to be confident that such sites do not pose a risk. However, the presence of urine reduces the viability of *M. paratuberculosis* (Table 1) so sheep camps and shed/yard environments may not be favourable environments despite high levels of faecal contamination. The risks posed by water draining from affected properties remain uncertain.

The sampling interval used in this study corresponded to a full summer and part autumn. The results suggest, as far as can be assessed microbiologically, that a 5 month period of decontamination provides a strong likelihood that pasture grazing sites will be free of infection. This probably would not apply to sheep camps, which were not specifically investigated, nor to low-lying drainage areas, where the starting levels of contamination could be higher.

The results on property 5, where there were several possible sources of reintroduction of infection (water, cattle) highlight the need for stringent assessment of risk when planning property disease eradication programs.

Caution is needed in application of these results because of the insensitive nature of culture (see Part 1 this report) and the relatively low intensity of sampling used in the study - i.e. only a small sample of the micro-environments present on these farms was sampled.

Table 2.1. Culture positive site descriptions and sample identification for Property 1 (Vittoria, NSW). Results are weeks to GI >999 for PCR-REA +ve samples

Site No.	Slope °	Aspect of slope °	Pasture	Site description		Sampling date		
						17.11.97	16.04.98	08.12.98
2	0			Further along ridge beneath gum tree on a sheep route. Dry soil.	Occasional piles of pellets.	12 (GI 74)	-, -	-, -
14			Long grass	Rim of dam, 10 m s.e. of dam. Soil very dry.	Fresh faeces within the site	14	-, -	-, -
17				Exposed area on hill top. Soil very dry. Pellet in the sample.	Present	10	-, -	-, -
Y1				Sediment from dam in paddock 27		3	-, -	-, -

Table 2.2. Culture positive site descriptions and sample identification for Property 2 (Borenore, NSW). Results are Weeks to GI >999 for PCR +ve samples

Site No.	Slope °	Aspect of slope °	Pasture	Site description	Sheep faeces	Sampling date		
						17.11.97	16.04.98	08.12.98
5	0			Open area near fence		12 (GI 881)	n.s.	-, -
6				On a small soak with green pick. Otherwise similar to 5		10	-, -	-, -
7				Within a small ditch near site 6		4	n.s.	n.s.
8				Site shaded in late afternoon by small gum tree		7	n.s	n.s.
10				Damp corner		14 (GI 766)	n.s.	-, -
17				1 m south of fence near road. Open area. Very dry. No pellets in sample	A few fresh pellets present	4	n.s.	-, -

n.s. - not sampled as site marker pegs had been removed or lost.

Table 2.3. Culture positive sites for Property 3 (Rockley, NSW). Results are weeks to GI >999 for PCR +ve samples

Site No.	Slope °	Aspect of slope °	Pasture	Site description	Sheep faeces	Sampling date		
						18.11.97	16.04.98	27.11.98
3	7.5	100		further down gully from site 2	scattered pellets	14 (GI 391)	-, -	-, -
5	11	100		near sheep camp. Faeces in sample	scattered faeces	5	-, -	-, -
8	0		Grass and herbs	within drainage gully to dam, at edge of dam. A moist area near sedges.		5	-, -	-, -
11	slight			Open area 1 m from old tree stump. Compacted soil (vehicle track?)	Nil	14 (GI 597)	-, -	-, -
12			Grass	Open area 2 m south of large tree. Very dry. Faeces in the sample.	Fresh and dry faeces	4	-, -	-, -
19			Grass	Open, exposed dry area 3 m east of fence. Very hard dry soil.	Nil	12 (754)	-, -	-, -
20				Dry open area, unshaded, 0.5 m east of gate, 1 m north of fence.	Nil	14	-, -	-, -

Table 2.4. Culture positive sites for Property 4. (Limekilns, NSW). Culture results are weeks to GI >999 for PCR-REA +ve samples

Site No.	Slope °	Aspect of slope °	Pasture	Site description	Sheep faeces	Sampling		
						18.11.97	16.04.98	27.11.98
4	5	east	nil	Sheep camp in light timber, shaded. Faeces in sample	Extensive build up old and fresh faeces	14 (GI 578)	-, -	-, -
8	7		scant	open hillside in full sun	Scattered dry	6	-, -	-, -
12	0			As for 11, 2.5 m south west of large tree, partially shaded	Dry pellets	7	-, -	-, -
13			grass	open, exposed area in a slight gully that might collect water, 1 m south of fence (cattle nearby)	Nil	7	-, -	-, -
18			patchy grass	adjacent deep gully in open dry area with tree 20 m west, 5 m south of fence. Very dry soil	Nil	12 (GI 256)	-, -	-, -

Table 2.5. Culture positive site descriptions from the primary collection and subsequent collection details for Property 5 (Carcoar, NSW). Culture results are weeks to GI >999 for PCR-REA +ve samples

Site No.	Slope °	Aspect of slope °	Pasture	Site description	Sheep faeces	Date of sampling				
						01.09.97	06.04.98	05.05.98	01.06.98	03.11.98
12	12	80	Very short green (bowling green)	20 m uphill of site 10; 1 m downhill of old sheep carcase	Pellets everywhere	8 (GI 350)	-,-	-, 9 (871)	n.s.	-,-
16	0		ditto	Sheep camp; on south west side of tree. Peg 156° 6 m from vertical part of trunk	very heavy pellets	7 (GI 745)	-, -	-, -	n.s.	-, -
24	5	45	Short green pick	Pool of 10 soil cores from south western inlet area to dam, collected linear fashion rather than from a grid	nil - destocked area	4	-, -	-, -	n.s.	n.s.
24B				Opposite end of dam in another inlet area, sampled on 01.06.98 only					-, 5	n.s.
29				50 faecal pellets from sheep camp collected 30.08.97.	pooled faeces	5 (GI 371)				
30				Adjacent to dam at site 24	nil	n.s.	n.s.	n.s.	n.s.	-, -
31				Adjacent to dam at site 24	nil	n.s.	n.s.	n.s.	n.s.	-, -
32				Adjacent to dam at site 24	nil	n.s.	n.s.	n.s.	n.s.	-, -
33				Adjacent to dam at site 24 - duck faeces	nil	n.s.	n.s.	n.s.	n.s.	-, -
34				Adjacent to dam at site 24	nil	n.s.	n.s.	n.s.	n.s.	-, -
35				Adjacent to dam at site 24	nil	n.s.	n.s.	n.s.	n.s.	-, -
36				Adjacent to dam at site 24	nil	n.s.	n.s.	n.s.	n.s.	-, -
37				Adjacent to dam at site 24	nil	n.s.	n.s.	n.s.	n.s.	-, -
38				Adjacent to sdam at site 24 - rabbit faeces	nil	n.s.	n.s.	n.s.	n.s.	-, -

n.s. not sampled

6.0 Part 3. Survival of the organism in pasture plots and boxes

6.1 Background

During the planning stages of this project it was decided that survival needed to be studied in the OJD endemic region, preferably on infected farms, using a sheep strain of *M. paratuberculosis* and in such a manner that the experimental design resembled a real world situation. This would complement the artificial environment room study being undertaken at the Victorian Institute of Animal Science (TR.055) with the cattle strain. Consequently experimental sites were found near Orange, a simple plot and box design was implemented, contamination was done using faeces from infected sheep at levels representative of those seen in the field, and basic observations were made. The results taken at face value are fairly easily understood, but require close consideration.

The primary reason for undertaking the experiments was to enable rational decontamination intervals to be prescribed for eradication of OJD. Since the project began there has been evolution in thinking about OJD control in Australia and it is now clear that the information will be of general benefit in developing strategies for farm management so that the risks and consequences of infection are reduced on properties that, for whatever reason, will "live with the disease". Complementary strategies of pasture management, vaccination and other aspects of flock management may be effective in reducing losses due to OJD on affected farms.

6.1.1 Assessing the duration of survival

There are several ways of looking at the results of experiments to determine the survival of *M. paratuberculosis*. There are advantages and disadvantages with each. In summary:

1. Observed duration of survival. This is the time elapsed between contamination of a plot or box and the collection of the sample with the last positive culture result. This is a simple observation and the one most readily made from the experiments in this project but it ignores the starting level of contamination. The greater the level of contamination at the start the longer the duration of survival that would be expected. This is because bacterial populations generally decline at a more or less steady rate. The observed duration of survival is also dependent on the sensitivity of the culture method. As described in Part 1 this is imperfect, especially for cultures from soil. This will cause underestimation of the duration of survival.

2. Decay rate. The decimal decay rate or D-value is a term used to describe the decline in bacterial viability as a result of exposure to heat (specified temperature and time, for example pasteurisation) or other factors. A D-value of 3 minutes means that a 1 $\log_{10}$ reduction in the bacterial population occurs after each 3 minutes of treatment. This is the same as a 90% reduction in the bacterial population. Knowledge of the decay rate is potentially more useful than simple observations of survival because it can be extrapolated to deal with situations in which the level of bacterial contamination varies. Another advantage is that its measurement is not affected greatly by the sensitivity of the culture method. However, measurement of the decay rate is difficult, very expensive and sometimes very hard to standardise, even for conceptually simple processes such as pasteurisation of milk. The concept of D-value can be used to describe declines in bacterial populations due to any cause or combinations of causes.

In this project both these approaches were used to obtain an impression of the survival potential of the organism in the region of NSW where OJD is most prevalent.

6.1.2 Assessing factors that affect survival in the environment

Having assessed the duration of survival and decay rate, it is useful to understand what might affect these values. Many factors may influence the survival of bacteria in the environment. These include temperature, moisture levels, incident radiation, particularly ultra-violet radiation but also visible light, presence of organic matter, competition from other microbes, stimulation from other microbes, pH and oxygen tension. Some of these factors can be measured and were monitored throughout the experiments in this study. Some were specifically included as treatment effects in the experiments (incident radiation, moisture levels, soil pH alteration). Another important consideration is location of the organism - is survival similar in faecal pellets and in soil?

6.2 Materials and methods

6.2.1 Collection of OJD affected sheep for provision of infected faeces

Sheep were selected on three occasions (January 1998, October 1998 and September 1999) from a heavily infected property at Goulburn in the Southern Highlands of NSW. At each visit faecal samples and blood samples were taken from mature wethers from infected mobs. The animals were individually identified. ZN-stained smears were prepared from faeces and AGID tests were undertaken. Animals with acid fast bacilli in faeces and or positive results in the AGID were purchased off shears, transported to EMAI, Camden, housed in a biosecure animal house and fed lucerne pellets, lucerne hay, chaff and oats. Canvass faecal collection bags were attached to the sheep to allow bulk collection of faeces (Figure 3.1). The faeces from each animal from each day were collected into plastic bags and held at 4°C. Although an occasional sheep lost its bag overnight, clean samples of approximately 300 - 1000g faeces per sheep per day were collected. The amounts of faeces collected and the OJD status of animals is provided in Appendix II. Additional sheep selected in January 2000 were unsuitable for the trial, necessitating use for the January 2000 contamination of faeces that had been stored at -80°C since November 1999. As will be shown, there was no detectable loss of viability.

6.2.2 Preparation of bulk faecal mixtures for contamination of plots and boxes

January 1998

Faeces from sheep 2, 7, 9, 25 and 27 that were collected between 13.01.98 and 27.01.98 inclusive were pooled on 27.1.98 and mixed in a cement mixer. Faeces from animal 2, which was of soft formed consistency rather than pelleted, was first mixed with chaff at a rate of 4L chaff per kg faeces to obtain discrete masses of faeces. 12.4 kg of the mixed faeces was removed from the total pool and 50L of chaff was added and mixed thoroughly in the cement mixer. The resulting mixture was divided into 12 equal portions of 1.5 kg and stored in sealed plastic bags at 4°C (labelled - 'pellet mixture').

500 ml of milli Q purified water was added per kg of the remaining mixture of faeces and mixed until a crumbling texture was obtained. 2.5L of chaff per kg faeces was then added and mixed until a dry, flaky, free-falling mixture was obtained; this still contained some faecal pellets, but the majority had been broken down. The mixture was divided into 30 equal portions of 3.2 kg and stored in sealed plastic bags at 4°C (labelled - 'slurry mixture').

20 g of pellet mixture and 20 g of slurry mixture were retained for control cultures and enumeration of *M. paratuberculosis*.

November 1998

Faeces from animals 247 and 253 that were collected between 20.10.98 and 2.11.98 inclusive were pooled on 2.11.98. The total weight of faeces was 12.6 kg. 10L chaff was added and the material was mixed thoroughly in a cement mixer so that pellets from individual sheep and individual days were completely dispersed; pellets were not broken-up in the process. 3 kg of the mixture was removed for contamination of pasture boxes at EMAI. Approximately 50 g of the pellet mixture was placed in a sterile jar and stored at -80°C for control cultures and enumeration of *M. paratuberculosis*. The remainder was divided into 6 aliquots of 2 kg.

For both of the above contaminations the pre-prepared faecal mixes were placed on wet ice and transported the same day to the experimental sites at Orange.

November 1999 and January 2000

Six sheep had variable numbers of acid fast bacilli in their faeces on one or more occasions. For the November contamination, the daily faeces from sheep 3604, 3607, 3609, 3611, 3612 and 3613 from 26.10.99 to 2.11.99 (8 consecutive days) were pooled and placed at 4°C. On 02.11.99 the bulk faeces from sheep with a soft or soft-formed stool in which individual pellets were not present was first rendered a semi-dry and semi-pelleted mixture by blending by hand with lucerne chaff and water in large stainless steel bins. The mixture was then blended by hand and with a shovel with pelleted faeces from the other sheep to form an homogenous sample. The mixture consisted of faeces 42.0 Kg, chaff 6.0 Kg and water 2.0 Kg (84% faeces by weight). A sample of 100 g was retained at -80°C for enumeration of *M. paratuberculosis*. The remainder was dispensed by weight into plastic bags for contamination of plots, boxes and water troughs (see Part 4). The faecal mixture was placed at 4°C and kept chilled during transport.

For the January 2000 contamination faecal material was collected from the above sheep between 9.11.99 and 19.11.99 (7 non-consecutive days) and placed at -80°C each day after collection. The faeces were removed from storage at -80°C and placed at 4°C on 21.01.2000. The thawed faeces were blended with lucerne chaff as described above on 24.01.2000. The mixture consisted of faeces 31.4 kg and chaff 3.5 kg (90% faeces by weight). A sample of 100 g was retained at -80°C for enumeration of *M. paratuberculosis*. The mixture was placed at 4°C and transported on wet ice.

6.2.3 Plan of field sites, plots and treatments

There were two experimental field sites, one at Borenore and the other at Carcoar in the Central Tablelands district of NSW within the OJD endemic area. Both sites were on OJD affected properties. A shade house was constructed at each site. Each was fully-covered with 70% shade cloth and measured approximately 10 x 6 x 2.4 m (l x b x h). Each was surrounded by a secure perimeter fence and one had an earth mound to prevent surface run-off (Figures 3.1 - 3.3). Micro-irrigation sprayers were installed to provide water to selected plots. Irrigation was conducted each night for 15 mins to provide constant moist soil conditions. Prior to application of faecal material, fine agricultural lime was applied to 'low lime' and 'high lime' treatment plots at rates of 50 g/m^2 and 250 g/m^2 (0.5 and 2.5 tonnes/Ha) respectively.

Seven treatment plots were marked out on each site for contamination in January 1998 and an additional plot at each site was later marked out for the contamination in November 1998. (Table 3.1). Treatment plots consisted of three adjacent replicates each 1.5 x 1.5 m and were separated from adjacent plots by walkways 0.5 m wide. Each plot was marked out and numbered clearly with pegs. Replicate 'A' was closest to a marker post, 'B' was in the middle while 'C' was most distant from a marker post. Four additional plots at the Borenore site were marked out for contamination in November 1999 and January 2000. There were 4 replicates labelled A, B, C and D in each plot, each 1.1 m x 1.1 m. These new plots (9, 10, 11, 12) did not overlap earlier plots to which lime had been applied and in which pH had increased above background levels.

Table 3.1. Description of treatments for experimental plots at Carcoar and Borenore

Plot	70% Shade	Water	Lime	Vegetation cut and removed to simulate grazing	Contaminated with	Contamination date
1	No	No	No	No	slurry mix	Jan 1998
2	Yes	No	No	No	slurry mix	Jan 1998
3	Yes	Yes	No	No	slurry mix	Jan 1998
4	Yes	Yes	Low	No	slurry mix	Jan 1998
5	Yes	Yes	High	No	slurry mix	Jan 1998
6	Yes	Yes	No	No	pellet mix	Jan 1998
7	No	No	No	No	pellet mix	Jan 1998
8	No	No	No	Yes	pellet mix	Nov 1998
9*	Yes	No	No	Yes	pellet mix	Nov 1999
10*	No	No	No	Yes	pellet mix	Nov 1999
11*	Yes	No	No	Yes	pellet mix	Jan 2000
12*	No	No	No	Yes	pellet mix	Jan 2000

* Borenore site only

The existing pasture at each site was allowed to grow from the first contamination in January 1998 until the second in November 1998 but was carefully mown and vegetation removed thereafter to simulate grazing. Mowing was undertaken immediately before the contaminations in November 1998, November 1999 and January 2000.

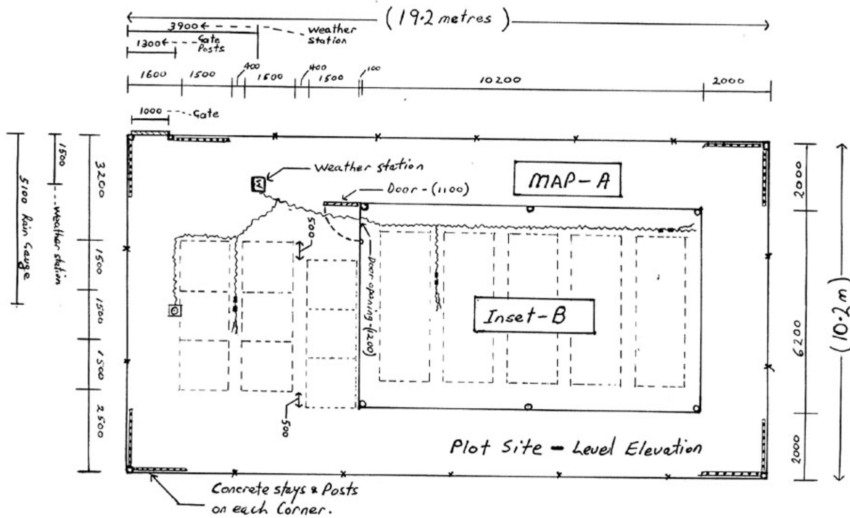
An experimental site was constructed at EMAI, Camden for the November 1999 and January 2000 contaminations (Figure 3.4). Pasture boxes were placed on trestle tables in an unshaded exposed location with northerly aspect. Boxes were placed also on a concrete verandah with northerly aspect. It was covered by a high tin roof. The sides and front of the verandah were fully enclosed in 70% shade-cloth. A third set of boxes was placed in a breeze-way between two buildings, fully shaded, with southerly aspect and designated as 100% shade. The eastern side of the breezeway was covered by 70% shade cloth. The entire area was surrounded by a animal-proof fence and human access was strictly controlled.

Automatic weather data loggers (Easydata Mk4, Environdata Australia Pty. Ltd., Warwick, QLD) were installed at each site to record dry bulb air temperature, soil temperature at 1 cm depth, soil moisture by gypsum block at 1 cm depth, relative humidity, UV radiation (290-400 nm), solar radiation (500-1000 nm with correction to encompass 400-3000 nm), rainfall and wind speed. Daily maximum, minimum and average dry bulb air temperature, relative humidity, soil temperature, soil moisture, and total daily rainfall, solar radiation, UV radiation and evaporation were recorded or were derived from these measurements. From November 1999 the weather logger channels at Borenore were reallocated to record soil temperature inside the shade house and outside the shade house in both boxes and plots and soil moisture probes were removed as they were unreliable.

The weather data logger at Carcoar was relocated to EMAI in October 1999 to record standard parameters as well as soil temperature in a box and the water trough inside the 70% shade enclosure (see Part 4), in the trough on the verandah and in a box in the 0% shade treatment. A separate data logger was used to record soil temperature in a box in the 100% shade treatment.

Figure 3.1.
1. Borenore site ; 2. Irrigated plots within shade house; 3. External plots marked out prior to contamination; 4. Means of collection of bulk faeces from donor sheep

Figure 3.2. Plan of experimental plot site at Carcoar



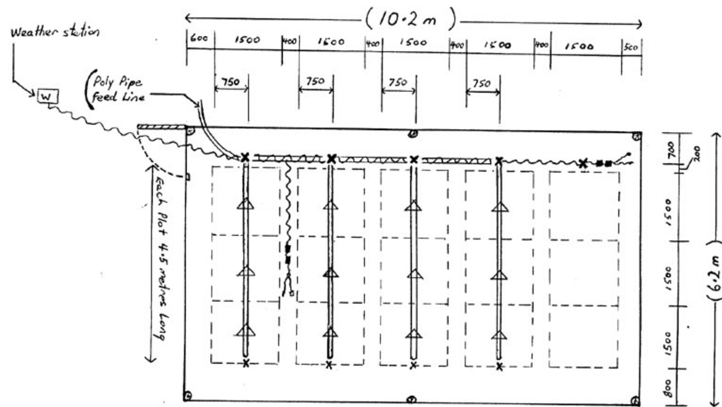
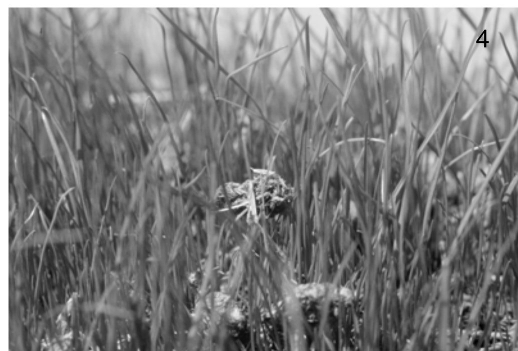


Figure 3.3. Plan of experimental plot site at Borenore

Figure 3.4.
1. **Grass growth in pasture boxes contaminated in November 1998;**
2. **Camden site, pasture boxes in unshaded location inside perimeter fence in early November 1999. Note the lids on the boxes to be contaminated in January 2000;**
3. **Camden site, pasture boxes 70% shade in February 2000 with browned-off grass in November boxes (front) and standing green grass in January boxes (back);**
4. **Camden site in unshaded location - note faecal material elevated from soil surface by vigorous growth of grass**



6.2.4Contamination of pasture plots

January 1998

Plots were contaminated on 28.01.98. Plots 6 and 7 were contaminated with pellet mix at a rate of 1.033 Kg per 1.5 m x 1.5 m plot replicate, which equates to 0.046 g per cm^2. This is equivalent to a pellet every few square centimetres which is how the ground generally looks on moderately to heavily stocked sheep farms in the Central Tablelands district of NSW. The remaining plots (plots 1-5) were contaminated with slurry mixture at a rate of 1.505 Kg per 1.5 m x 1.5 m plot replicate, which equates to 0.067 g per cm^2. Again this represents a meaningful level of faecal contamination in the context of stocking rates in the endemic OJD area. The faecal mixture was scattered carefully across each replicate plot by hand using a side to side motion. There was sufficient slurry material to completely cover each replicate plot, and remaining faecal pellets in this mixture were partially broken down by hand as it was spread so that there were few intact pellets visible on these plots. For plots contaminated with pellets the distribution of material was done as evenly as possible.

November 1998

Each plot was mown on 02.11.98 and the vegetative matter removed. The Borenore site (plot 8) was contaminated on 02.11.98. The remaining aliquots of faeces were held overnight at 4°C and the Carcoar site (plot 8) was contaminated on 03.11.98. The faecal pellet mixture (2 Kg) was scattered carefully across each 1.5 x 1.5 m replicate plot by hand using a side to side motion.

November 1999 and January 2000

Samples of 2 kg of faecal mixture were transported to Orange by car and applied evenly to each 1.1 m x 1.1 m plot at Borenore on 03.11.1999. For the January contamination, faecal mixture was transported to Orange by car on 24.01.2000 and placed at 4°C overnight. On 25.01.2000 samples of 2 kg were applied evenly to each 1.1 m x 1.1 m January plot.

6.2.5 Collection and handling of samples from pasture plots

Unless otherwise stated, samples of faecal material and underlying soil were collected using the grid sampling method described in the general materials and methods. As each pellet was removed the underlying soil core (2cm deep) was collected into a separate jar. Soil cores from plots contaminated with slurry included the slurry material on the surface of the soil. Post-contamination time zero samples were collected to confirm successful contamination of all plots and boxes.

January 1998

Just before contaminating the plots, two series of 10 cores were collected from each replicate in plots 6 and 7 as negative controls for soil inside and outside the shade house. Immediately after contamination, two series of 10 cores from each replicate in plots 1-5 were collected as a time zero positive control for plots contaminated with slurry. In addition, two series of 10 faecal pellets were collected from each replicate in plots 6 and 7 as a time zero positive control. Samples were then collected from each replicate plot at monthly intervals: plots1 - 5, soil cores, 2 series of 10; plots 6 - 7 soil cores, 2 series of 10 and 2 series of 10 pellets. Collection of pellets from plots 6 - 7 was discontinued when pellets were no longer recognisable on the pasture, but collection of soil cores continued.

Deep soil cores were collected from selected plots between 10 to 17.11.98. Metal sampling corers were used to obtain 2 pools of 10 cores from each replicate of plots 1, 7, 2, 3 and 6. The core was laid out on a board and the top 2 cm of soil was cut away and placed in tubes. This comprised the material for the routine monthly culture. The core was cut again at the 3 cm and 5 cm depth marks and the 2 cm long core segments (i.e. 3 - 5 cm depth) were placed in a separate jar, labelled 'deep soil core'.

November 1998

Before contaminating plot 8, two series of 10 cores from each replicate were collected as a negative control. Immediately after contamination, two series of 10 pellets were collected from each replicate in plot 8 as a time zero positive control. Disturbance to the pellets on the plots was avoided.

Sampling continued from plots 1-7 at monthly intervals but was brought forward by a week to avoid Christmas and was synchronised with that from plot 8 from the earliest possible date. Sampling from plot 8 was at weekly intervals for 5 weeks then monthly. Sampling of pellets was discontinued when they were no longer recognisable on the pasture, but soil collection continued.

November 1999 and January 2000

Time zero samples were collected from the plots at Borenore on 03.11.1999. Samples were then collected at 2 week intervals until 12 weeks and then at monthly intervals as given in the results. Close to the end of the project in February 2001 a final sample was collected from each plot: two sets of 10 soil cores were collected from each replicate (A,B,C,D) so that there were the usual number of samples overall.

6.2.6 Sample handling

Sample vials were labelled with the following details: site - plot - replicate - series - date - soil/pellets for example '1-1-A-1, 28.1.98, soil'.

Samples were transported on wet ice packs to EMAI by overnight courier and were either cultured upon receipt or held at -80°C. The material remaining after culture was held at -80°C although samples were progressively displaced to storage at -20°C by the need to store samples received more recently. Some of these stored samples were later subjected to enumeration *of M. paratuberculosis*.

6.2.7 Pilot pasture box experiment at Camden - November 1998

Ten expanded polystyrene (styrofoam) boxes each measuring 58 x 38 x 23 cm (l x b x h) were set up on 22.10.98 on the covered verandah of a biosecure animal house at EMAI, Camden. This was before installation of shade cloth. The boxes were not subjected to direct sunlight except during the winter period when the angle of the sun was such that it came beneath the eaves of the verandah.

Each box was filled to a depth of 20 cm with commercial "topsoil" (clay loam, probably sourced from the Nepean River flood plain at Elderslie). A sample of 2 Kg of soil was retained for chemical analysis. Boxes were labelled 1 -10. The soil was watered down lightly to compact it. An even cover of a commercial mixed species grass seed (Budget Lawn Grass, Arthur Yates, Milperra NSW: couch 20%, chewings fescue 10%, perennial ryegrass 70%) was sown on 30.10.98 with a light dressing (10 g / box) of fertiliser (4.8% nitrogen as ammonium, 5.7% phosphorus, 5.9% potassium chloride, 12.6% sulphur and 12.4% calcium - Lawn Planting Fertiliser, Brunnings) and re-sown on 02.11.98 and watered in each time.

An even cover of the pellet mix produced for the November 1998 plot contamination was applied to the boxes on 02.11.98 coincident with the contamination of the plots in the Central Tablelands, at a rate of 300 g pellet mix per box. Boxes were then lightly watered as required to maintain the viability of the grasses, generally at a rate of > 0.5L per box per week. Watering was discontinued after 3 months and boxes were then allowed to become dry. As they were protected from natural rainfall the soil and pellets dried out completely.

The daily maximum and minimum shade temperatures in the immediate environment on the verandah of the building were recorded.

Each box was regarded as having equal 2 segments for the purposes of sampling and was used for two consecutive samplings. Time zero positive control faecal pellet samples were collected from

boxes 1 and 10 in week 0. Pellet and deep soil core samples were collected from the boxes each week for 7 weeks then fortnightly until 24 weeks after contamination and then monthly. Two pools of 10 pellets and two pools of the underlying soil cores were collected. The cores were extruded from the collection tube carefully to separate samples according to depth: top 2 cm, 3-5 cm and 6-8 cm . In addition, a sample of grass was collected with scissors, cutting at a height so as to avoid contamination with faeces or soil. Close to the end of the project in February 2001 a final sample was collected from each box: segments were ignored and one set of 10 pellets and two sets of 10 soil cores were collected so that there were 8 pellet samples and 16 soil core samples, i.e. 8-16 times the usual sampling rate for this experiment.

Run-off water was collected weekly from a drainage tube in the base of box 10 after over-watering this box only. About 50 ml of water was collected on each occasion for culture.

6.2.8 Pasture box experiment - November 1999 and January 2000

Preparation of pasture boxes

A mixture of 9 parts "topsoil" from the same source as previously and 1 part "brickies sand" was prepared at EMAI and used to fill polystyrene vegetable boxes measuring 58 x 39 x 26 cm (l x b x h) to a depth of 20 cm. 1 Kg of soil was retained for analysis. Drain holes were placed in each box. The boxes for the November contamination were seeded with mixed grasses (perennial rye grass, fescue, couch and bluegrass) and a light dressing (10 g / box) of fertiliser (4.8% nitrogen as ammonium, 5.7% phosphorus, 5.9% potassium chloride, 12.6% sulphur and 12.4% calcium - Lawn Planting Fertiliser, Brunnings) on 29.10.1999 and watered heavily. The boxes were seeded again on 1.11.99. Boxes for the January contamination were not seeded at this time but lids were placed on these boxes. The lids were removed from these boxes on 20.1.2000 which were then sown with grasses and watered as described above. The 0% shade boxes at Borenore were covered with hessian to facilitate germination. Boxes at EMAI were re-sown at the time of contamination.

Each box was regarded as having 3 equal segments (A,B,C) for sampling purposes.

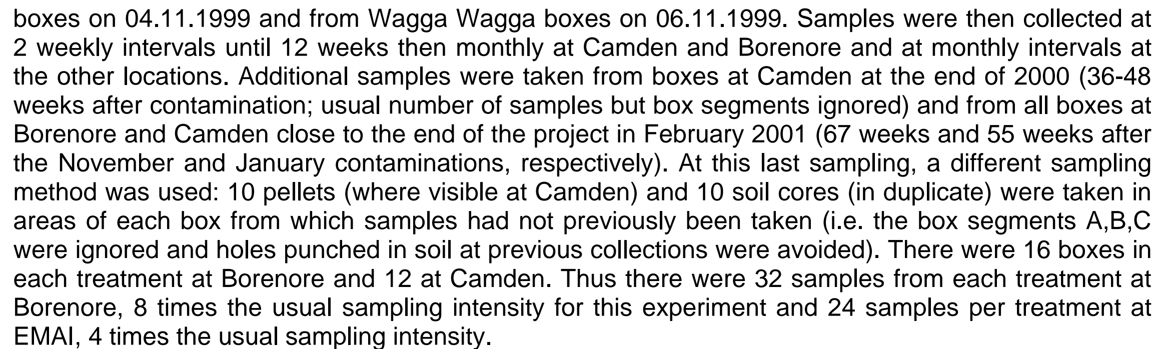
Contamination and transport of pasture boxes and sample collection

Samples of 300 g of faecal mixture were applied evenly to all the November contamination boxes at Camden on 03.11.1999 and these boxes were then transported to Orange on a tray-top truck and thence to their intended destinations by car, or directly from Camden to Goulburn and Armidale by car. Samples of 300 g of faecal mixture were applied evenly to each January box at Borenore on 25.01.2000. The January boxes had been transported to Borenore and put in place the previous November. Samples of 300 g of faecal mixture were applied evenly to all January boxes at Camden on 24.01.2000. These contamination events coincided with the contamination of field plots at Borenore.

Upon arrival at final destinations in November 1999 the soil in the boxes was compacted and/or had shifted in transit as muddy marks were visible on the sides of the boxes to a height of 50 mm above the soil level and the faecal material in some boxes was coated in mud and in some cases had shifted to one end of the box. There was also pooling of water on the surface in some boxes. Faecal material was manually redistributed where there was clear evidence that it had shifted to one end of a box. These factors appeared to adversely influence seed germination (see results). However, the boxes set up at EMAI were not disturbed in any way.

One segment of each of 3 boxes (at EMAI) or of 2 boxes (at Borenore and other locations) was designated for sampling at each time point so that there were 3 replicates at EMAI and 2 at the other locations. Duplicate samples were taken from each replicate.

Time zero samples positive control samples were collected from all Camden and Goulburn boxes on 02.11.1999, from Borenore, Cobar, Armidale and Broken Hill boxes on 03.11.1999, from Bourke



boxes on 04.11.1999 and from Wagga Wagga boxes on 06.11.1999. Samples were then collected at 2 weekly intervals until 12 weeks then monthly at Camden and Borenore and at monthly intervals at the other locations. Additional samples were taken from boxes at Camden at the end of 2000 (36-48 weeks after contamination; usual number of samples but box segments ignored) and from all boxes at Borenore and Camden close to the end of the project in February 2001 (67 weeks and 55 weeks after the November and January contaminations, respectively). At this last sampling, a different sampling method was used: 10 pellets (where visible at Camden) and 10 soil cores (in duplicate) were taken in areas of each box from which samples had not previously been taken (i.e. the box segments A,B,C were ignored and holes punched in soil at previous collections were avoided). There were 16 boxes in each treatment at Borenore and 12 at Camden. Thus there were 32 samples from each treatment at Borenore, 8 times the usual sampling intensity for this experiment and 24 samples per treatment at EMAI, 4 times the usual sampling intensity.

6.2.9 Direct-PCR analysis of pellet samples

The recently-described method of Marsh and Whittington[14] was used to confirm the presence of *M. paratuberculosis* DNA directly from pellet samples from the November 1999 contamination of Camden boxes in 100% shade. The samples of pellets from weeks 0, 10, 12 and 32 were tested. These included both culture positive (T=0 & 10) and culture negative (T = 12 & 32) pellet samples (Table 3.10).

6.2.10 Soil analysis

Methods for analysis of soil are given in Appendix V. Soil used for pasture box experiments was well-mixed and 1 kg samples were submitted for analysis. Soil samples from pasture plots were collected in two ways. "Surface samples" consisted of the top 50 mm of the soil profile. Two samples, each about 180 g, collected at opposite sides of the square area, were taken from each replicate so that a total of over 1 kg was collected per plot. "Standard samples" were collected using a corer 2cm diameter x 10 cm depth. Twelve cores were collected in a grid pattern from each replicate, i.e. 36 cores per plot. Samples were well-mixed before analysis.

6.2.11 Statistical methods

Culture results were analysed using logistic regression for binomial data. For the 1998 contamination data were pooled within two time intervals (1 - 3 months and 4 - 9 months after contamination) and expressed as the percentage of samples that were culture positive. For the 1999-2000 contamination data were similarly pooled and expressed (2 - 12 weeks and 16 - 36 weeks after contamination). The 1998 data were also analysed using a Chi square test for differences in proportions after pooling data from site and type of sample. Counts of the number of viable organisms per gram in environmental samples collected over time were plotted using curve fitting software (Prism, Graphpad Software Incorporated). Linear regression was used to estimate decay rates per unit time.

Note: statistical analyses are on-going and those completed are only for major effects. Further logistical regression analyses for shorter time intervals will be conducted to further elucidate the effects of various treatments.

6.3 Results

6.3.1 Soil analysis

The composition of the soil mixture used in pasture boxes and present in pasture plots is given in detail in Appendix V. The mixture used in pasture boxes was a dark yellow-brown, light, sandy loam with low organic matter content. It had been selected to maximise the chance of recovering the organism (see Part 1), had pH of 5.8 to 6.1 and iron levels of 12 to 30 mg/kg.

The soil in pasture plots at Borenore and Carcoar was a brown clay loam and had a higher organic matter content than that in pasture boxes, was slightly acidic pH (5.1 to 6.7 across plots), and had iron levels of up to 180 mg/kg. The application of lime resulted in an increase in pH of about 0.5 unit for low lime and 1.0 unit for high lime at Borenore and 0.2 unit for low lime and 0.7 unit for high lime at Carcoar in surface soil (0 to 5 cm depth), measured 18 months after application. The high lime plots at both Borenore and Carcoar had a pH of 7.4 in surface samples. An increase in pH due to lime was also noted in the standard soil samples which extended from the surface to a depth of 10 cm.

6.3.2 Enumeration of *M. paratuberculosis* in faecal mixtures

The estimated viable counts of *M. paratuberculosis* in faecal mixtures used to contaminate plots and boxes and the estimated counts per cm^2 soil at the start of each experiment are given Tables 3.2 and 3.3.

Each of the faecal mixtures used between November 1998 and January 2000 contained 10^5 - 10^6 viable organisms per gram. The rates applied to pasture plots and pasture boxes ranged from 10^4 - 10^5 per cm^2 except for the slurry mix applied in November 1998 which resulted in about 1 $\log_{10}$ greater contamination. The faecal mixtures used to contaminate the pasture plots and boxes in November 1999 and January 2000 were enumerated together in April 2000. The count of the January mixture was the same as that for the November mixture.

Table 3.2. Estimated viable counts of *M. paratuberculosis* in faecal mixtures used to contaminate pasture plots and boxes

Contamination date	Sample	Count per gram of faecal mixture
January 1998	Slurry mixture	5.00×10^6
January 1998	Pellet mixture	5.00×10^5
November 1998	Pellet mixture	1.20×10^6
November 1999	Pellet mixture	1.58×10^5
January 2000	Pellet mixture	1.58×10^5

Footnote: these counts were obtained using PBS as the diluent. For comparison, counts 1 $\log_{10}$ higher were obtained for the November 1999 and January 2000 samples using PBS with 0.1% Tween 80 diluent.

Table 3.3. Estimated viable counts *M. paratuberculosis* applied per unit surface area of pasture plots and boxes

Contamination date	Sample	Count per cm^2 soil
January 1998	Slurry mixture on plots	7.11×10^5
January 1998	Pellet mixture on plots	3.34×10^4
November 1998	Pellet mixture on plots	1.07×10^5
November 1998	Pellet mixture on boxes	1.63×10^5
November 1999 & January 2000	Pellet mixture on plots	3.16×10^4
November 1999 & January 2000	Pellet mixture on boxes	2.10×10^4

see footnote to table 3.2; the same applies to these figures

6.3.3 Duration of survival of *M. paratuberculosis*

Detailed culture results for each sample at each time point are provided in Appendix III. A summary of these results is provided in Tables 3.4 - 3.11. In each table black shading is used to show time points where positive cultures were obtained so that the data can be scanned quickly like a horizontal bar graph. Also shown is the incubation period in weeks required to reach peak growth index in BACTEC culture, which is directly proportional to the number of viable organisms in the sample.

January 1998 contamination of pasture plots

Survival was observed for up to 7 months after the contamination in January 1998 at both Borenore and Carcoar (Tables 3.4 and 3.5). There were several trends:

i. The proportion of replicate samples that were culture positive declined over time so that by 3-4 months after contamination usually only 1 of 6 replicates was positive.
ii. Reinforcing the first trend, the incubation time required for cultures to reach peak growth index tended to increase over time, consistent with declining numbers of viable organisms in the samples.
iii. Positive outcomes became intermittent for all plots at both sites from 1 to 2 months after contamination.
iv. Cultures at 5 months were uniformly negative at both sites, only to be followed by positive results at 6 months and 7 months, with growth occurring more quickly in the 7th month than at earlier time points.
v. There was no obvious pattern in the results suggesting that any of the treatments had an effect on duration of survival. There was survival for a similar period regardless of the degree of shade, moisture or application of lime. For example in the absence of shade there was survival in slurry mix applied to soil and in soil beneath faecal pellets for 7 months. However, there was a tendency for a larger proportion of samples from shaded plots to be culture positive compared to unshaded plots. This is evident in cumulative totals of culture positive soil and pellet/slurry samples grouped according to the degree of shade and adjusted for sample size (there were twice as many samples from shaded plots compared to unshaded plots) at both Borenore and Carcoar (Figure 3.5).

Statistically, the prevalence of culture positive samples at Borenore was significantly greater than at Carcoar in the first 3 months ($P<0.001$) but there was no difference between sites in the next 6 months. The prevalence of positive cultures for soil samples collected from plots contaminated with slurry was greater than for pellet samples from the plots contaminated with pellets ($P<0.01$). This is attributable to the 1 $\log_{10}$ higher contamination rate in plots contaminated with slurry and the fact that soil samples from the plots contaminated with slurry included slurry on the surface of the soil sample. The prevalence of culture positive samples for soil from beneath pellets was intermediate between soil from slurry plots and pellets; this implies that the organism moved out of the pellets into the adjacent soil.

Allowing for variation due to site (Borenore, Carcoar), type of sample (soil, pellet) and time period (1-3 mths, 4-9 mths) there was no significant effect of treatment (shade, moisture etc) in logistic regression analysis. However, there was a significant effect of shade in the first 3 months ignoring differences between type of sample ($P<0.05$). Fifty two of 216 samples from shaded plots were culture positive compared to 15 of 108 samples from unshaded plots (Chi square 4.6, $P=0.03$) (Figure 3.5).

It is important to note that vegetation was not removed from the plots so that by 5 months after contamination there was complete vegetation cover of the soil even in outside unshaded plots (see below for further descriptions of plots). This would have provided shade so that differences between shaded and unshaded treatments are likely to have been masked. This was addressed in later experiments.

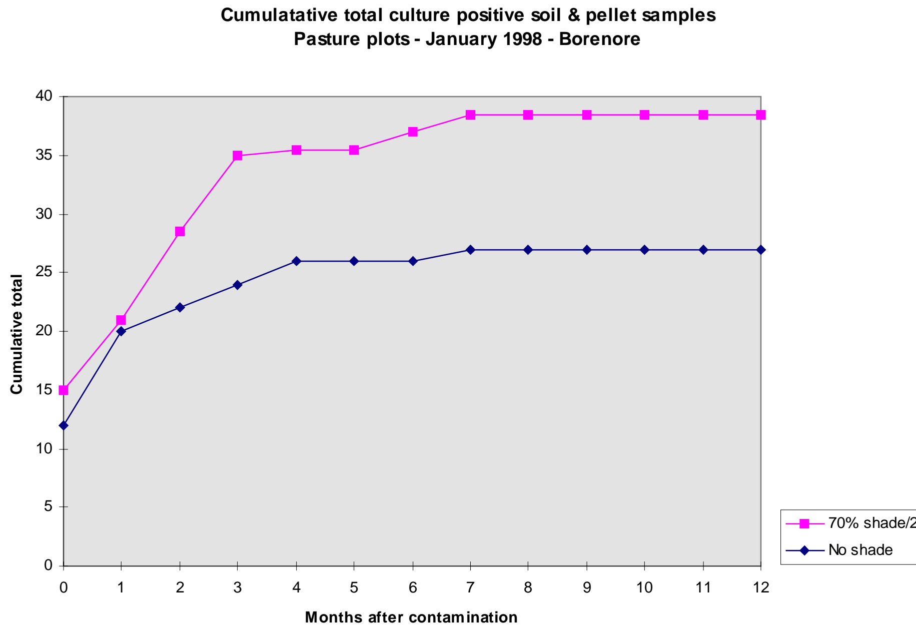
Table 3.4. Number of culture positive replicate samples at the Borenore site after the January 1998 contamination. Black shading indicates a positive culture and the figures within indicate the number of replicates (n) that were culture positive. -ve indicates negative culture result from week 0 control soil sample

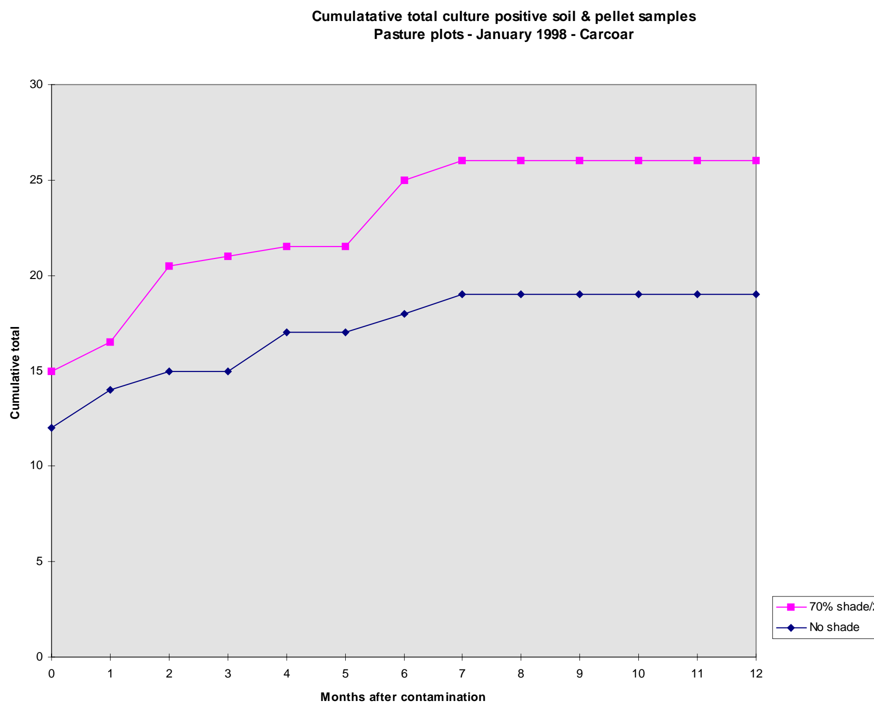
Plot	Treatment	Contaminated with	Culture of	n	Time of sample collection (months after contamination)																	
					0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	No shade	faecal slurry	soil	6	6	5		1	1			1										
2	Shade	faecal slurry	soil	6	6		2	3			1	1										
3	Shade + water	faecal slurry	soil	6	6	4	3	3			2	1										
4	Shade + water + low lime	faecal slurry	soil	6	6	3	2	2														
5	Shade + water + high lime	faecal slurry	soil	6	6	2	4	3				1										
6	Shade + water	faecal pellets	soil	6	-ve	1	2		1													
			pellets	6	6	2	2	2														
7	No shade	faecal pellets	soil	6	-ve	2	2		1													
			pellets	6	6	1		1														
Time to maximum growth index in weeks (range)					3-4	5-12	3-12	3-18	8-11		8-11	5-9										

Table 3.5. Number of culture positive replicate samples at the Carcoar site after the January 1998 contamination. Black shading indicates a positive culture and the figures within indicate the number of replicates (n) that were culture positive. -ve indicates negative culture result from week 0 control soil sample

Plot	Treatment	Contaminated with	Culture of	n	Time of sample collection (months after contamination)																	
					0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	No shade	faecal slurry	soil	6	6	1			1		1											
2	Shade	faecal slurry	soil	6	6	1	2		1		1	1										
3	Shade + water	faecal slurry	soil	6	6	1	2	1			3											
4	Shade + water + low lime	faecal slurry	soil	6	6						1											
5	Shade + water + high lime	faecal slurry	soil	6	6	1	3				1	1										
6	Shade + water	faecal pellets	soil	6	-ve		1				1											
			pellets	6	6																	
7	No shade	faecal pellets	soil	6	-ve	1	1		1			1										
			pellets	6	6																	
Time to maximum growth index in weeks (range)					3-4	8-11	3-10	13	8		7-10	6-8										

Figure 3.5. Cumulative total culture positive soil and pellet/slurry mix samples for the January 1998 contamination adjusted for sample size





November 1998 contamination - pasture plots and pilot box experiment

Survival in pellets applied to plots at Borenore and Carcoar was compared to survival in pasture boxes on a shaded verandah at EMAI, Camden (Tables 3.6 and 3.7). There was survival to 5 weeks after contamination on the plots in the central tablelands compared to 10 weeks after contamination in the boxes at Camden. Survival in pellets appeared to be greater than survival in soil beneath pellets, perhaps because there was little movement of the organism into the soil over this time frame. The proportion of culture positive replicates decreased over time and the time required for positive cultures to reach peak growth index generally increased with time, consistent with decreasing numbers of viable organisms over time.

Samples of grass that grew through the contaminated soil surface in the pasture boxes were culture positive each week up to and including week 4 after contamination while run-off water was culture positive to week 3. The time for grass samples to reach peak growth index was similar to that of pellets implying substantial contamination rates.

The duration of survival after the November 1998 contamination was considerably less than that after the January 1998 contamination despite heavier contamination. As the summer solstice occurs about December 22nd the November contamination was followed by increasing levels of exposure to solar radiation while the January contamination was followed by decreasing levels, suggesting that the degree of exposure to solar radiation may have been important. This also appeared to be a potential factor in explaining the greater length of survival in the shaded environment at Camden compared to exposed plots in the central tablelands and was consistent with the difference observed between cumulative results for the shaded and unshaded plots at Borenore and Carcoar in the January 1998 contamination. These observations led to a third experiment in which shade was included as a major treatment.

November 1999 and January 2000 contamination - pasture plots and boxes

To test the difference in effect of exposure of faecal mixtures to a full summer compared to a part summer and to evaluate the effect of shading, field plots and pasture boxes were contaminated with infected faeces in early November 1999 and late January 2000 at Borenore. Pasture boxes were contaminated with the same infected faeces at Camden. Two levels of shade were provided at Borenore and three at Camden. Contaminated pasture boxes were deployed also to Goulburn, Wagga Wagga, Broken Hill, Cobar, Bourke and Armidale in November 1999 and placed in exposed locations. Unfortunately the summer was extraordinarily wet, with pasture boxes being inundated early in the trial.

All sites were culture positive immediately after contamination. The prevalence of positive cultures was higher in the first period (2-12 weeks) than the second period (16-36 weeks) ($P<0.001$) as would be expected due to progressive loss of the organism.

Effect of shade

Cumulative totals of culture positive samples are given in Figure 3.6. The effect of shade on survival can be appreciated readily. At both sites and after both contamination times there were a greater total number of culture positive samples from shaded locations, with a graded shade effect at Camden, and the duration of survival was also greater in these shaded locations. Averaged over both the Borenore and Camden sites and over plots and boxes there was greater survival in 70% and 100% shade than in 0% shade in the first 12 weeks ($P<0.001$). In the following 24 weeks there was greater survival in 100% shade than in 70% shade or 0% shade ($P<0.01$). Note that in the first period survival in 100% shade was not different from that in 70% shade ($P>0.05$) and in the second period survival in 70% shade and 0% shade were not different ($P>0.05$). This means that moderate degrees of shade were significant when organisms were most numerous but after they decline in numbers a higher level of shade was required to prolong their survival.

Table 3.6. Number of culture positive replicate samples at the Borenore and Carcoar sites after the November 1998 contamination. Black shading indicates a positive culture and the figures within indicate the number of replicates (n) that were culture positive. nt, not tested

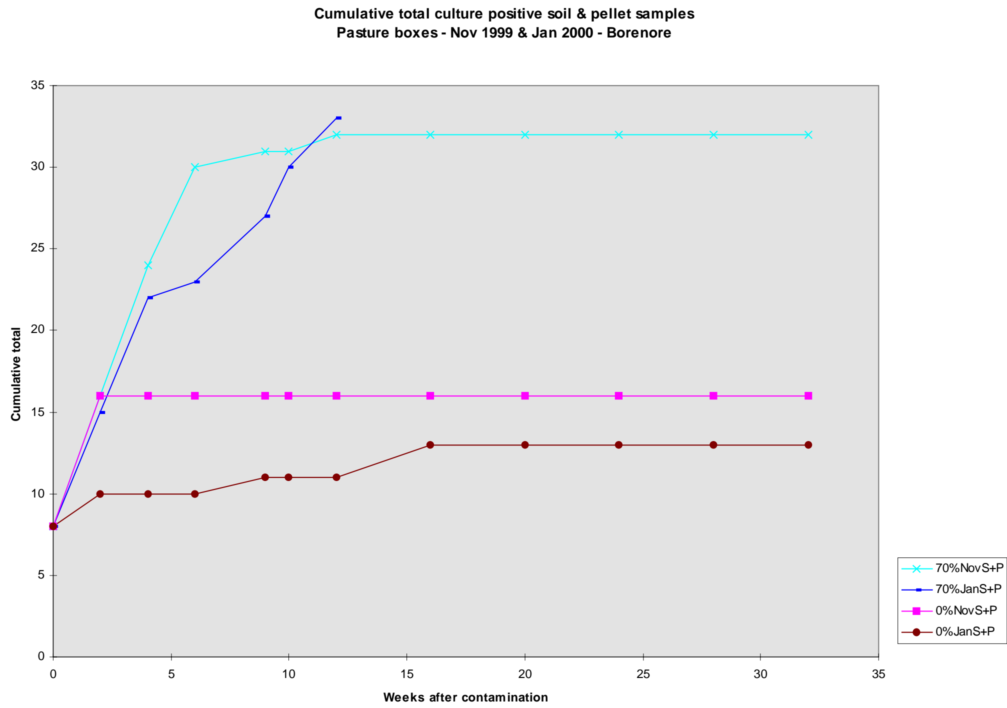
Plot	Treatment	Contaminated with	Culture of	n	Time of sample collection (weeks after contamination)												
					0	1	2	3	4	5	9	14	17	21	25	29	33
8	No shade Borenore	faecal pellets	soil	6	nt			1									
			pellets	6	6	6	1	1									
8	No shade Carcoar	faecal pellets	soil	6	nt	4	2										
			pellets	6	6	6	4	1	2	1							
Time to maximum growth index in weeks (range)					4	7-10	6	7-8	9	9							

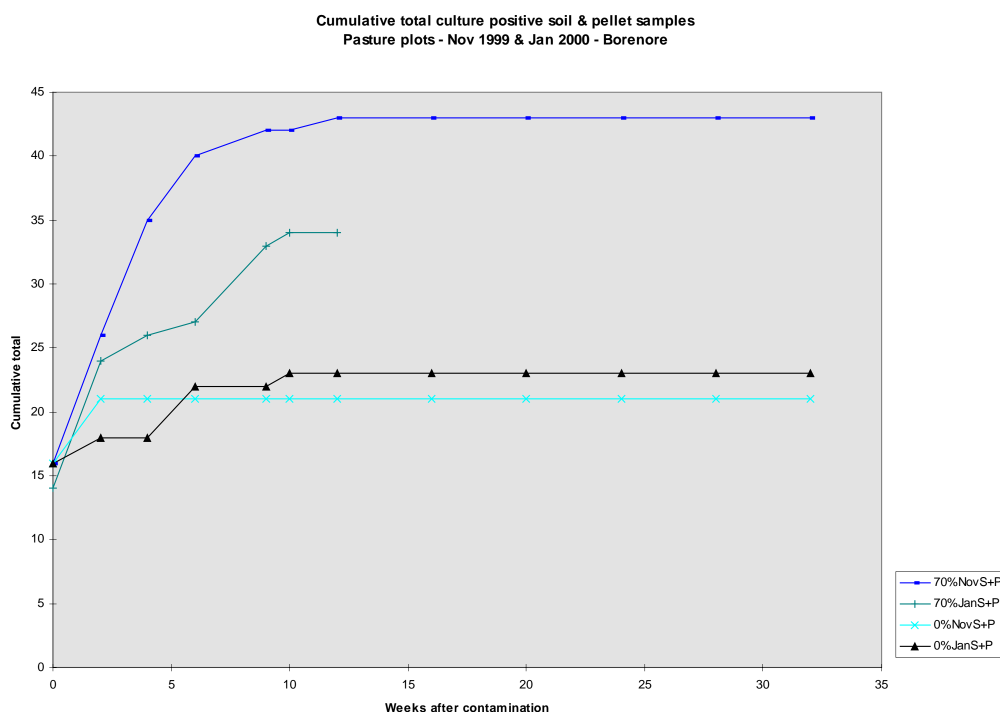
Table 3.7. Number of culture positive replicate samples in pasture boxes at Camden in a shaded location after the November 1998 contamination. Black shading indicates a positive culture and the figures within indicate the number of replicates (n) that were culture positive. nt, not tested

Box	Treatment	Contaminated with	Culture of	n	Time of sample collection (weeks after contamination)																			
					0	1	2	3	4	5	6	7	9	10	12	14	16	18	20	22	24	29	33	117*
1-10	Pasture boxes on shaded verandah	faecal pellets	soil 2cm	2	2	2	2	1					1											
			pellets	2	2	2	2	2	2	2	2	1	1	1										
			grass	1	nt	1	1	1	1												nt	nt	nt	nt
			run-off water	1	nt	1	1	1																nt
Time to maximum growth index in weeks for pellets (range) [Grass 5-8 weeks; run-off water 5-11 weeks]					4-5	5	8	8	6	7-8	8	9	12	11										

* at this time point n = 16 soil and n = 8 pellets; the pellet samples were blended with 20 ml saline but cultured with the standard protocol.

Figure 3.6. Cumulative total numbers of culture positive soil and pellet samples for the November 1999 - January 2000 contamination





The duration of survival
Survival in plots at Borenore after the November contamination extended to 12 weeks in 70% shade compared to 2 weeks in full sun (Table 3.8). The same results were obtained in pasture boxes (Table 3.9). After the January contamination, survival was detected in plots to 10 weeks in both 70% shade and full sun. However, survival in pasture boxes was more prolonged - 24 weeks in 70% shade and 16 weeks in full sun. Declining numbers of culture positive replicates and increasing incubation periods were seen over time, consistent with declining levels of residual contamination (Tables 3.8 & 3.9).

Similar results were obtained in pasture boxes at Camden for full-sun and 70% shade with survival to 12 weeks after the November contamination and 16 weeks after the January contamination (Table 3.10). However, survival in 100% shade was prolonged, extending to 28 weeks after the November contamination and at least 55 weeks after the January contamination (Table 3.10).

Survival in pasture boxes deployed to various sites in NSW in November was relatively short, there being no positive cultures from pellets or soil after week 9 post contamination with most being negative after 4 weeks (Table 3.11).

The intermittent nature of culture positive results from plots and boxes was similar to that observed in previous experiments.

The short apparent duration of survival from pasture boxes set up in November 1999 and coinciding with very heavy rainfall events was of interest. Rainfall recorded at Camden and Borenore amounted to 125 mm and 225 mm, respectively, up until week 10 after the November contamination and heavy rain was recorded across much of NSW at that time. It is possible that the plots and boxes were disturbed by rainfall. Boxes deployed to other sites in NSW, which were also exposed to very heavy rainfall, had mostly had considerably greater periods in transit and may have been affected to a greater degree by water pooling caused by vibration; certainly pasture germination was poor in many of these boxes.

The organism was recovered from grass samples from the pasture boxes at Camden for up to 24 weeks after the January contamination in 100% shade and 9 weeks in 70% shade (Tables 3.9 - 3.10). There were few positive cultures from grass from pasture boxes at Borenore, but survival was found at

9 weeks after the November contamination in 70% shade. The shaded boxes at Camden were not exposed to natural rainfall and were watered very carefully by hand to avoid "washing" the grass. This probably explains the higher rate of recovery of the organism from grass at Camden.

The data did not support the hypothesis that survival after a late summer (January) contamination would be significantly greater than survival after an early summer (November) contamination. Based on logistic regression analysis of the proportions of positive cultures in each period, the month of contamination did not affect the outcome. However, visual examination of data in Tables 3.4 - 3.10 would suggest an effect of month on the ultimate duration of survival, albeit at a very low level for the more lengthy observations. The maximum duration of survival observed at each site is listed by month below and this aspect will be addressed again in the discussion.

Month	Site	Source	Shade	Maximum duration of survival (weeks)	Source of data
January 1998	Borenore	plots	partial	28	Table 3.4
January 1998	Carcoar	plots	partial	28	Table 3.5
November 1998	Borenore	plots	nil	3	Table 3.6
November 1998	Carcoar	plots	nil	5	Table 3.6
November 1998	Camden	boxes	partial	10	Table 3.7
November 1999	Borenore	plots	70%	12	Table 3.8
January 2000	Borenore	plots	nil & 70%	10	Table 3.8
November 1999	Borenore	boxes	70%	12	Table 3.9
January 2000	Borenore	boxes	70%	24	Table 3.9
November 1999	Camden	boxes	100%	28	Table 3.10
January 2000	Camden	boxes	100%	55	Table 3.10

Movement of organisms from faecal pellets to soil

There was evidence of a movement of organisms from pellets to soil as would be expected during breakdown of the pellets (Tables 3.8 - 3.10). The prevalence of positive cultures from pellets was higher than that in soil (P<0.001) in the first 12 weeks but not generally in the later period (P>0.10) except at Camden (P<0.01) where in the shaded boxes the pellets remained intact due to lack of water, thereby providing little opportunity for movement of organisms into soil.

Boxes as experimental units

Boxes were simpler to prepare and maintain than plots. The results from boxes were similar to those from plots although there were some significant differences in the proportions of positive cultures in the November 1999 and January 2000 contaminations. There were more positive cultures from Borenore boxes than Camden boxes and Borenore plots (P<0.05) but Camden boxes and Borenore plots were not significantly different. On balance boxes were a very useful experimental system.

Table 3.8. Number of culture positive replicate samples at the Borenore site after the November 1999 and January 2000 contamination. Black shading indicates a positive culture and the figures within indicate the number of replicates (n) that were culture positive. nt - not tested as pellets not visible on plots

Plots 0% shaded - November

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	67
Pellet	8	8	4								nt	nt	nt	nt
Soil	8	8	1											
Wks to max GI		3-8	7-8											

Plots 70% shaded - November

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	67
Pellet	8	8	8	5	5	1				nt	nt	nt	nt	nt
Soil	8	8	2	4		1		1						
Wks to max GI		3-6	6-8	6-9	6-9	8-12		12						

Plots 0% shaded - January

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	55
Pellet	8	8	1		3					nt	nt	nt	nt	nt
Soil	8	8	1		1		1							
Wks to max GI		3-8	5-8		4-9		12							

Plots 70% shaded - January

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	55
Pellet	8	8	7	1		5		nt	nt	nt	nt	nt	nt	nt
Soil	8	6	3	1	1	1	1							
Wks to max GI		3-6	6-10	6-7	12	6-11	8							

Table 3.9. Number of culture positive replicate samples in pasture boxes at the Borenore sites after the November 1999 and January 2000 contamination. Black shading indicates a positive culture and the figures within indicate the number of replicates (n) that were culture positive. nt - not tested as pellets not visible on plots

Boxes 0% shaded Borenore - November

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	67*
Pellet	4	4	4											nt
Soil	4	4	4											
Grass	2	nt												nt
Wks to max GI		3-5	6-8											

Boxes 70% shaded Borenore - November

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	67*
Pellet	4	4	4	4	4	1		1			nt	nt	nt	nt
Soil	4	4	4	4	2									
Grass	2	nt				2								nt
Wks to max GI		3-7	4-7	5-7	6-10	9-10		8						

Boxes 0% shaded Borenore - January

Week	n	0	2	4	6	8	10	12	16	20	24	28	32	55*
Pellet	4	4	2			1			1			nt	nt	nt
Soil	4	4							1					
Grass	2	nt												nt
Wks to max GI		3-8	6-7			10			9-12					

Boxes 70% shaded Borenore - January

Week	n	0	2	4	6	8	10	12	16	20	24	28	32	55*
Pellet	4	4	4	4	1	4	3	2	nt	nt	nt	nt	nt	nt
Soil	4	4	3	3				1			1			
Grass	2	nt												nt
Wks to max GI		4-6	4-7	5-8	8	6-7	5-8	8-9			8			

* n = 32 for this time point

Table 3.10. Number of culture positive replicate samples in pasture boxes at the EMAI site after the November 1999 and January 2000 contamination. Black shading indicates a positive culture and the figures within indicate the number of replicates (n) that were culture positive. . nt - not tested as pellets not visible on plots. Samples from week 48 (November contamination) and week 36 (January contamination), which was a late extension to the project design, were cultured using method variation 5, as part of the study described in detail in Part 5 of this report. Pellet samples from weeks 67 (November contamination) and 55 (January contamination) were blended with 20 ml saline but cultured with the standard protocol

Boxes 0% shaded EMAI - November

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	48	67*
Pellet	6	6						3							nt
Soil	6	6	3												
Grass	3	nt												nt	nt
Wks to max GI		3-7	8-10					6							

Boxes 70% shaded EMAI - November

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	48	67*
Pellet	6	6	6	4	6	1		4							
Soil	6	6	2	1	4	1									
Grass	3	nt	1	3										nt	nt
Wks to max GI		3-7	8-12	6-8	6-8	7-8		6-7							

Boxes 100% shaded EMAI - November

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	48	67*
Pellet	6	6	5	6	6	5	3		6		1	1			
Soil	6	6	4	5	6										
Grass	3	nt	3	2		3	1							nt	nt
Wks to max GI		3-6	6-10	6-9	6-10	7-10	7-9		5-6		10	8			

* n = 24 at this time point

Boxes 0% shade EMAI - January

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	36	55*
Pellet	6	6													nt
Soil	6	6						1							
Grass	3	nt												nt	nt
Wks to max GI		4-8						2							

Boxes 70% shaded EMAI - January

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	36	55*
Pellet	6	6	6	2	1	5		1	1						
Soil	6	5	6	2		2									
Grass	3	nt		2		2								nt	nt
Wks to max GI		3-8	5-11	7-9	7	6-10		8	7						

Boxes 100% shaded EMAI - January

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	36	55*
Pellet	6	6	6	6	4	3			2		3	2	2	2	1
Soil	6	5	6	2		1						1			
Grass	3	nt		1	1	2		1	2		1			nt	nt
Wks to max GI		4-6	5-8	6-9	8-11	6-14		10	6		6-8	7-9	6-7	7-8	8

* n = 24 at this time point; the culture result from this time point was obtained too late to permit enumeration by MPN

Table 3.11. Number of culture positive replicate samples in pasture boxes at various sites after the November 1999 contamination. Black shading indicates a positive culture and the figures within indicate the number of replicates (n) that were culture positive. nt, not tested

Boxes 0% shaded Wagga Wagga - November

Week	n	0	4	9	12	16	20
Pellet	4	4	1				
Soil	4	4					
Grass	2	nt					
Wks to max GI		4-7	7				

Boxes 0% shaded Broken Hill - November

Week	n	0	4	9	12	16	20
Pellet	4	4	2				
Soil	4	4	1				
Grass	2	nt					
Wks to max GI		3-7	6-7				

Boxes 0% shaded Bourke - November

Week	n	0	4	9	12	16	20
Pellet	4	4					
Soil	4	4		2			
Grass	2	nt					
Wks to max GI		4-7		3-8			

Boxes 0% shaded Cobar - November

Week	n	0	4	9	12	16	20
Pellet	4	4	1				
Soil	4	4					
Grass	2	nt					
Wks to max GI		3-6	8				

Boxes 0% shaded Armidale - November

Week	n	0	4	9	12	16	20
Pellet	4	4	3				
Soil	4	4	2				
Grass	2	nt					
Wks to max GI		3-7	7				

Boxes 0% shaded Goulburn - November

Week	n	0	4	9	12	16	20
Pellet	4	4					
Soil	4	3	1				
Grass	2	nt					
Wks to max GI		3-7	10				

Decay rate of the organism

Retrospective enumeration of *M. paratuberculosis* in selected culture positive samples was undertaken on a batch basis after completion of most of the cultures in each experiment. This enabled selection only of culture positive samples for enumeration, it being impossible to attempt enumeration on all samples. Most of the samples enumerated were pellets because it was considered more likely that low counts could be detected in pellets compared to soil samples (see Part 1). The data are shown in Tables 3.12 - 3.14.

Counts and D-values

Plots of the data are given with linear regression analyses in Figures 3.7 to 3.9. In each case there was an initial phase of relatively rapid decline in bacterial count followed by a phase of relatively slow decline. In addition, in both the November 1998 and January 2000 data these phases were separated by a phase of apparent increase in bacterial count (Figures 3.8 & 3.9). In the latter case there was significant non-linearity in the decay curve caused by a large apparent increase in bacterial count in weeks 16 and 24 after successive weeks with negative cultures (Figure 3.7 upper panel) and it was most appropriate to fit two lines, one for the rapid decay phase and the other for the slow decay phase (Figure 3.7 lower panel). Note that cultures were negative in weeks 10 and 12.

The rates of decline are listed below according to the length and period of observation. The results are expressed in lunar months for consistency.

Period of observation	D-value	Source
0 - 1.5 months	1.7 logs/month	Figure 3.9
0 - 2 months	0.8 logs/month	Figure 3.8
0 - 7 months	0.5 logs/month	Figure 3.7
4 - 9 months	0.4 logs/month	Figure 3.9
0 - 9 months	0.2 logs/month	Figure 3.9
4 - 13 months	0.2 logs/month	Figure 3.9*

*the positive culture result for week 55 was obtained at the end of the project which prevented enumeration of this sample by MPN. The time to peak growth index for this sample suggested that there were similar numbers of viable organisms present in it as there were in the samples at week 36. This being the case the slope of the regression line in the lowest panel of Figure 3.9 would be -0.06 logs/week.

There is a clear pattern in these results - the greater the period of observation the lower the D-value. This is due to the two phase decline. The longer the period of observation (or the further out in the trial the period of observation) the greater the influence of the second slow decline phase.

Figure 3.7. Most probable number count estimates of *M. paratuberculosis* and linear regression analysis for samples collected from the January 1998 contamination

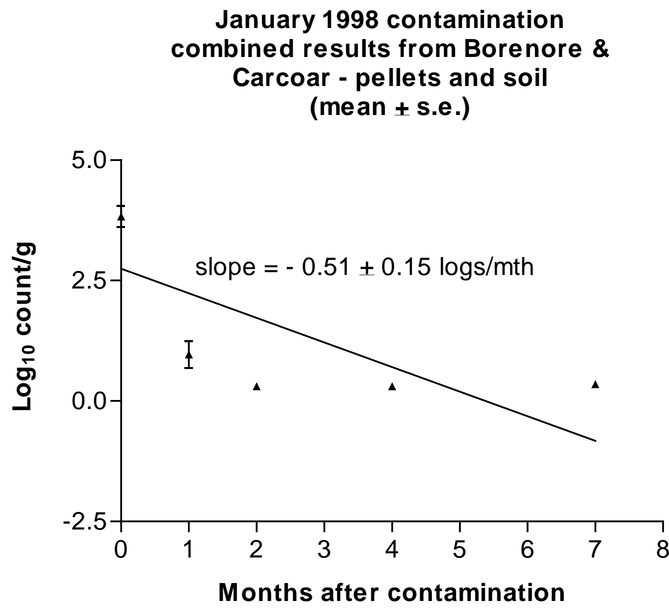
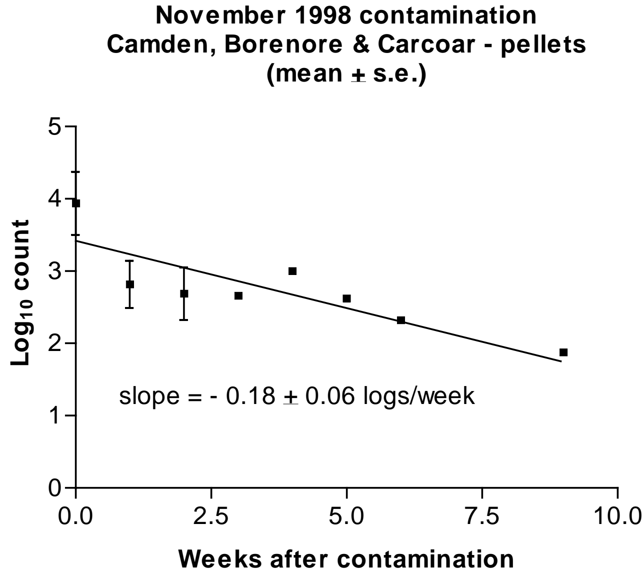


Figure 3.8. Most probable number count estimates of *M. paratuberculosis* and linear regression analysis for samples collected from the November 1998 contamination. Upper panel, data pooled from 3 sites; lower panel, data from one site



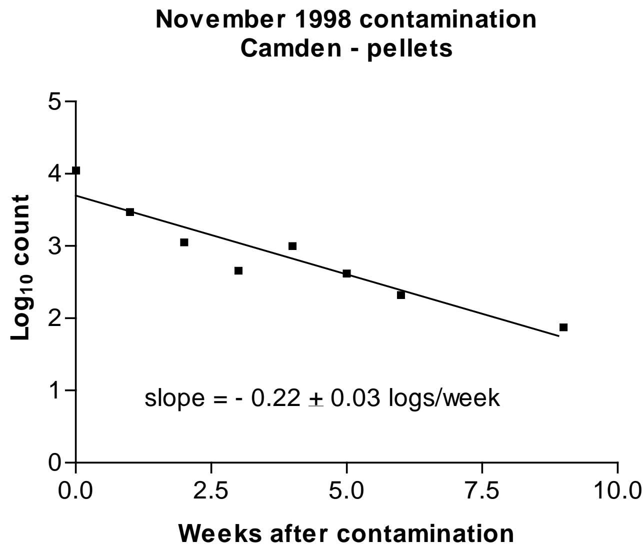


Figure 3.9. Most probable number count estimates of *M. paratuberculosis* and linear regression analysis for samples collected from the January 2000 contamination. Upper panel, a single regression line is shown which does not accurately represent the data. Middle panel, separate regression lines represent the two phases of the decay pattern. Lowest panel, the regression line for the second period is shown with a count for week 55 estimated from time to peak growth index

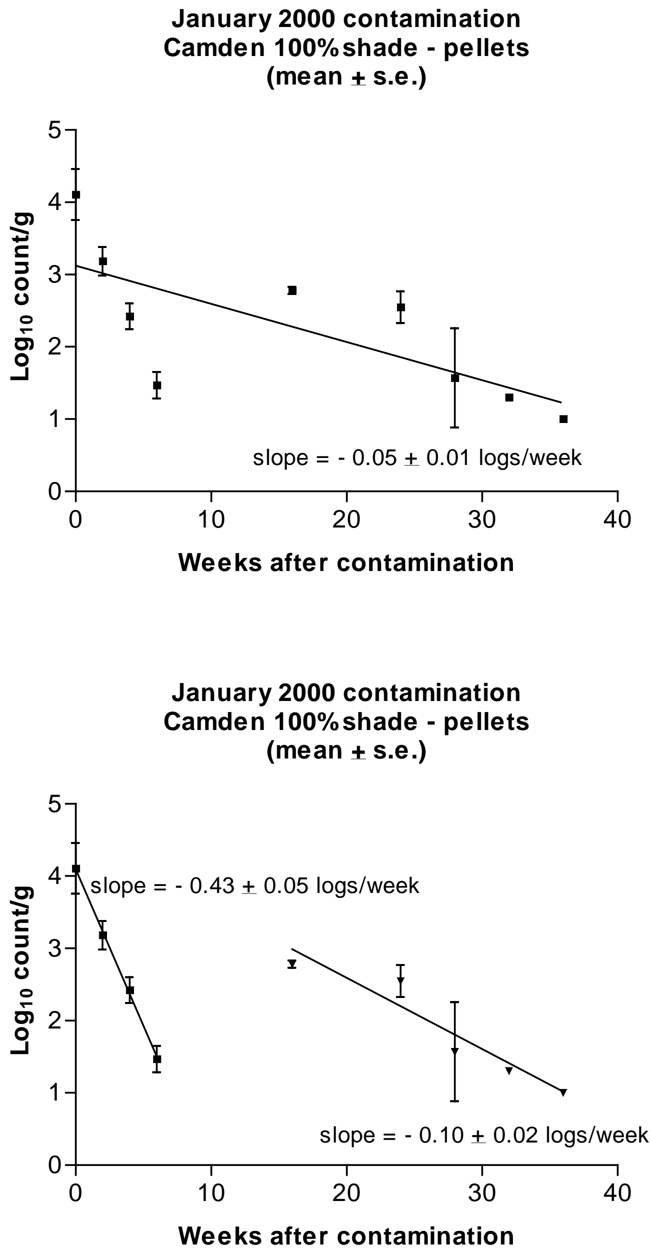


Figure 3.9 continued

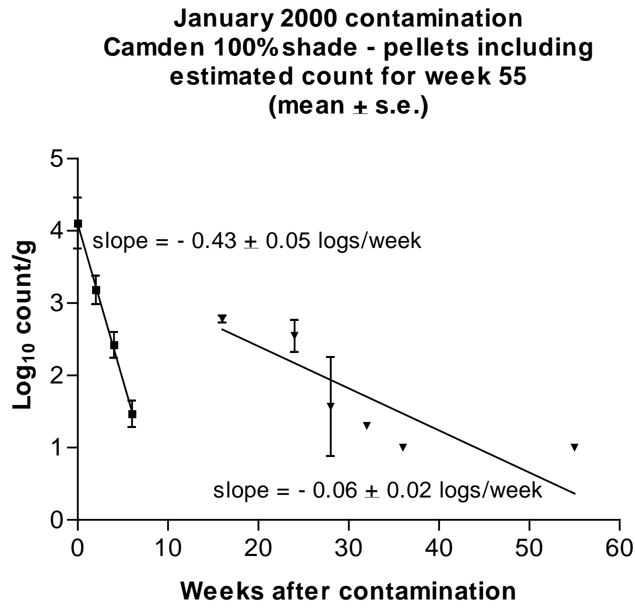


Table 3.12. Enumeration of *M. paratuberculosis* in selected culture positive samples from the contamination in January 1998. Each result is for a single sample (n=10 pellets or soil cores, mixed) tested at the time points shown after storage of samples at -80°C for up to 21 months until testing in July-August 1999. (count/g)

Months after contamination	Borenore		Carcoar	
	Pellets	Soil	Pellets	Soil
0	6.81×10^4 1.00×10^3 5.58×10^3 2.50×10^3		4.78×10^3 1.47×10^4 3.16×10^4 2.09×10^3	
1	8.00×10^1	0 1.05×10^2 0		0 7.50×10^1 3.00×10^1 3.50×10^1
2	0	0 0 2.00×10^1		0 0 2.00×10^1 2.00×10^1
3				
4		0 0 2.00×10^1		0 0 0
5				
6				0 0 0 0
7		0 0 2.50×10^1 2.00×10^1		0

These counts were undertaken using PBS as diluent and a starting dilution of 10^{-1}

Table 3.13. Enumeration of *M. paratuberculosis* in selected culture positive faecal pellet samples or grass from the contamination in November 1998. Each result is for a single sample (n=10 pellets, mixed) tested at the time points shown after storage of samples at -80°C for up to 19 months until tested in July-August 1999. (count/g)

Weeks after contamination	Pellets in pasture boxes at Camden	Grass in pasture boxes at Camden	Pellets in Plot 8 at Borenore	Pellets in Plot 8 at Carcoar
0	1.12×10^4		1.36×10^3	4.28×10^4
1	2.96×10^3	2.00×10^1	3.00×10^2	3.2×10^2
2	1.13×10^3		nt	2.1×10^2
3	4.57×10^2		0	0
4	1.00×10^3	0		0
5	4.20×10^2			0
6	2.10×10^2			
7	0			
8	0			
9	7.5×10^1			

These counts were undertaken using PBS as diluent and a starting dilution of 10^{-1}

Table 3.14. Enumeration of *M. paratuberculosis* in culture positive faecal pellets from the 100% shade pasture boxes at Camden that were contaminated in January 2000. Each result is the mean count of replicate samples (each sample containing n = 10 pellets, mixed) tested after storage for up to 13 months until tested in March 2001

Weeks after contamination	Mean count/g	No. replicates	Storage temperature of sample °C
0	2.4×10^4	6	- 20
2	2.4×10^3	6	- 20
4	4.4×10^2	6	- 80
6	3.8×10^1	4	- 80
9	0	3	- 80
16	6.1×10^2	2	- 80
24	4.7×10^2	3	- 20
28	9.4×10^1	2	- 80
32	2.0×10^1	2	- 80
36	1.0×10^1	2	- 20
55*	not tested	1	-

These counts were undertaken using PBS Tween 80 as diluent and starting dilutions ranging from undiluted to 10^{-2} depending on the expected counts; * the count for this sample can be assumed to be the same as that for week 36 based on time to peak growth index (Table 3.10)

6.3.4 Direct-PCR examination of pellet samples

Both culture positive and culture negative pellet samples from the November 1999 contamination of Camden boxes in 100% shade contained *M. paratuberculosis* DNA. Six of 6 samples from T=0, 6 of 6 samples from T=10, 4 of 5 samples from T=12 and 6 of 6 samples from T=32 were positive in direct-PCR. These results indicate that *M. paratuberculosis* cells persisted in pellets even though the organism could not be isolated in vitro at T = 12 and T = 32.

6.3.5 Appearance of plots

The appearance of vegetation on plots is given in Appendix IV. Note that vegetation would have provided shade as well as other changes to the micro-environment.

January 1998 and November 1998 contaminations

At Borenore in January 1998 there was little vegetative cover (2-3 cm high, 20% cover) on the unshaded plots compared to 10 cm high vegetation inside the shade house. Conditions at Carcoar were harsher, with no vegetative cover on unshaded plots and 4-6 cm high vegetation inside the shade house. There was a mixture of native and introduced grasses which changed in composition with the season, together with broadleaf weeds and clover. By mid June there was prolific cover both inside and outside the shade house at both sites, with heavy growth of *Echium plantagineum* at Carcoar. The vegetative cover was allowed to persist at both sites and provided additional shade to the plots until the November 1998 contamination.

Vegetation was mowed prior to the November 1998 contamination of plot 8 at both sites. Cover remained at <10 cm height thereafter, but there was almost complete coverage of the ground at Borenore and good coverage at Carcoar.

November 1999 and January 2000 contaminations

Plots

Vegetative cover ranged between 40 and 85% in the shaded plots compared to 50-95% in the unshaded plots and was maintained at a height generally less than 15 cm in both cases. The vegetation was grass dominant, with broad leaf weeds and clover.

Pasture boxes

By 1 week after the November contamination (10.11.1999) boxes at Camden had an even cover of green grass to 75 mm. Faecal material was visible on the surface and in some cases had been pushed up off the soil surface by grass shoots and was held in the air several centimetres above the ground.

Germination was delayed in boxes at Orange and other sites due to the disturbance of transport and excessive wetting. These boxes had variable grass cover, pellets and faecal material was still visible but generally depressed partly into the surface of the soil or was covered in mud. In some of these boxes there had been partial failure of seed germination. Heavy rain had fallen at most sites soon after deployment of the boxes. Later at Broken Hill a dried muddy crust containing faecal material was lifted into the air by germinating grass. There was subsequently reasonable germination at Orange and Armidale with an even cover developing in most boxes. At other sites grass cover was mostly uneven, patchy and light.

There was a good even germination within a week of the January contamination in boxes at Camden and Orange.

It was difficult to maintain similar watering conditions in boxes under different shade treatments at Camden due to protection from rainfall of boxes under 70% and 100% shade. Rainfall in 0% shade boxes maintained grass growth whereas grass was eventually allowed to brown off in the shaded boxes. Watering at Orange ceased as soon as grasses had reached the desired height. Growth continued only as a result of natural rainfall.

6.3.6 Weather records

Apart from premature failure of the soil moisture gypsum block sensors rendering data in those leads of no use, occasional electrical short circuits at connectors particularly affecting soil temperature readings, failure of the UV sensor on one unit, accidental severing of one soil temperature lead, failure of a laptop computer during data download causing loss of about 4 weeks data from one site and loss of calibration of the UV sensors in the last year of the project, useable weather data were obtained from both sites for relatively long periods.

Weekly maximum, minimum and average temperatures for air (dry bulb) and soil are provided in the following figures together with weekly total rainfall and weekly total solar radiation and UV radiation (Figures 3.10 - 3.14). The time point closest to each contamination date is shown in the date label on the X axis on each figure (Con). Data outages are indicated by breaks in the lines in each figure.

Rainfall was evenly distributed at Carcoar, Borenore and Camden, with periodic heavy falls of up to 100 mm per week at any time of the year. Maximum dry bulb air temperatures approached 40°C at Carcoar and Borenore and 45°C at Camden and minima fell below 0°C at each site.

Total weekly solar radiation levels exceeded 200 MJ/m^2 in summer and were as low as 25 MJ/m^2 in winter while total weekly UV levels were 5-7 W/m^2 in summer and 0.5 - 1 W/m^2 in winter.

Manual calibration of the UV radiation sensors was conducted using a hired UV radiometer in January 2000. Readings on the radiometer were taken hourly throughout the day and compared to hourly

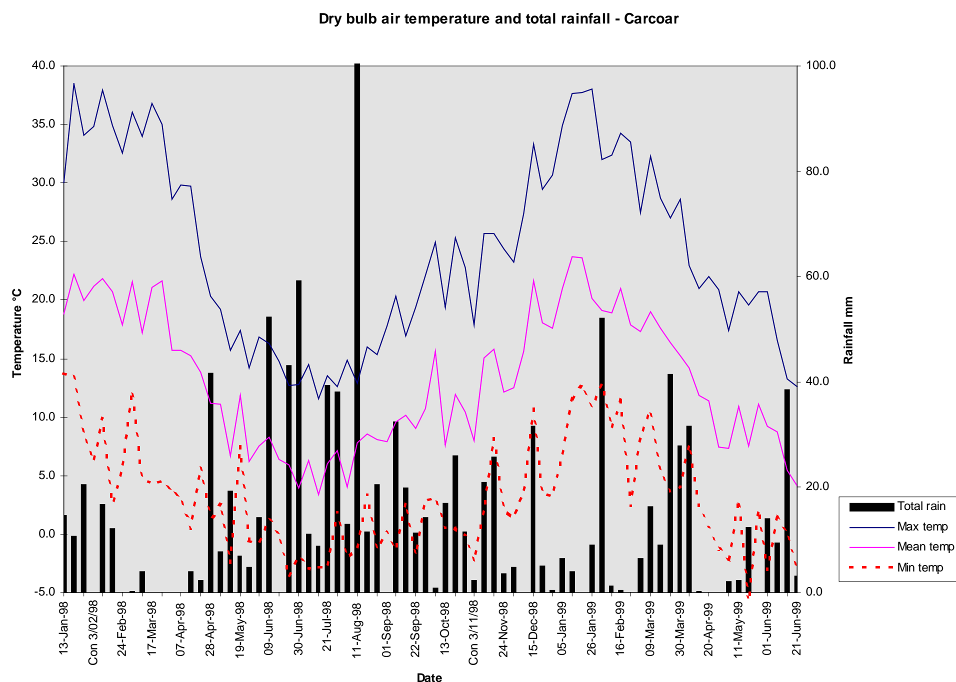
averages from the weather station. Readings from the radiometer were approximately half those from the weather station UV sensors at both Camden and Borenore at certain times of the day. Readings were difficult to take and compare due to the great sensitivity of the radiometer to incident angle. In addition software in the weather stations applied corrections due to time of day. However, using the hand held radiometer we were able to confirm that 70% of incident radiation was absorbed by the shade cloth at each location and that there was little or no UV radiation in the 100% shade area at Camden. The calibration of the weather station radiation sensors drifted during the trial, evidenced by increasingly lower UV and solar radiation readings at Camden compared to Borenore after January 2000. At the time of writing (June 2001) these issues are being pursued with the manufacturer of the weather stations with the aim of developing correction factors to facilitate analysis of the recorded data.

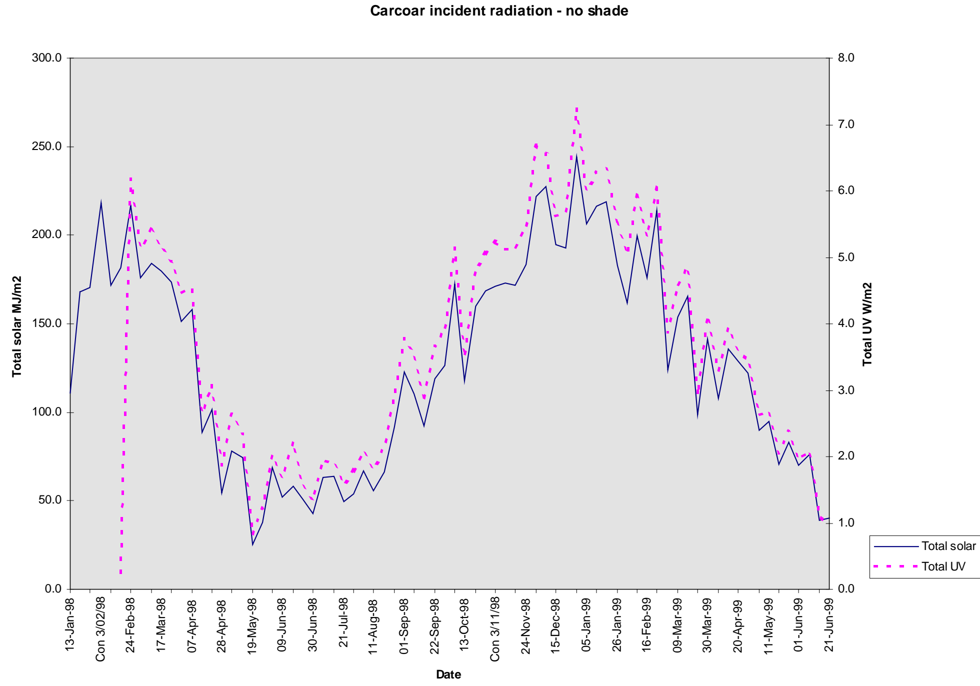
Soil temperature at a depth of 1 cm in unshaded plots or boxes ranged from about 50°C in summer to just above 0-5°C in winter at Carcoar and Borenore. In shaded plots and boxes the maximum was about 40°C. Maximum soil temperatures approached 60°C in unshaded boxes at Camden and conditions were less extreme in shaded locations - to 38°C in 70% shade and 30°C in 100% shade. The differences in temperature ranges between the shade treatments were striking.

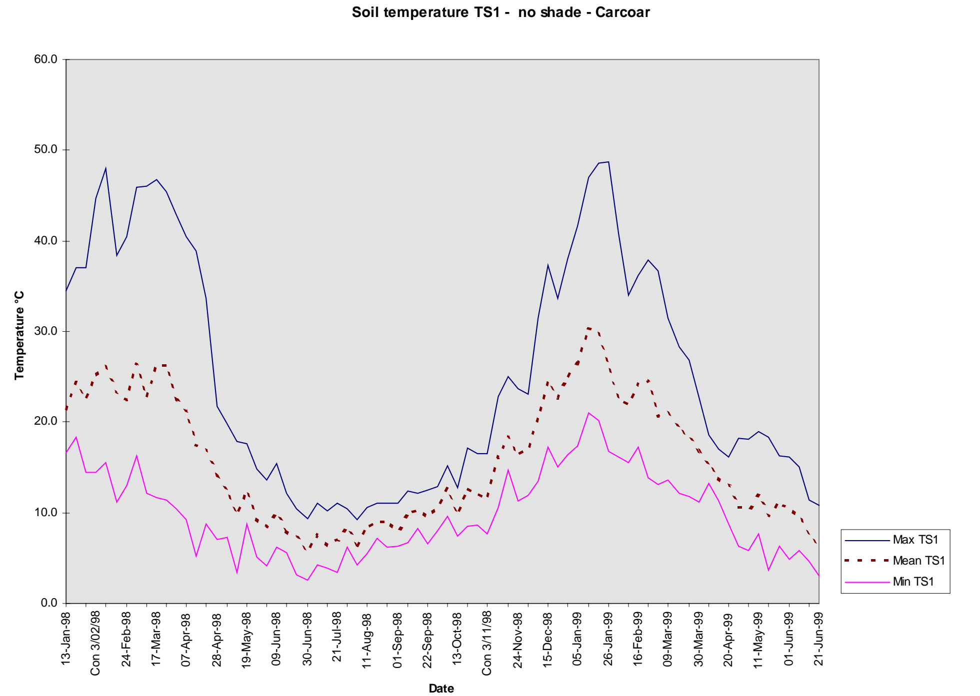
Because the 70% shade and 100% shade locations at Camden were also covered by a verandah roof, these sites were protected from natural rainfall.

Further statistical analysis of weather data beyond shade gradations in relation to culture outcomes depends on correction of raw data required due to drifting UV calibration. This and other relevant analyses will require considerable biometrical input and will be completed after the termination of the project.

Figure 3.10. Weather data for the January 1998 and November 1998 contaminations - Carcoar




Carcoar incident radiation - no shade
Total solar MJ/m2
Total UV W/m2
Date
Total solar
Total UV


Soil temperature TS1 - no shade - Carcoar
Temperature °C
Date
Max TS1
Mean TS1
Min TS1

Soil temperature TS3 - 70% shade, irrigated - Carcoar
Temperature °C
60.0
50.0
40.0
30.0
20.0
10.0
0.0
13-Jan-98
Con 3/02/98
24-Feb-98
17-Mar-98
07-Apr-98
28-Apr-98
19-May-98
09-Jun-98
30-Jun-98
21-Jul-98
11-Aug-98
01-Sep-98
22-Sep-98
13-Oct-98
Con 3/11/98
24-Nov-98
15-Dec-98
05-Jan-99
26-Jan-99
16-Feb-99
09-Mar-99
30-Mar-99
20-Apr-99
11-May-99
01-Jun-99
21-Jun-99
Date
Max TS3
Mean TS3
Min TS3

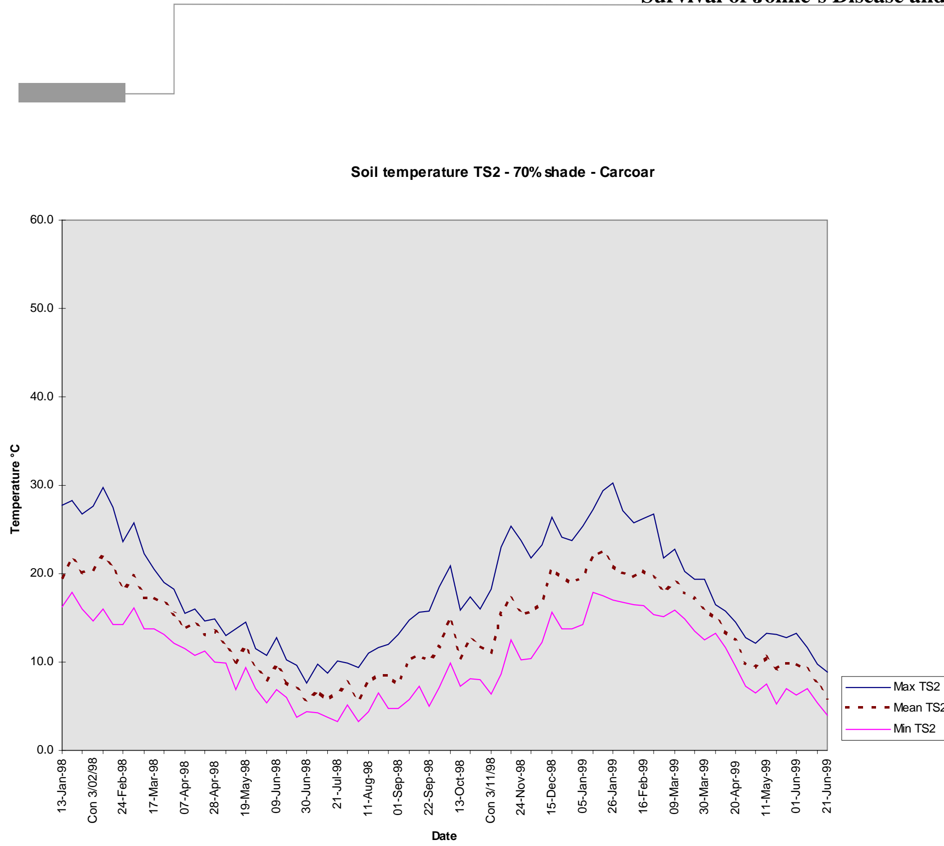
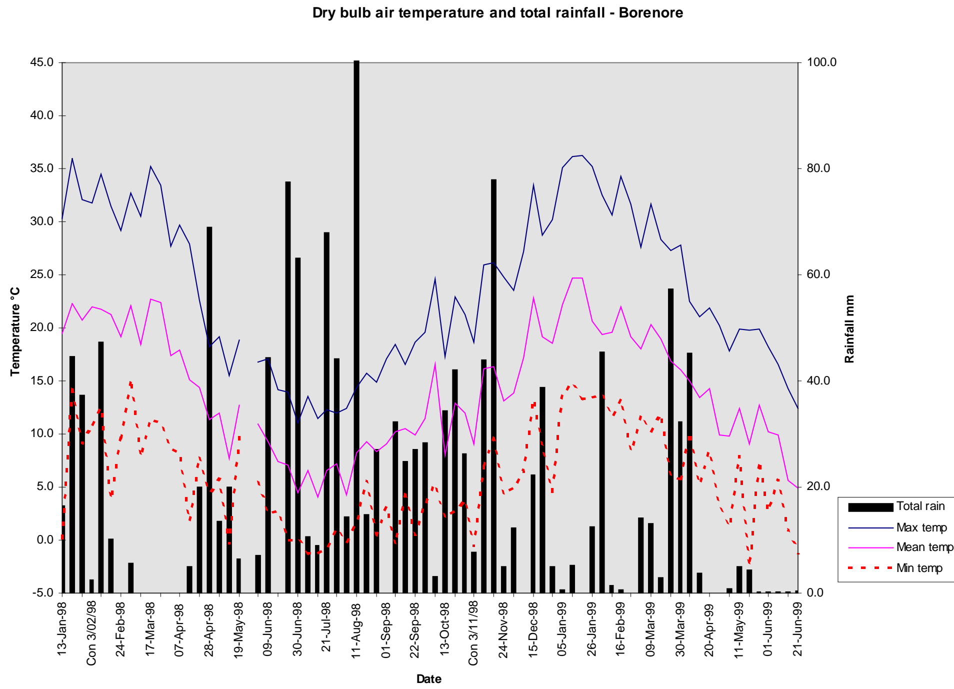
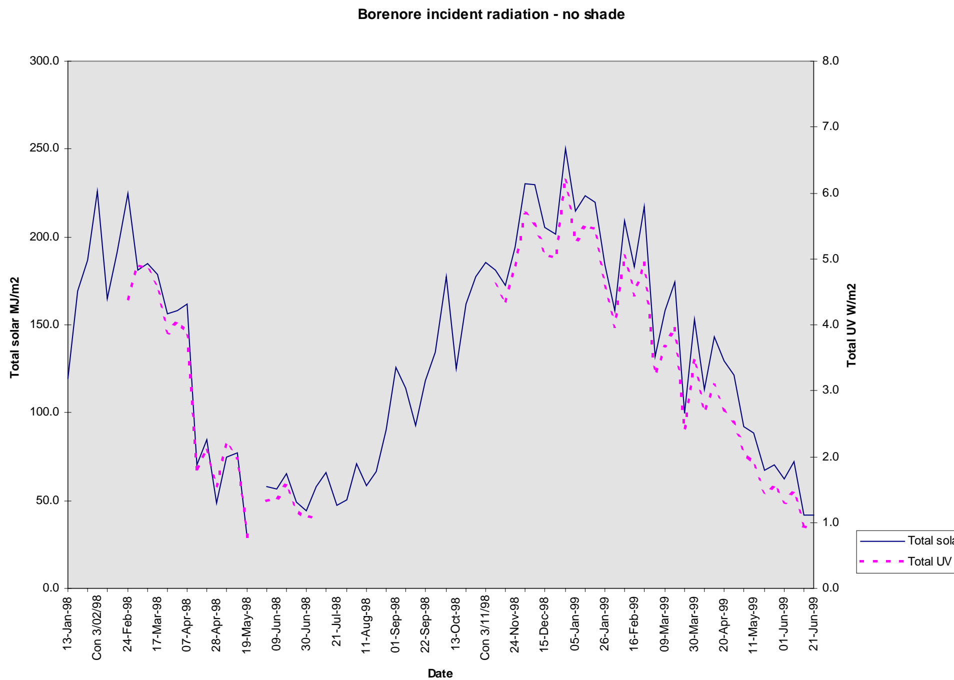
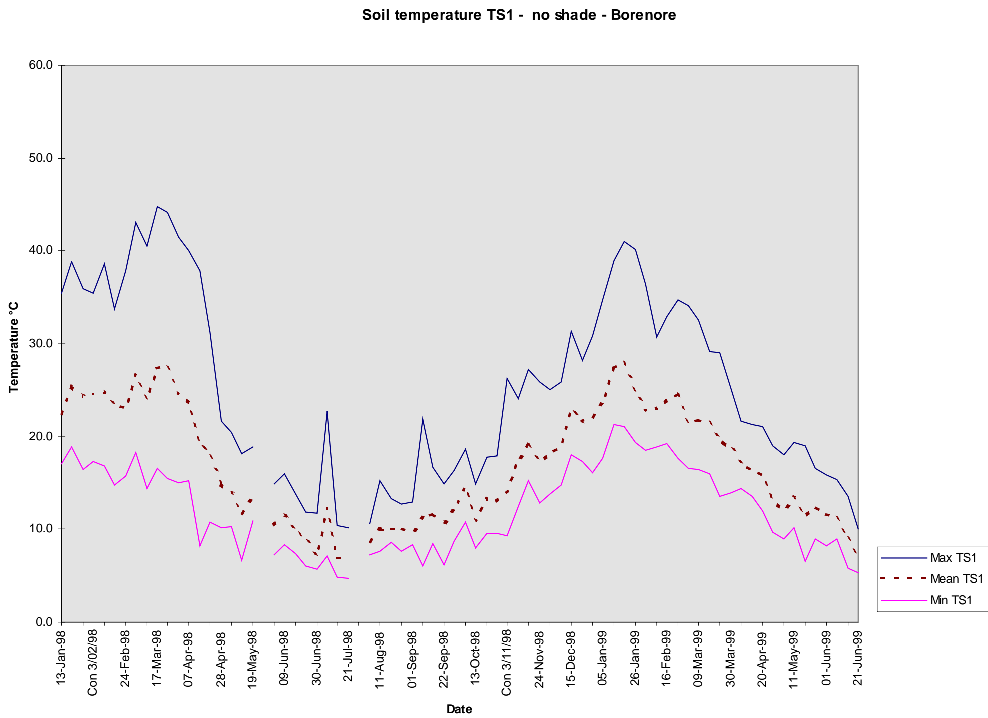
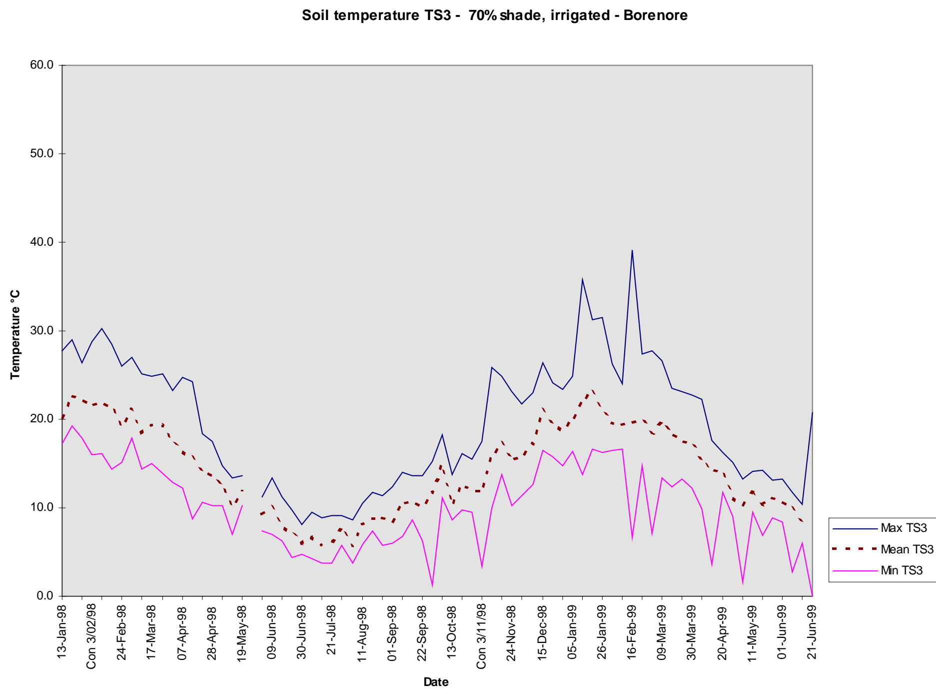


Figure 3.11. Weather data for the January 1998 and November 1998 contaminations - Borenore


Dry bulb air temperature and total rainfall - Borenore
Temperature °C
Rainfall mm
Date
Total rain
Max temp
Mean temp
Min temp


Borenore incident radiation - no shade
Total solar MJ/m2
Total UV W/m2
Date
Total solar
Total UV


Soil temperature TS1 - no shade - Borenore
Temperature °C
Date
Max TS1
Mean TS1
Min TS1


Soil temperature TS3 - 70% shade, irrigated - Borenore
Temperature °C
Date
Max TS3
Mean TS3
Min TS3

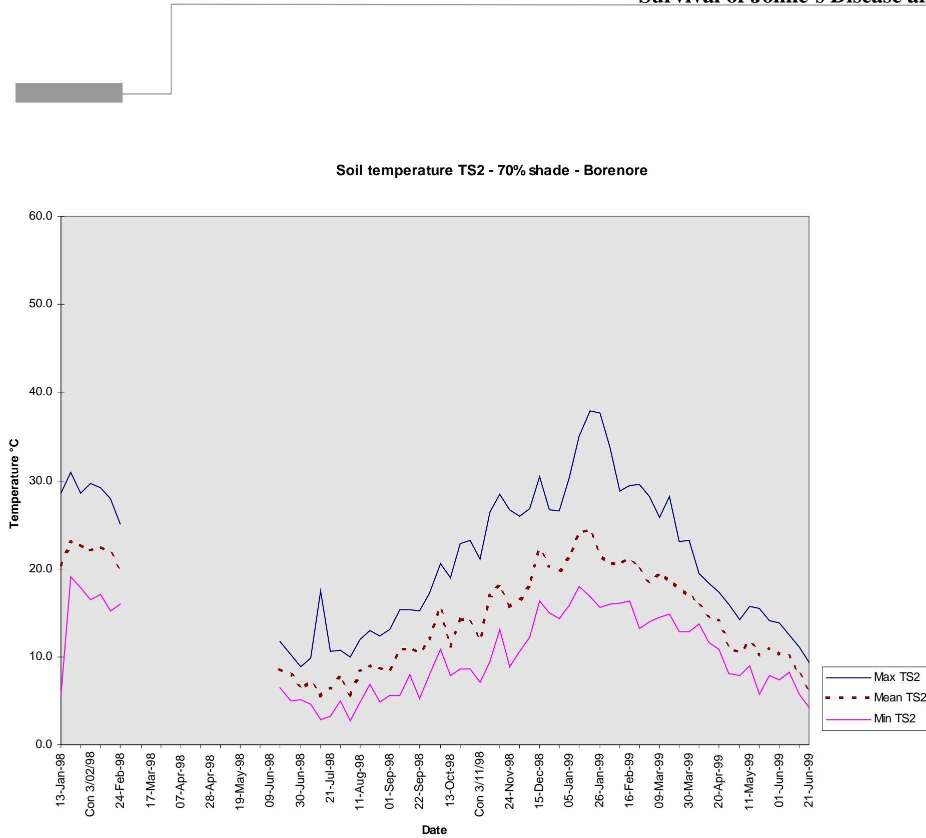


Figure 3.12. Weather data for the November 1998 contamination of pasture boxes at Camden. Data are weekly maximum, minimum and the calculated average temperature

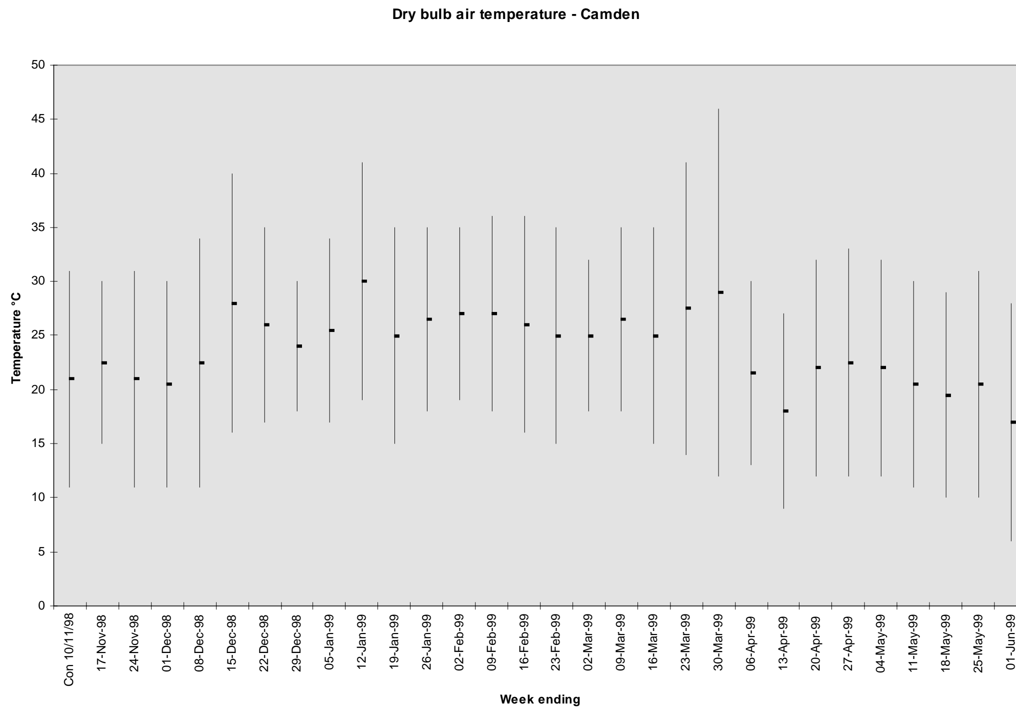
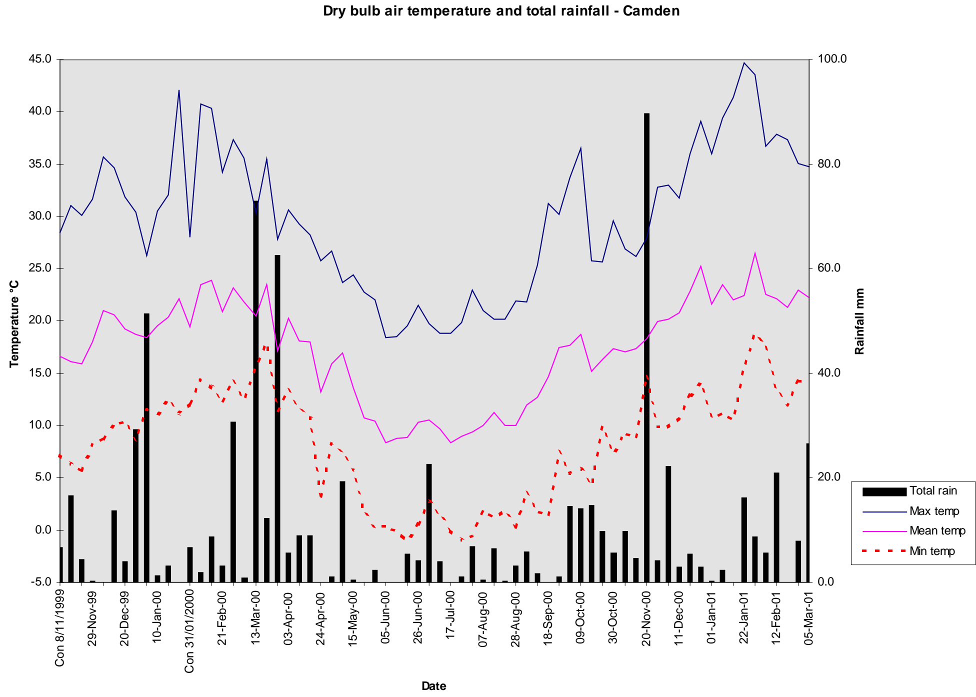
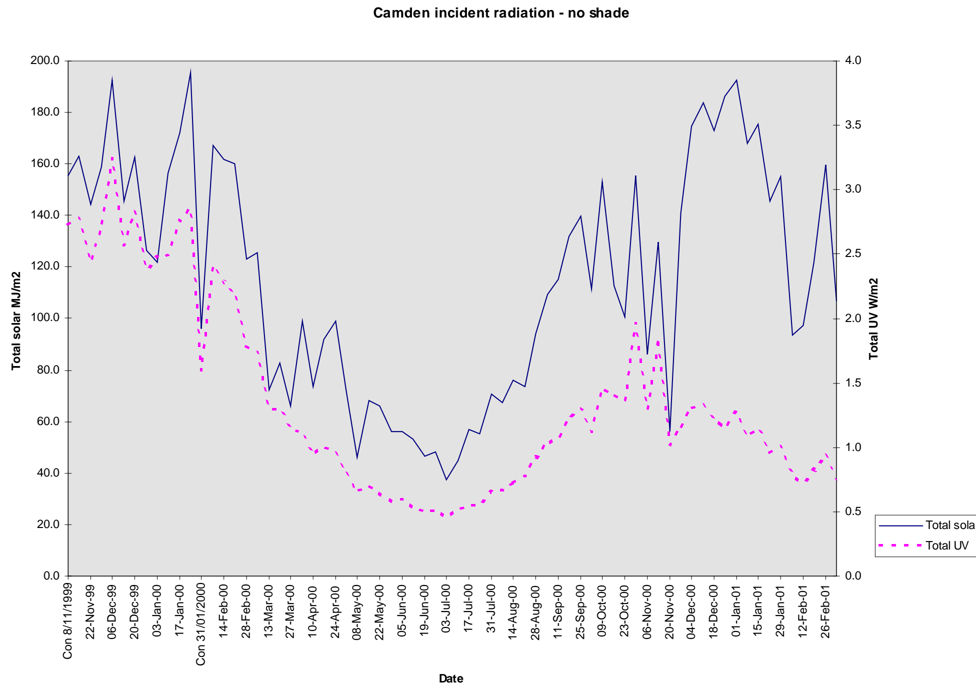
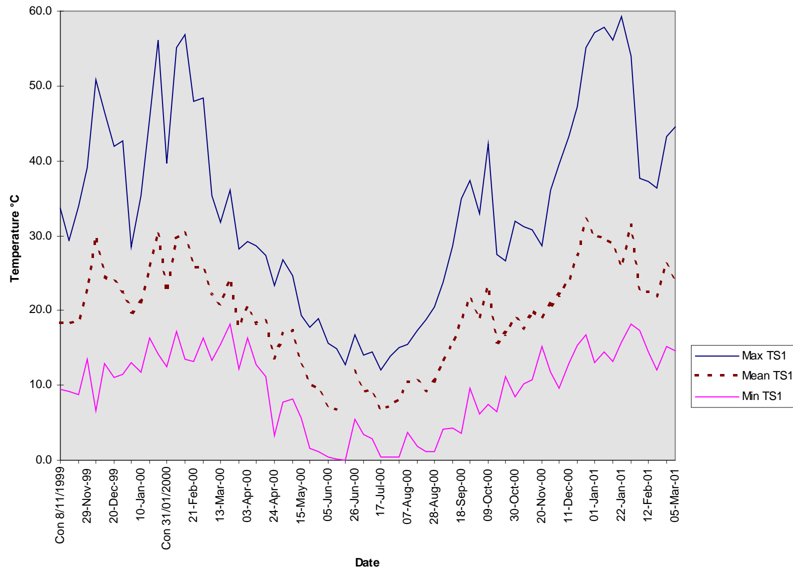
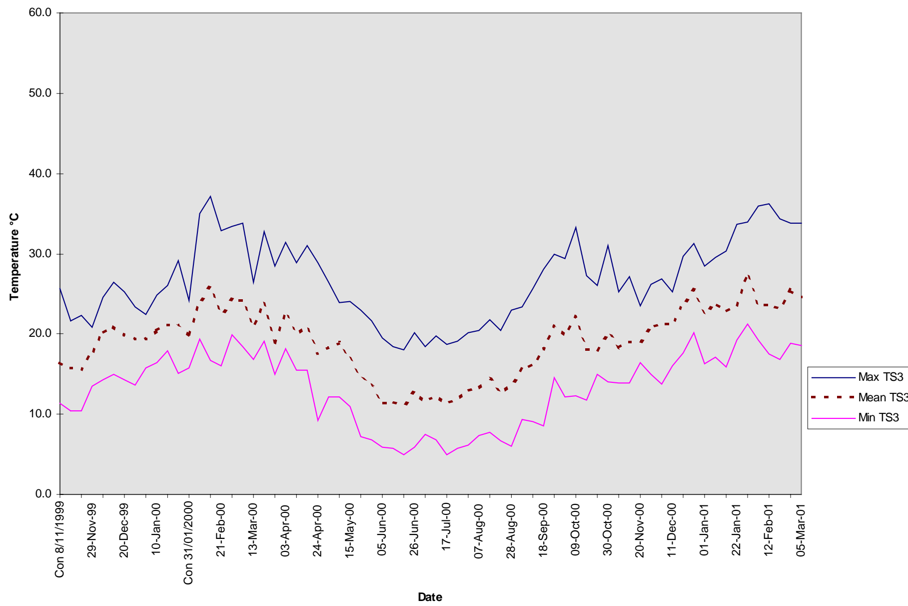


Figure 3.13. Weather data for the November 1999 and January 2000 contaminations - Camden






Soil temperature TS1 - pasture boxes - no shade - Camden
Temperature °C
Date
Max TS1
Mean TS1
Min TS1


Soil temperature TS3 - pasture boxes - 70% shade - Camden
Temperature °C
Date
Max TS3
Mean TS3
Min TS3

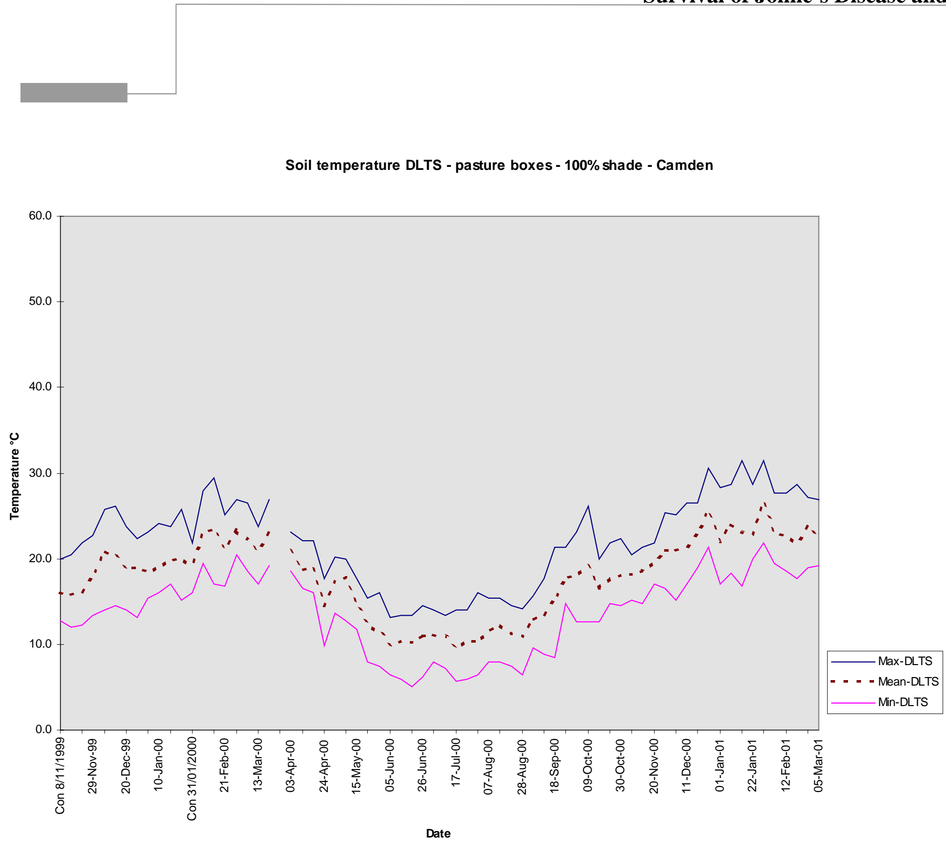
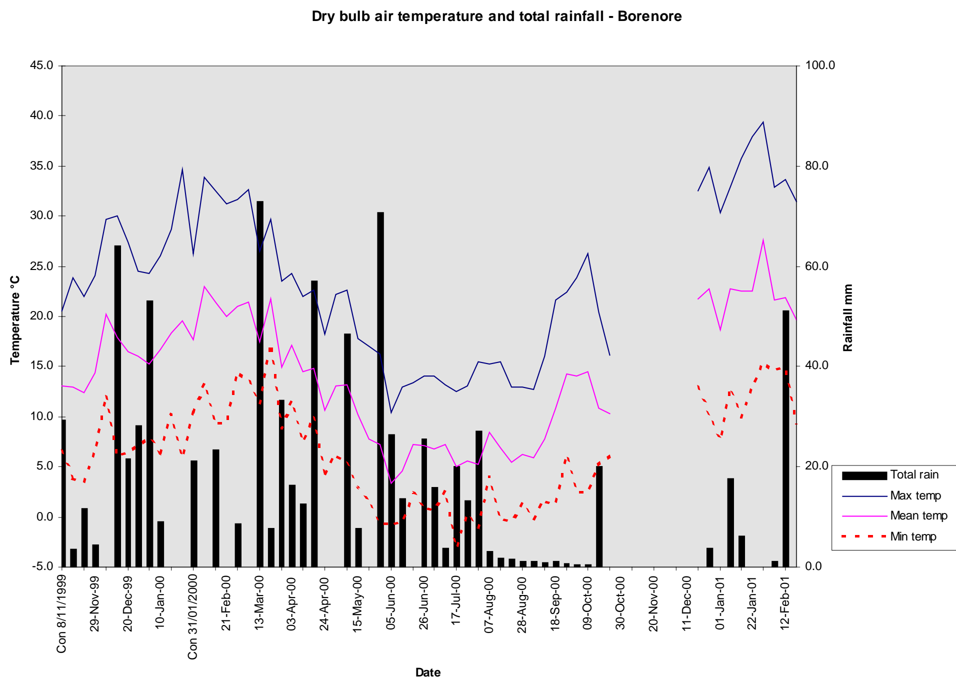
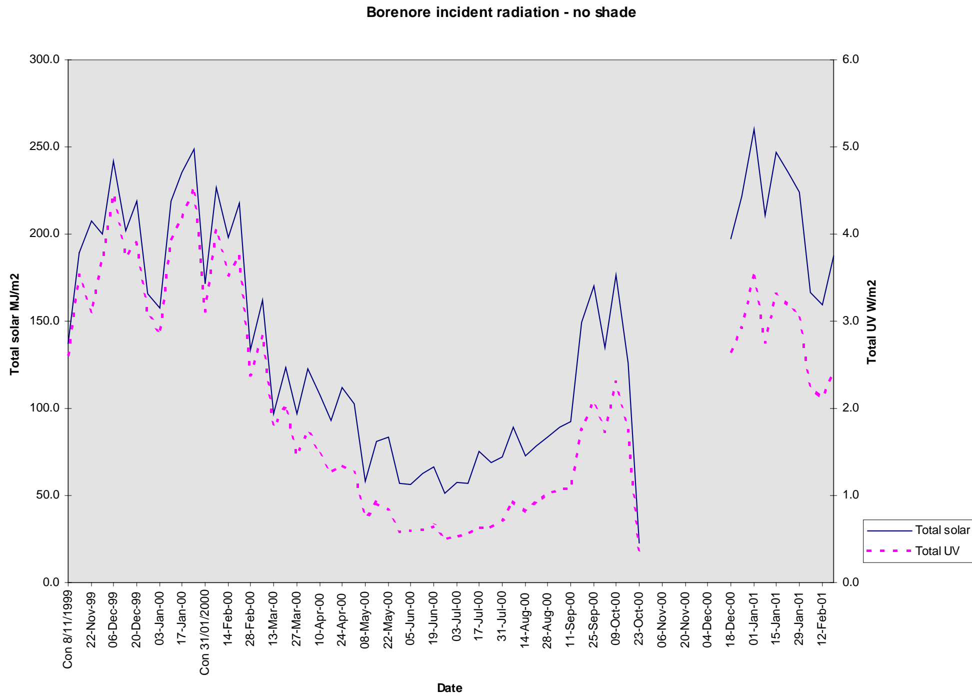
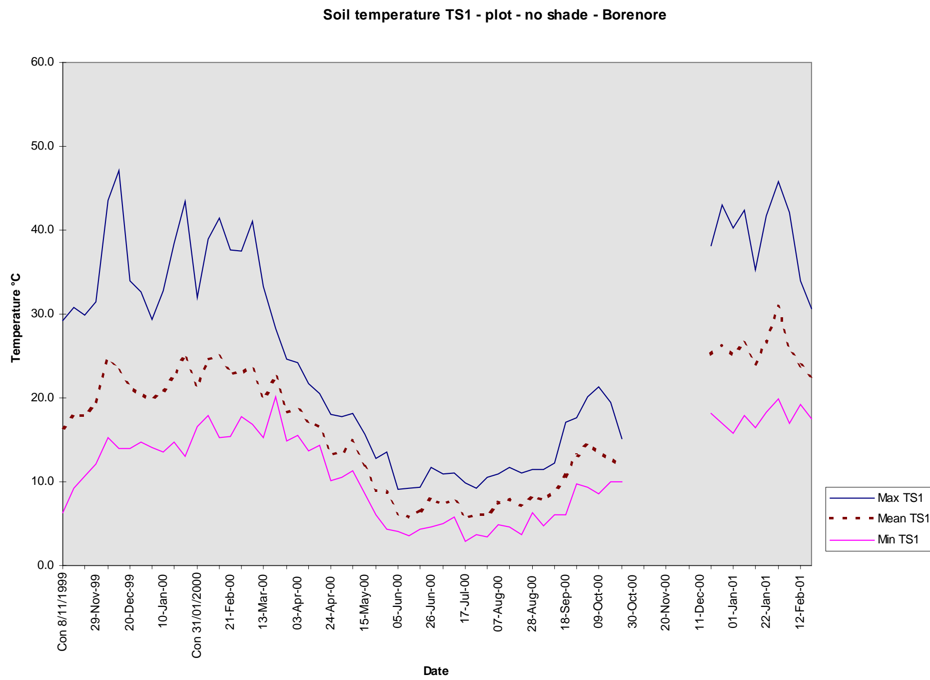
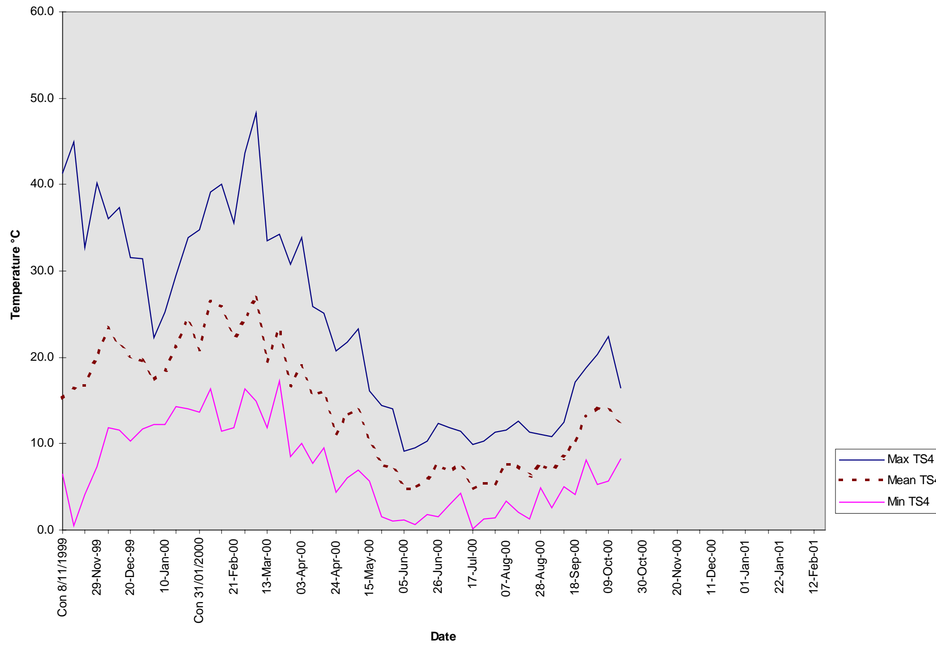


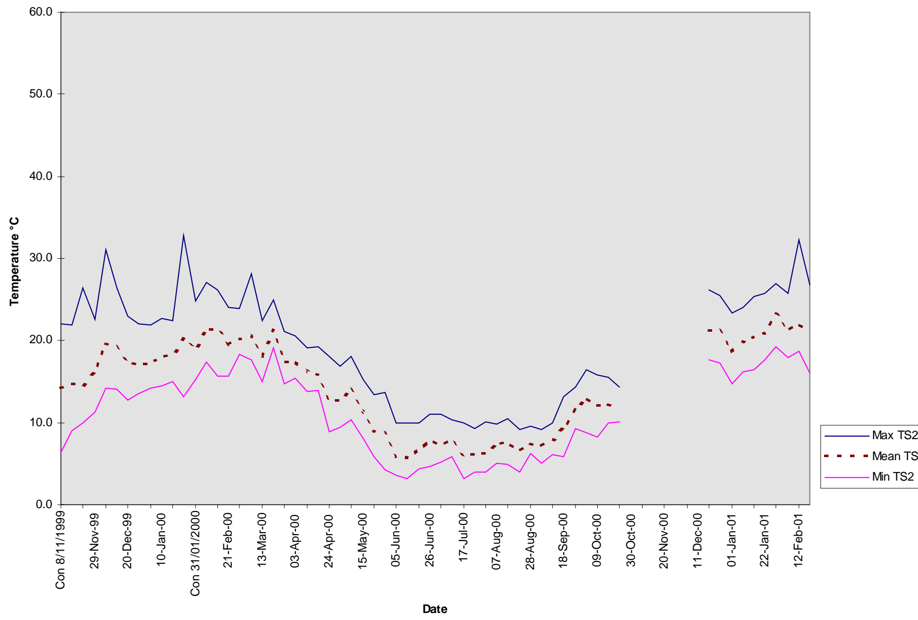
Figure 3.14. Weather data for the November 1999 and January 2000 contaminations - Borenore

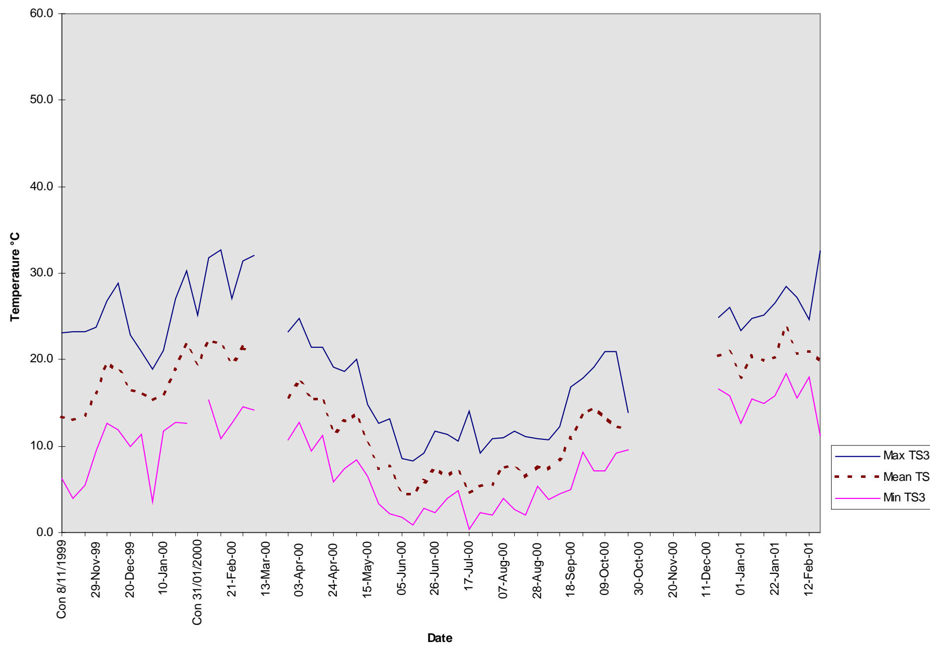



Borenore incident radiation - no shade
Total solar MJ/m2
Total UV W/m2
Date
Total solar
Total UV
Con 8/11/1999
22-Nov-99
06-Dec-99
20-Dec-99
03-Jan-00
17-Jan-00
Con 31/01/2000
14-Feb-00
28-Feb-00
13-Mar-00
27-Mar-00
10-Apr-00
24-Apr-00
08-May-00
22-May-00
05-Jun-00
19-Jun-00
03-Jul-00
17-Jul-00
31-Jul-00
14-Aug-00
28-Aug-00
11-Sep-00
25-Sep-00
09-Oct-00
23-Oct-00
06-Nov-00
20-Nov-00
04-Dec-00
18-Dec-00
01-Jan-01
15-Jan-01
29-Jan-01
12-Feb-01


Soil temperature TS1 - plot - no shade - Borenore
Temperature °C
Date
Max TS1
Mean TS1
Min TS1
Con 8/11/1999
29-Nov-99
20-Dec-99
10-Jan-00
Con 31/01/2000
21-Feb-00
13-Mar-00
03-Apr-00
24-Apr-00
15-May-00
05-Jun-00
26-Jun-00
17-Jul-00
07-Aug-00
28-Aug-00
18-Sep-00
09-Oct-00
30-Oct-00
20-Nov-00
11-Dec-00
01-Jan-01
22-Jan-01
12-Feb-01


Soil temperature TS4 - pasture box - no shade - Borenore
Temperature °C
Date
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Con 8/11/1999
29-Nov-99
20-Dec-99
10-Jan-00
Con 31/01/2000
21-Feb-00
13-Mar-00
03-Apr-00
24-Apr-00
15-May-00
05-Jun-00
26-Jun-00
17-Jul-00
07-Aug-00
28-Aug-00
18-Sep-00
09-Oct-00
30-Oct-00
20-Nov-00
11-Dec-00
01-Jan-01
22-Jan-01
12-Feb-01
Max TS4
Mean TS4
Min TS4


Soil temperature TS2 - plot - 70% shade - Borenore
Temperature °C
Date
Max TS2
Mean TS2
Min TS2
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Con 8/11/1999
29-Nov-99
20-Dec-99
10-Jan-00
Con 31/01/2000
21-Feb-00
13-Mar-00
03-Apr-00
24-Apr-00
15-May-00
05-Jun-00
26-Jun-00
17-Jul-00
07-Aug-00
28-Aug-00
18-Sep-00
09-Oct-00
30-Oct-00
20-Nov-00
11-Dec-00
01-Jan-01
22-Jan-01
12-Feb-01


Soil temperature TS3 - pasture box - 70% shade - Borenore
Temperature °C
Date
Max TS3
Mean TS3
Min TS3
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Con 8/11/1999
29-Nov-99
20-Dec-99
10-Jan-00
Con 31/01/2000
21-Feb-00
13-Mar-00
03-Apr-00
24-Apr-00
15-May-00
05-Jun-00
26-Jun-00
17-Jul-00
07-Aug-00
28-Aug-00
18-Sep-00
09-Oct-00
30-Oct-00
20-Nov-00
11-Dec-00
01-Jan-01
22-Jan-01
12-Feb-01

6.4 Discussion and conclusions

6.4.1 Duration of survival

The maximum duration of survival of the organism in faecal material applied to soil in pasture plots or boxes in experiments commencing between January 1998 and January 2000 was at least 55 weeks. The most prolonged survival occurred in a fully shaded environment at Camden. The faecal material was not exposed to sunlight and was protected from extremes of temperature and from natural rainfall, which at times was extremely heavy. For most of this period the faecal material was dry, in fact very dry, as the pasture boxes were not watered after the initial period. Therefore moisture is not a factor promoting survival in a shaded environment. This conclusion was reached also from statistical analysis of the results of the January 1998 contamination experiment in which some plots were irrigated. Observations from the shade treatments can be considered to be a worst case scenario. The duration of survival was less in faecal material exposed to some sunlight and less still in faecal material fully exposed to the extremes of weather in the same trial; these results were statistically significant. The components of sunlight responsible for the effect are uncertain but could include infra-red (heat), ultra-violet and visible light. In shaded environments temperature per se, fluctuations of temperature, evaporation and ultra-violet radiation levels were lower than in exposed environments. Rainfall may have caused some leaching of bacteria from faecal material, but we were unable to significantly reduce the contamination levels in faecal material in a laboratory trial in which a rainfall event of 400 mm over 4 days was simulated (see Part 5). Therefore it is unlikely that the organism was eluted completely from faecal material exposed to natural rainfall in pasture boxes and for this reason sunlight is believed to be the main factor influencing survival.

This result is in direct contrast to results of the study on survival of the cattle strain of *M. paratuberculosis* in soil conducted at the Victorian Institute of Animal Science[5]. The reasons lie in experimental design. In the Victorian study, contaminated soil samples were exposed to UV light from fluorescent tubes. As UV light does not penetrate beyond the immediate surface layer of soil this treatment was ineffective in sterilising the entire soil sample. Even though the soil was mixed after each weekly sampling for 8 weeks, surface exposure to UV sterilisation would not have made much impact on the total bacterial count in the mixed soil. In a recent American study of the effect of UV light on the cattle strain of *M. paratuberculosis* the organism was irradiated while suspended in distilled water[15]. The organism appeared to be no more resistant than many other bacterial species. There were other differences between the present study and the Victorian study that make it difficult to compare results, for example the use of constant soil temperatures in the latter.

Although the maximum duration of survival was at least 55 weeks in the shade at Camden, survival for much shorter times was also observed. In pasture boxes exposed to full sunlight at both Camden and Borenore, the duration of survival was 12-16 weeks after the November 1999 and January 2000 contaminations in which pasture was removed to simulate grazing. Shading from growth of vegetation may explain the observation of survival for 7 months after the January 1998 contamination in plots that were not otherwise shaded.

The value of pasture box experiments conducted in Wagga Wagga, Goulburn, Cobar, Armidale and Broken Hill was discounted by the adverse effect of transportation of the boxes with pooling of water and partial burying of faecal material, and soon thereafter by extremely heavy rainfall. Nevertheless survival at these locations was at least 4-9 weeks but less than 12 weeks after the November 1999 contamination.

The duration of survival assessed in boxes was comparable to that observed in plots although there were some differences, generally favouring survival in boxes. Boxes are therefore a useful substitute for plots and may be used to advantage in future studies because they are simple to set up and maintain and the contamination can be safely and readily contained.

Overall, the results indicate potential for long term survival of the organism, particularly when it is clear that the culture method detects less than 10% of the viable organisms present in soil. The observed durations of survival were therefore underestimates; given observed decay rates the durations

observed were probably underestimated by several months. In addition the observed durations of survival depend on the levels of contamination applied at the start of each experiment and do not indicate the maximum potential duration of survival. If higher levels of contamination than were used in these trials exist on OJD affected farms, survival will be longer than observed in these trials. For this reason the results of experiments to measure decay rate are important, because these can be extrapolated to situations with different levels of contamination.

6.4.2 Patterns of recovery of the organism

In general, cultures were positive from all samples collected soon after contamination (T=0), indicating even spread of contaminated faecal material and adequacy of the sampling methods, and then for a variable period afterwards, with a general reduction over time in the number of culture positive replicates, and a prolongation in the incubation period required to reach peak growth index. These factors are consistent with a gradual decline in the viability of the organism.

However, there were unexpected features in the data. Firstly, the time required to reach peak growth index tended to stabilise at less than 12 weeks, often at around 9 weeks of incubation, with subsequent cultures being negative. With a progressive decline of bacterial numbers this interval would have been expected to have increased to the maximum allowed (20 weeks). That it did not do this suggests a sudden reduction in viability or ability to culture. Secondly, there was recovery of the organism from soil and faecal pellets after negative culture results from earlier time points. This gave the appearance of intermittent recovery of the organism. Thirdly, associated with renewed recovery of the organism sometimes there was a reduction in the time required for cultures to reach peak growth index compared to earlier time points, and in a few cases there was also a sudden increase in the proportion of culture positive replicates (eg November 1999 contamination, EMAI boxes, 100% shade, 16 weeks, Table 3.10). This is summarised in the Table 3.15.

Table 3.15. Intermittent culture positive outcomes

Experiment	Time when all cultures negative	Time when cultures first again positive	Time to peak GI relative to previous time points
January 1998, Borenore plots	5 months	6-7 months	similar or reduced
January 1998, Carcoar plots	5 months	6-7 months	similar or reduced
November 1998, Borenore & Carcoar plots	4-9 weeks	not applicable	
November 1998, Camden, boxes	4-12 weeks	not applicable	
November 1999, Borenore plots	10 weeks	12 weeks	similar
January 2000, Borenore plots	4-9 weeks	6-10 weeks	reduced or increased
November 1999, Borenore boxes	10 weeks	12 weeks	reduced
January 2000, Borenore boxes	4-16 weeks	8-24 weeks	similar or increased
November 1999, Camden boxes	4-12 weeks	12-16 weeks	reduced
January 2000, Camden boxes	2-10 weeks	12-16 weeks	similar or reduced

Coincident with reductions in time to peak growth index an increase in the viable count of the organism was measured by most probable number methods. For example the rise in the decay curve in Figure 3.8 between weeks 3 and 5 coincides with a reduction in time to peak growth index from 8 to 6 weeks (Table 3.7). Similarly the rise in the decay curve in Figure 3.9 coincides with a reduction in time to peak growth index from 10 weeks to 6 weeks (Table 3.10).

There are several possible reasons for these observations:

1. Uneven contamination - i.e. faecal material was not evenly distributed in the plots and boxes so that it was not included in all samples. This is unlikely because the methods used to contaminate plots and boxes involved relatively even dispersal of material. This was confirmed by checking samples collected at T=0. The faeces were well-mixed and in any case were collected from a number of sheep with multibacillary disease, so that the component faeces in the mixture all had high levels of contamination (differences between sheep with pelleted and non-pelleted faeces are discussed in the section below on validity of most probable number counts). This would tend to overcome any deficiencies in mixing of faeces prior to contamination. Further evidence for even

contamination came from the demonstration of intact *M. paratuberculosis* DNA in culture negative faecal pellet samples.

2. Sampling effect. The sampling method involved collection of material from 20 sites (2 replicates of 10 per pool) per plot or box each time. In the case of plots, sampling sites were chosen randomly, and for pellet samples, pellets were taken if visible anywhere by selecting them from the cell nearest the one that was randomly selected. T=0 samples were consistently culture positive. If the intermittent culture results always involved small numbers of positive replicates sampling could be a factor but in some instances a large proportion of replicate samples were culture positive after previously testing negative. Further evidence for the adequacy of sampling came from the demonstration of intact *M. paratuberculosis* DNA in culture negative faecal pellet samples. Therefore sampling effects are most unlikely to explain the observation.

3. Laboratory error. Systematic variation in the sensitivity of laboratory culture due to batch testing of samples is a theoretical consideration (media effect, operator effect). However, there was little or no overlap in testing of batches of samples from the relevant time points across the various experiments. In other words, samples collected at 10 weeks in different experiments were tested at different times using different batches of media, often by different technicians. That media and laboratory techniques were not responsible for the observations is indicated also where groups of samples were culture positive at time points where other groups of samples were culture negative, i.e. where the samples were tested using the same batch of media by the same technician - for example at 10 weeks for the November 1999 contamination of boxes at EMAI.

4. An environmental effect that causes the organism to change its binding properties with faecal material or soil components so that its availability in the culture system changes over time. In Part 1 of this report data were presented to suggest an association of the organism with soil particles that results in 90 to 99% of the organisms in a sample being excluded from the culture system. This phenomenon is poorly understood and may not have fixed properties. However, it is difficult to imagine that such changes could occur within faecal pellets in a protected, dry location and for this reason it is very difficult to explain the data in Table 3.10 or Figure 3.9 in this way.

5. A biological effect. The organism may be able to enter a dormant or viable-non-cultivable state and later revert to a vegetative form. It may also be able to replicate outside the mammalian host. Intact DNA of the organism was demonstrated in pellet samples at time points where all samples were culture negative. While the DNA could just as easily have been from dead bacteria its presence clearly establishes the continuing presence of intact bacterial cells (or the DNA would have been degraded by ubiquitous DNAases of other organisms present in faeces).

Based on the consideration of these factors, the data are most consistent with a biological effect.

6.4.3 Biological effects to explain patterns of recovery of the organism

Neither dormancy nor environmental replication are biological properties that have been reported for *M. paratuberculosis*. However, the patterns of recovery of *M. paratuberculosis* from soil and faeces over time, which were seen consistently across experiments in this project, are typical of those observed for the so-called enteric bacteria *Escherichia coli* and *Salmonella* serotypes in experiments to evaluate their survival in sewage by-products applied to agricultural soils in NSW[16]. The findings have been interpreted as environmental regrowth of the organisms and have been observed in other studies. Suggestions that *M. paratuberculosis* is capable of similar behaviour is likely to be a controversial finding and will stimulate considerable debate. However, it should be remembered that *M. avium* subsp. *avium*, the organism believed most closely related to *M. paratuberculosis* is an environmental organism with full replicative capacity outside the host.

Dormancy is defined as the state permitting survival of a non-sporing bacterial cell for a period without requiring replication of the cell. It is a genetically programmed function and is reversible. It is induced by an unfavourable environment, classically when an essential nutrient required for growth becomes limiting. The evidence for dormancy is inability to culture the organism, i.e. the organism can no longer form colonies on conventional media[17]. When the environment again becomes favourable the cells

divide and become detectable. In rapidly growing bacterial species dormancy is associated with expression of specific genes, at least some of which are known in mycobacteria. Altered gene expression leads to increased ability to scavenge limiting nutrients (e.g. carbon, iron, phosphate, nitrogen) by acquiring increased affinity for the nutrient. Energy expenditure is required during dormancy, acquired by metabolism of stores of polycarbon molecules and by degradation of ribosomal RNA.

The evidence for dormancy among mycobacterial species is scant and mostly indirect but is now being mentioned in reviews of the growth of mycobacteri**a**[18]. The evidence began as an extension of the concept of latency of clinical infection which is well-known in mycobacterial disease and was probably associated in the past with the concept that organisms "persisted" in lesions. There is clinical evidence of dormancy in leprosy when disease occurs years or decades after exposure, although this discounts the issue of variable and prolonged incubation period per se. Similarly in tuberculosis, an old sub-clinical lesion, walled off in the lung by inflammation and no-longer shedding organisms into respiratory secretions, may be reactivated after many years. Perhaps more convincing is the failure to cultivate mycobacteria from diseased humans or animals after several months of antibiotic treatment, despite indirect evidence of persistence of the microbes in tissues (eg PCR detection), followed by recrudescence of infection detected by culture[19]. But even this kind observation could simply be due to culture methods being insensitive. There is no suggestion that dormancy as such occurs within animals with *M. paratuberculosis* infection.

Better evidence of dormancy in mycobacteria comes from in vitro studies. In the case of *M. tuberculosis*, for example, oxygen depletion of cultures can lead to reduced growth rate[20] and increased resistance to antibiotics[21] and prolonged in vitro culture with reduced growth rate is associated with expression of heat shock proteins in the stationary phase of culture[22]. Dormancy induced by oxygen depletion has been shown to occur now also in cultures of *M. bovis*[23] and *M. smegmatis*[24]. *M. tuberculosis/M. bovis* (slow growing pathogen) and *M. smegmatis* (fast growing saprophyte) are not closely related and yet their physiological response to low oxygen tension is very similar. This adds considerable weight to the proposition that *M. paratuberculosis* is capable of dormancy.

The results of the present study where *M. paratuberculosis* was seeded into pasture plots and went through stages characterised by being initially recoverable, then declining in abundance, then no longer being recoverable, then again being recoverable in culture is strongly suggestive of dormancy.

The stimulus for dormancy in the present study is unclear but by inference from studies with other organisms there must have been an environmental stimulus such as depletion of an essential growth factor or oxygen tension declining. Similarly, there must have been an environmental stimulus favourable for reversion to the vegetative state. Two hypotheses that can explain the observed growth pattern of *M. paratuberculosis* will now be considered, the first where there was one stimulus for dormancy, the second where there were multiple stimuli.

Hypothesis 1

M. paratuberculosis progressively dies off, the evidence being a progressive decline in the proportion of culture positive replicate samples and a progressive increase in the time required to obtain growth in cultures, until such time as all samples are culture negative, but dormant cells remain (T=D). The initial rapid decline from T = 0 in the number of viable cells would be expected to leave a minority of cells relative to those present at T=0 being capable of displaying dormancy. Thus the level of growth after reversion to the vegetative state (T=V) would be lower than that at T=0 and at the same or a lower level than that seen immediately prior to the dormant state (T=D-1). However, as discussed, growth rates at T=V were sometimes greater than at T=D-1. Given only one environmental stimulus for dormancy, environmental replication of the organism after reversion from the dormant to the vegetative state is required to explain the observations.

Hypothesis 2

In the second hypothesis, a series of different environmental stimuli for dormancy are present, each acting on a different sub-population of *M. paratuberculosis*, perhaps at different times. These sub-populations enter the dormant state at different times, creating the effect of steadily declining bacterial

recovery rates over time, until cultures become negative. Later, more than one sub-population is reactivated synchronously by changing environmental conditions, leading to the observation of apparently increased recovery rates at T=V compared to T=D-1. Environmental replication is not required to explain the observations.

Both scenarios require genetic diversity in the population of *M. paratuberculosis*, the second scenario requiring a very diverse population with genes responding to multiple stimuli for dormancy. As genetic diversity within *M. paratuberculosis* is believed to be minimal, the second scenario seems less likely than the first. It is difficult to avoid the possibility that environmental replication might have occurred during these experiments.

Further experiments are required to evaluate dormancy and environmental replication in *M. paratuberculosis*. However, notwithstanding the possibility of dormancy and environmental replication, under the conditions of the present study survival was finite.

6.4.4 Decay rate of the organism

Counting methods

The most common method of counting *M. paratuberculosis* in published studies is by simple colony counts. The appropriate method for doing this, known as the Miles and Misra drop plate technique, requires use of multiple dilutions and replication but seems seldom to have been used. An alternative method involving 50% end-point titration can be applied to organisms in broth cultures and provides an estimate of the most probable number of organisms present because colonies are not observed. It has not been used to enumerate *M. paratuberculosis* until recently. It is the most appropriate method for the sheep strain which grows more readily in broth culture than on solid media. Most probable number counts are time consuming and expensive to perform because they require multiple dilutions to ensure an end-point is reached and replication to ensure accurate estimation of that end point. For example the data in Table 3.14 are the net result of 900 individual BACTEC cultures.

Validity of most probable number estimates

Absolute counts do not affect the estimation of decay rate provided that the same factors equally influence all samples because only the slope of the decay curve is required. Two possible anomalies were noted in the data and were carefully considered.

Firstly, in the case of samples from the January 1998 and November 1998 contaminations, many previously culture positive samples had apparent zero counts. End point dilution assays for these samples all commenced at the 10^{-1} dilution, which reduced sensitivity by 1 log compared to the original cultures. Additional freeze-thaw cycles also applied to the samples for enumeration (see below).

Secondly, the retrospective counts for T=0 samples were up to 2 logs lower than the initial counts from the faecal mixtures. T = 0 samples when subjected to enumeration had already been frozen and thawed a number of times (prior to their original culture and after their original culture, and possibly also during freezer failures or transfers) whereas the samples of faecal mixtures for enumeration were frozen only once. Loss of viability during storage of samples at -80°C is considered to be unlikely based on identical counts for the November 1999 and January 2000 contamination mixes and on other unpublished data. Sampling effects may also explain the anomaly. The faecal mixture consisted of naturally formed pellets plus pellets "manufactured" from soft faeces using chaff as a binder; the natural pellets probably contained fewer organisms as they were from animals in an earlier stage of disease; sampling from plots/boxes would probably have been biased to naturally formed pellets as they were easier to identify visually, were denser and less likely to break down over time.

The validity of counts is further supported by the pattern of counts matching the changes in incubation time required to reach peak growth index observed when the samples were cultured originally as discussed above under patterns of recovery of the organism.

Are D-values sufficiently representative to use in estimating risk?

The D-values were measured using selected culture positive samples. For later time points these samples were found among culture negative samples. For example in the January 2000 contamination,100% shade pellet series (Figure 3.9) at the 36 week time point there were 2 culture positive samples while 10 were culture negative. Thus positive samples represented 20% of the samples present and counts averaged across all samples would have been lower. For this reason the D-values reported may tend to underestimate the true D-value. However, the magnitude of this effect was fairly small where a two stage decline was fitted (Figure 3.9) as all samples in the second phase were affected similarly and the slope of the line would not have changed had this correction been applied.

Choice of D-value for estimation of duration of survival on farm

As noted in the results, D-value estimates depend on the period of observation. This is reiterated in Table 3.15. The trial circumstances are also given to assist interpretation. In each case the estimates were based on samples from fully shaded or partially shaded treatments. As shade was shown to be a significant factor in survival of the organism these D-values can be assumed to be the worst case scenario for terrestrial locations. Decay rates for unshaded locations are proposed later in this report.

The rapid and slow decay phases observed in this study were noted by the early workers who studied the cattle strain of *M. paratuberculosis*[4,6,7].

Table 3.15. Decay rate estimates of *M. paratuberculosis* from three experiments

Period of observation (months)	D-value (logs/month)	Shade	Sample	Start time	Source of data
0 - 1.5	1.7	full[1]	pellets from boxes	January 2000	Fig 3.9
0 – 2	0.8	partial[2]	pellets from boxes & plots	November 1998	Fig 3.8
0 – 7	0.5	partial[3]	pellets & soil from plots	January 1998	Fig 3.7
4 – 9	0.4	full	pellets from boxes	January 2000	Fig 3.9
0 – 9	0.2	full	pellets from boxes	January 2000	Fig 3.9
4 – 13	0.2	full	pellets from boxes	January 2000	Fig 3.9

[1]100% shade
[2]shaded verandah boxes and exposed plots
[3]pasture growth provided additional shade

Estimating the duration of survival of the organism on farm according to purpose

The total duration of survival of the organism on farm will depend on the starting level of contamination, the decay rate, the occurrence and duration of the putative dormant phase (when cultures are negative), and the occurrence and magnitude of putative environmental replication of the organism. Some of these factors can be estimated but choice of D-value may be problematical. A single average D-value could be selected from Table 3.15 or different values could be used to account for the rapid and slow decline phases.

For the purpose of eradication of OJD by destocking and pasture decontamination conservative estimates for D-value should be used. Shaded macro- and micro-environments are present on all farms. Therefore when considering eradication of OJD it is recommended that the range of D-values for shaded environments (Table 3.15) should be used. As survival was demonstrated for > 9 months during these studies the values for long-term observations are most relevant. A single conservative D-value of 0.37 logs/month could be used, this being the average figure derived from long-term observations in two experiments (0 to 9 months, Table 3.15). This takes account of the rapid decay phase, the observed periods of putative dormancy, the observations of putative environmental replication and the slow decline phase. Its use is not dependent on developing understanding of these phenomena, although clearly this would be desirable.

For the purposes of mitigating against the impact of losses of OJD on affected farms by managing pasture and grazing rotations, D-values for shorter terms are relevant. An average D-value of 1.3 logs/month could be used for partially shaded pastures (Table 3.15). However, higher implied decay

rates than this were observed in unshaded treatments in this study. If the duration of survival in exposed non-shaded plots and boxes is used as a guide, decay rates ranging from 1.3 logs/month to as high as 5-6 logs/month were implied (Table 3.16). It must be emphasised that this is based on inference not measurement and it would be sensible to use the lower figures or an average, for example 3 logs/month (the average of those in Table 3.16).

For the purposes of managing the losses due to OJD it would appear that useful degrees of decontamination can probably be achieved within the periods of pasture preparation (spelling, grazing by animals of low susceptibility) normally recommended for control of nematode parasites (i.e. 3-6 months). This is discussed further below.

Table 3.16. Decay rates of *M. paratuberculosis* inferred from unshaded locations where pasture was either light or was removed to simulate grazing

Site	Starting time	Source	Results from	Starting concentration in pellets	Observed duration of survival* (assumed value)	Implied decay in logs/mth	Data from
Borenore	Nov 1998	Plots	Both soil & pellets	1.2×10^6	$\geq$ 3, < 4 weeks (1)	6	Table 3.6
Carcoar	Nov 1998	Plots	Pellets	1.2×10^6	$\geq$ 5, < 9 weeks (2)	3	Table 3.6
Borenore	Nov 1999	Plots	Both soil & pellets	1.6×10^5	$\geq$ 2, < 4 weeks (1)	5	Table 3.8
Borenore	Nov 1999	Boxes	Both soil & pellets	1.6×10^5	$\geq$ 2, <4 weeks (1)	5	Table 3.9
Camden	Nov 1999	Boxes	Pellets	1.6×10^5	$\geq$ 12, <16 weeks (4)	1.3	Table 3.10
Borenore	Jan 2000	Boxes	Both soil & pellets	1.6×10^5	$\geq$ 16, < 20 weeks (5)	1	Table 3.9
Borenore	Jan 2000	Plots	Soil	1.6×10^5	$\geq$10, < 12 weeks (3)	1.7	Table 3.8
Camden	Jan 2000	Boxes	Soil	1.6×10^5	$\geq$ 12, <16 weeks (4)	1.3	Table 3.10

*assumes lunar months and survival to a month closer to the later observation point when the cultures were negative

6.4.5 Factors that affect survival

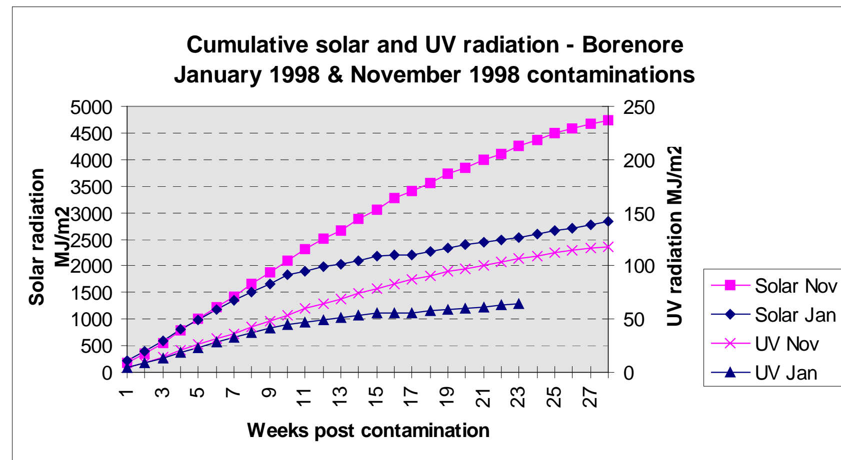
The results clearly indicate that shade is an important factor influencing the survival of the organism. Shade is a proxy for a number of effects - incident electromagnetic radiation over a wide range of wavelengths including UV, visible and infra-red. Thus a shaded environment has less UV radiation, lower degrees of heating and less evaporation than a sunny one. The shade treatments used in this study also protected from cooling, and overall there was a lower temperature range in the shaded environments compared to the exposed environments. In addition, and particularly at Camden where shade included an impervious roof as well as shade cloth, shade protected faecal material from rainfall. Although the results of laboratory experiments (see Part 5) suggested that inundation did not elute the organism completely from pellets, there may have been some direct losses of bacteria from pellets by "washing" as well as other effects associated with natural rainfall that were not measured, such as mixing of the surface layers of pellets with soil particles leading to binding of organisms to adjacent soil. Moisture did not appear to be a significant factor in the January 1998 experiment.

The components of shade that favour survival cannot be identified at this time but the effect of shade has important practical implications for control and eradication of ovine Johne's disease. As mentioned in the results further statistical analysis of the data will be undertaken in an effort to understand the factors that affect survival of the organism. In the discussion that follows "radiation" is used loosely to mean whatever significant factors are present or missing in shaded environments that favour survival.

In logistic regression analysis of results from the November 1999 and January 2000 contaminations there was no statistically significant effect on the proportion of positive cultures due to a decontamination commencing in November compared to one commencing in January in the same summer. However there appeared to be a qualitative difference in the duration of survival of low numbers of organisms, this being longer for January contaminations than November contaminations. The lack of strong statistical effect of starting month is an apparent paradox given the significant effect of shade on survival. The explanation for this may lie in the normal pattern of solar radiation about the summer solstice combined with the rapid decline phase observed in the first period of the experiments. The cumulative doses of radiation measured at Borenore after the January 1998 and November 1998 contaminations were aligned from a common point - the week of contamination - and plotted (Figure 3.15). As can be seen there is very little difference in total doses of radiation in the two contamination periods until after about 7 weeks. This is because there is very little difference in solar

elevation or day length in the weeks preceding and following the solstice. Progressively greater daily changes in day length and solar elevation occur earlier and later.

Figure 3.15. Cumulative doses of solar radiation and UV radiation from early November and late January aligned according to the time of contamination



The rapid decline phase in viability of *M. paratuberculosis* occurred within the first 2 months after contamination (Figures 3.7 - 3.9). If radiation affects the rapid decline phase little difference would be seen in survival between the January and November contaminations because the levels of radiation are similar over this phase. During the rapid decline phase survival was prolonged by reductions in radiation levels of 70% or 100%. In the slow decline phase radiation reductions of 100% were required to significantly influence survival. In Figure 3.15 note that 26 weeks after contamination the total dose of radiation received by the January contamination plots was still more that 50% of that received by the November contamination plots and by this time day length was increasing for the former. Therefore 70% shade cloth was more effective in reducing radiation levels than season. This may explain why moderate to high levels of shade but not month of contamination were significant factors.

Unfortunately the minimum shade level investigated in this study was 70%, which exceeds the cumulative radiation reduction possible due to month. If <~50% reduction in radiation dose is sufficient to influence the rapid decline phase it might matter in which season a property decontamination commences, but if >~50% reduction is required to do this it would not matter much when decontamination commenced. This needs to be confirmed experimentally, for example by comparing survival and decay rates after June and December contaminations.

If one were to speculate on which components of shade influenced survival in faecal pellets the following would need to be considered: the lack of effect of evaporation except in cooling pellets as moisture levels of plots did not affect survival; the fact that UV radiation would not penetrate pellets, could only cause surface disinfection of pellets and could not affect the underside of pellets; the fact that pellets are dark objects and would therefore absorb radiant energy which would then be conducted to deeper regions of the pellet, and as dark objects would in turn radiate heat; the fact that striking differences were observed in daily temperature ranges between soils in the different shade treatments; the fact that temperature ranges in pellets on the surface would have been greater than those measured in soil at a depth of 1 cm. For these reasons temperature flux stands out as an obvious potential factor correlated with shade that could affect survival of the organism. This can and should be tested experimentally.

6.4.6 Assessing the risk of infection from environmental sources

Grazing sheep consume considerable amounts of soil[25]. Pasture plants become contaminated with soil by trampling, water splash and wind, the latter mechanism being especially important in dry conditions, low rainfall areas and extensive grazing conditions. Soil may comprise 6-8% of plant dry matter on average, but it may exceed 35% and it is unlikely that well grazed pastures are ever free of soil contamination. The amount of soil ingested depends on soil type (being least in well drained highly structured soils), as well as on stocking rates, earthworm activity, grazing management (short rotations increase) and season (highest in winter when pasture growth is poor). There is considerable variation between individuals in the amounts of soil ingested (and it is interesting to ponder whether this might be a major driver of OJD infection). In studies in both New Zealand and Canberra, sheep in winter ingested about 400 g of soil per day[25].

Using the data obtained in this study, published data and by a reasoned approach it is possible to develop a scenario of property contamination and risk associated with an OJD infected flock, and from this to develop a property decontamination plan. Factors that increase or decrease residual property contamination after destocking or pasture spelling are given in Table 3.17.

Table 3.17. Factors affecting the residual level of contamination on an infected farm

Factors increasing residual contamination	Factors decreasing residual contamination
Stocking rate	Time since destocking
Prevalence of faecal shedders	Season when destocking commences?
Stage of disease	
Amount of soil ingested	
High risk sites - shade, drainage areas, water	

The infectious dose of the sheep strain of *M. paratuberculosis* is uncertain but lies somewhere between 10^3 and 10^7 viable organisms administered as multiple oral doses over a period of weeks based on results from the tracer weaner pen challenge trial (Reddacliff and Whittington, unpublished).

The information summarised above will now be integrated into two scenarios as an example of how one might assess the risk of reinfection from the environment. The process of risk assessment outlined could be developed in spreadsheet format to simplify the calculations.

Scenario 1 - eradication from an endemically infected property by destocking

A 2000 head flock with 1600 head > 1 year old was set stocked at 5 DSE/Ha on 400 Ha undulating improved and unimproved pastures in the Central Tablelands district of NSW. There was light timber cover on ridges, vegetation corridors were established on several watercourses and there were some low lying pasture areas and dams draining large areas of pasture.

OJD was detected in the flock and based on PFC examination in a flock profile the average prevalence of faecal shedders in animals > 12 months old was estimated to be 10%. The producer wished to eliminate OJD by destocking adult sheep in November, and weaners the following autumn before they commence shedding.

Faecal output is assumed to be 750 g/sheep/day. Of the sheep shedding, 10% were assumed to be in advanced stages of OJD shedding 10^8 organisms per gram, and the rest shedding 10^5 per gram so that the average rate of faecal shedding is 10^7 per gram.

Total flock faecal shedding = 10% x 1600 x 10^7 organisms/g x 750g/day/400 Ha = 3 x 10^9 / Ha/day.

Assuming that contamination is eventually concentrated in 10% of the pasture area because of micro and macro drainage into lower lying areas, and that contamination might accumulate over a three month period (100 days) during winter and early spring in these sites before appreciable decay, the

potential contamination rate in high risk areas of the farm at the commencement of a decontamination period is:

100 days x 3×10^9/Ha/day x 10 = 3×10^{12}/Ha = $3 \times 10^4/cm^2$ (1 Ha=10,000m^2 ; 1m^2 =10,000cm^2)

Further, assuming that surface contamination becomes evenly mixed through the surface soil to a depth of 1 cm over the decontamination period, the soil contains:

3×10^4 viable organisms/cm^3 or /g.

Assuming that sheep graze this infected pasture for 10% of their grazing time, the number of organisms ingested is:

10% x 400g/day x 3×10^4/g = 1.2×10^6 organisms/day

This probably exceeds an infectious dose, resulting in disease transmission. The average long term decay rate of organisms is assumed to be about 0.37 log_{10}/month or about 1 log per 3 months. After 6 months decontamination the ingestion rate is expected to be 1.2×10^4/day, which is risky over a period of time as an infectious dose could be accumulated. A decontamination interval greater than 6 months is clearly required in this scenario. (This is also supported by the ability to isolate the organism for greater than 6 months from experimental pasture plots and boxes that were contaminated at comparable levels to those in this scenario; remember also that survival duration was underestimated). After 12 months decontamination the ingestion rate is expected to be 1.2×10^2/day, which is still risky, and to be more confident a full 15 months is recommended. Even at this time residual contamination could be expected.

Conceivably it might be possible to identify and exclude livestock from high risk areas, thereby reducing decontamination intervals. However, stocking rates and prevalence of faecal shedders in the OJD endemic areas are frequently higher than the values used in the above example and decay rates might be lower. For many properties the prevalence of faecal shedders is unknown. In addition it may be difficult or impossible to identify areas on a property that represent a greater risk than any other area.

Scenario 2 - coping with transient contamination on an otherwise clean property

Ten rams are introduced to a property and confined to 10 Ha. OJD infection is detected 3 months later as a result of a trace investigation. One ram had early histological lesions, was faecal culture positive and was assumed to be shedding 10^5 organisms per gram faeces.

The pasture contamination rate is = 1 ram x 10^5 organisms/g x 750g/day/10 Ha = 7.5×10^6/ Ha/day.

Assume that contamination accumulates over 100 days during winter and early spring before appreciable decay, and before detection of the rams. The potential contamination rate in the 10 Ha paddock at the commencement of a decontamination period is:

100 days x 7.5×10^6/Ha/day = 7.5×10^8/Ha (1 Ha = 10,000 m^2 ; 1 m^2 = 10,000 cm^2)
= $7.5 \times 10^0/cm^2$
= $7.5 \times 10^0/cm^3$ or /g

Assuming that clean sheep will later graze this infected pasture set stocked (which might not be done), the number of organisms ingested is:

400g/day x 7.5×10^0/g = 3×10^3 organisms/day

This might exceed an infectious dose, resulting in disease transmission. The average long term decay rate of organisms is assumed to be about 0.37 log_{10}/month or about 1 log per 3 months. After 6

months decontamination the ingestion rate is expected to be 3 x 10^1/day, and after a further 3 months 1.2 x 10^0/day which is unlikely to be significant.

Scenario 3 - pasture management to reduce losses due to OJD on an infected farm
Consider the first scenario as a starting point, where sheep were ingesting 1.2 x 10^6 organisms/day. The D-value in exposed (non-shaded) pastures can be assumed to be 3 logs per month so that after 2 months few organisms would be ingested. In shaded pastures or long or dense pasture the D-value can be assumed to be lower, say 1.5 logs per month so that 4 months decontamination would be required to minimise the risk.

6.4.7 Recommended destocking intervals with current knowledge

For the reasons given above it is difficult to be prescriptive about decontamination intervals on a given farm. The following might be used as a guide:

1. Where there has been transient contamination by low numbers of infected sheep, for example where young rams have been introduced, confined to a subsection of the farm, and later shown to be infected. Provided contamination is contained by topography or other effective barrier and there are no other means of spread the relevant area could be destocked and left for 9 months. The period could be reduced if the infected rams were faecal culture negative i.e. paucibacillary.

2. Where infection is endemic and eradication is the goal: the concept of a 4 month decontamination period for OJD eradication is not supported under any circumstances. A full 15 month decontamination is recommended. There might be some flexibility in when this can start provided that it is during the summer period.

3. Where infection is endemic and control/management of OJD is the goal: a short pasture spelling period may be used to advantage to reduce challenge doses and this might reduce the overall flock infection rate over a period of time. High values for decay rate can be assumed in the first two months. Decontamination intervals as short as 2 months will be beneficial for exposed pastures, and pasture preparation methods suggested for internal parasite control (eg 6 months spelling or grazing by adult sheep) will also benefit OJD control provide that any livestock used to prepare the pastures are not infected.

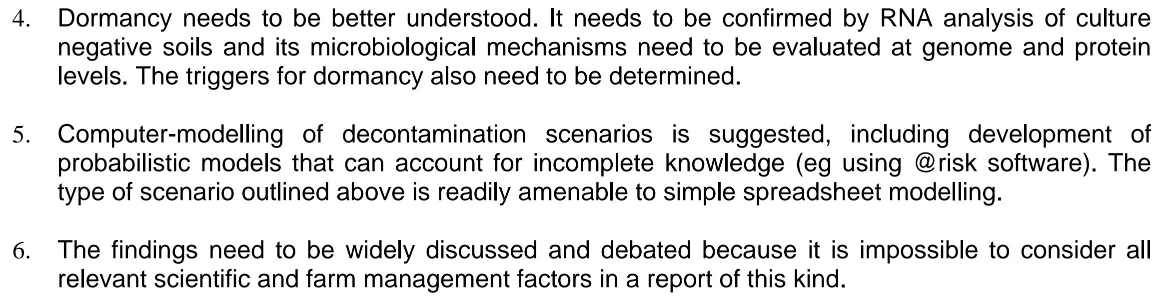
4. Water remains a potential threat to eradication because survival of the organism may be more prolonged in water than in soil. Access to potentially infected water bodies must be restricted. There may be less risk from flowing water sourced from a lightly or non-infected catchment where there would potentially be significant dilution rates.

6.4.8 Research that might lead to property-specific recommendations

1. There is uncertainty about age susceptibility of sheep, but if adult sheep were shown convincingly to be significantly less susceptible to infection than young sheep, shorter decontamination intervals could be specified where restocking was to be by adult sheep.

2. Greater use of flock prevalence profiling using PFC (assessing the prevalence of faecal shedding by age class or mob) on a whole-flock basis and enumeration of the organism in pooled faecal samples based on time to peak BACTEC growth indices may lead to better understanding of contamination rates on newly infected and endemically infected farms. This could allow decontamination intervals to be prescribed based on objective assessment of contamination rates.

3. Survival in water bodies needs to be evaluated more intensely than was possible in this study (see Part 4 for specific recommendations).



4. Dormancy needs to be better understood. It needs to be confirmed by RNA analysis of culture negative soils and its microbiological mechanisms need to be evaluated at genome and protein levels. The triggers for dormancy also need to be determined.

5. Computer-modelling of decontamination scenarios is suggested, including development of probabilistic models that can account for incomplete knowledge (eg using @risk software). The type of scenario outlined above is readily amenable to simple spreadsheet modelling.

6. The findings need to be widely discussed and debated because it is impossible to consider all relevant scientific and farm management factors in a report of this kind.

7.0 Part 4. Survival of the organism in water - pilot study

7.1 Background

Drinking water is required for stock on every farm and often is collected in dams draining infected pasture. Prolonged survival of *M. paratuberculosis* in water is clearly as significant as prolonged survival of the organism on pasture. Although not part of the original brief for this project the question of survival of the organism in water is important and a pilot study was endorsed by MLA in the third year.

7.2 Materials and methods

A small experiment was conducted using water troughs contaminated with faecal mixture.

The mixture of "top soil" and "brickies sand" that was used in the pasture box study was added to the base of two circular 370 plastic troughs to a depth of 120 mm and the troughs were then filled with water from a farm dam. Lids of 70% shade cloth were placed on the troughs to prevent bird access. One trough was placed within the 70% shade enclosure on the verandah of the high security block 14 at EMAI, Camden. The other trough was placed outside the shade enclosure on the same verandah. The weather logger at Carcoar was relocated to EMAI in October 1999 to record standard parameters as well as the water temperatures in the troughs.

A covered reservoir trough was maintained on the same verandah. The water in the reservoir was topped-up using water from the dam via a tanker in June (T32) and December 2000 (T54). On both occasions the experimental troughs were also topped up from the tanker to compensate for evaporation; the sediment was grossly disturbed, particularly in June (T32). The troughs were also topped up with large volumes from the reservoir using buckets in August (T40), October (T48) without gross disturbance to the sediment. Minor top-ups were performed at other times.

Dam water collected from the reservoir was submitted to the Environmental Centre of Excellence Laboratory, NSW Agriculture, Wollongbar for analysis in August 2000. pH and conductivity were corrected to 25°C; hardness was determined titrimetically; nitrate nitrogen, ammonia nitrogen and phosporus free reactive were determined colorimetrically and other parameters by spectroscopy.

The faecal mixture used for the November 1999 contamination of field plots and pasture boxes was also used to contaminate water troughs. Samples of 3.5 Kg of faecal material were added to the water troughs at EMAI on 02.11.1999.

Post contamination T = 0 water samples (1 L) and sediment samples (50 g) were collected from the water troughs at EMAI on 03.11.1999. Samples were then collected at 2 weekly intervals until 12 weeks then monthly or otherwise as given in the results.

The sediment samples were treated as faecal samples for culture. The water samples (1 L) were allowed to stand overnight at 4°C if visibly turbid. The supernatant was then carefully poured off and the sediment was cultured as for faeces. The supernatant was centrifuged at 10,000 rpm for 20 min at 4°C using a Beckman JA 10 fixed angle rotor, producing an average relative centrifugal force of 11,000 x g. The resulting pellet was cultured as for faeces.

Samples of dam water and sediment from the dam were collected at 10 sites around the periphery on 13.12.2000 and were culture negative. The soil-sand mix was cultured as part of the pasture box study and was culture negative.

7.3 Results

7.3.1 Chemical analysis of dam water

The dam water used in this experiment had pH 8.4, electrical conductivity 0.26 dSm^{-1}, hardness 81 mgL^{-1}, nitrate nitrogen 0.10 mgL^{-1} , ammonia nitrogen <0.03 mgL^{-1} , phosphorus <0.01 mgL^{-1}, boron <0.04 mgL^{-1}, calcium 22.1 mgL^{-1}, copper <0.02 mgL^{-1}, iron <0.2 mgL^{-1}, potassium 2.76 mgL^{-1}, magnesium 6.27 mgL^{-1}, manganese <0.02 mgL^{-1}, sodium 19 mgL^{-1}, phosphorus <0.6 mgL^{-1}, 0.53 sulphur mgL^{-1}, zinc 0.10 mgL^{-1}.

7.3.2 Survival of the organism

The faecal mixture used to contaminate the water in November 1999 was enumerated in April 2000. It contained 1.58×10^5 viable cells per gram of mixture, resulting in 1.58×10^6 viable cells per L of water in each trough at T = 0.

Survival in water troughs, both in the water column and in soil sediment appeared to be prolonged with survival evident at week 48 in the trough within the shade enclosure (Table 4.1). Sediment was culture positive for longer than water. Survival in the water and the sediments from troughs both outside and within the shade enclosure was much longer than in faeces and soil in pasture boxes kept in the same enclosure (maximum 12 weeks) (Table 3.8). Based on the incubation period required to achieve peak GI, there appeared to be a decline in the number of viable organisms from T = 0 (3-4 weeks to peak GI) to T = 9-10 weeks (4-8 weeks to peak GI), but after that the numbers of viable organisms appeared to stabilise or fluctuate.

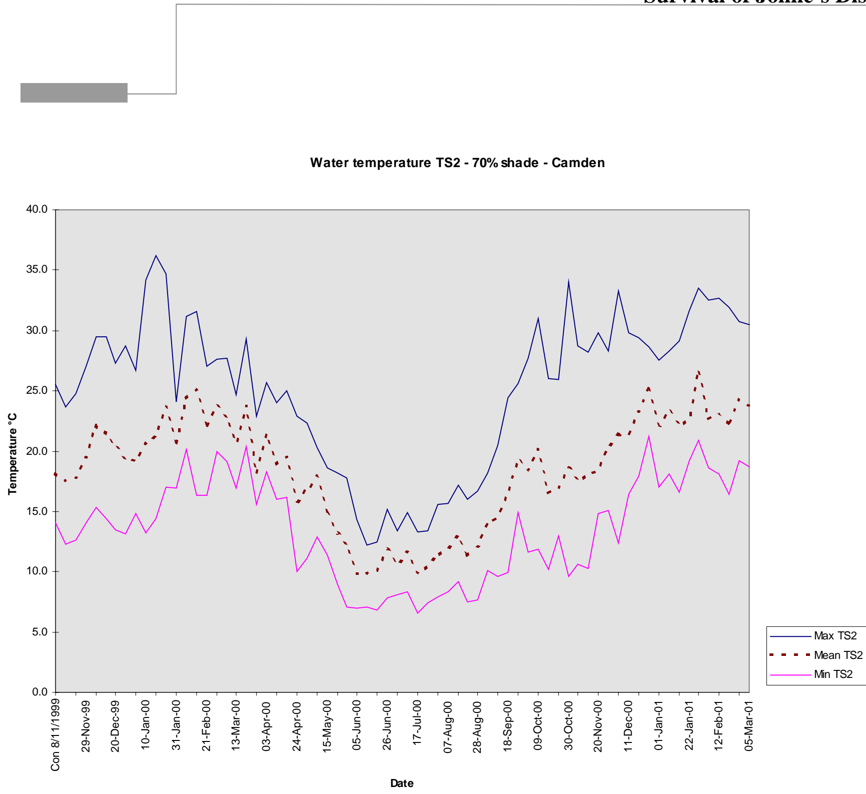
As in soil and pellets (Part 3) there was a period of apparent dormancy as cultures were negative for two or three successive months after week 20, but were followed by successive positive culture outcomes.

7.3.3 Observations on water in troughs

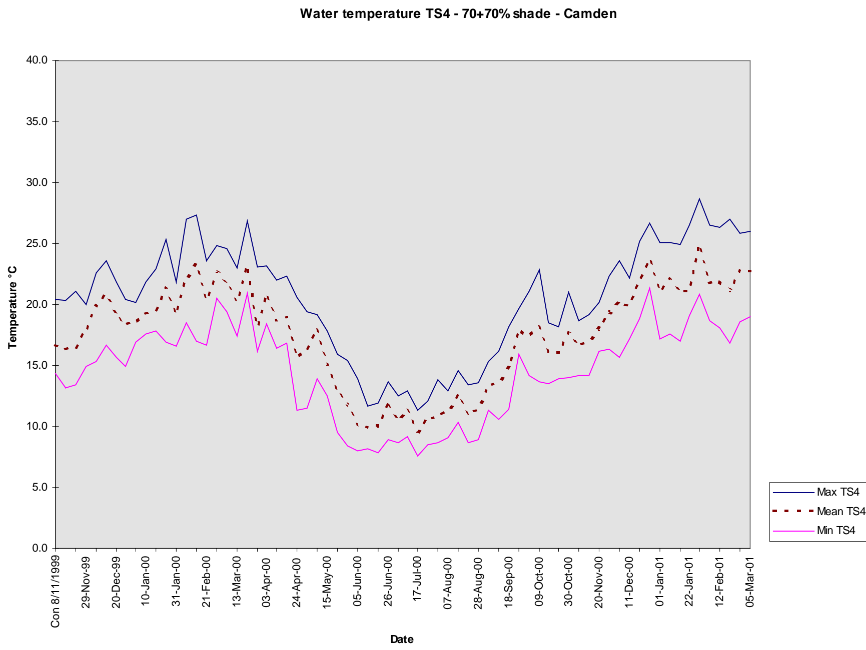
Water was initially slightly turbid but cleared upon standing. After addition of faecal mixture, an algal bloom developed in both troughs. This gradually subsided and a population of tiny invertebrates then took up residence. By August 2000 there was a stable population of tiny invertebrates. The water was murky but there was no evidence of algae.

7.3.4 Water temperature records

The following figures provide weekly maxima, minimum and average water temperatures in each trough. The trough in the shaded enclosure had a narrower temperature range throughout the period of study (about 7 to 28°C) compared to the trough outside the shaded enclosure (about 6 to 36°C). On 18.01.2000 the external (70%) trough was only 1/3 full due to evaporation and the temperature probe was out of the water.



Water temperature TS2 - 70% shade - Camden
Temperature °C
Date
Max TS2
Mean TS2
Min TS2



Water temperature TS4 - 70+70% shade - Camden
Temperature °C
Date
Max TS4
Mean TS4
Min TS4

Table 4.1. Number of culture positive replicate samples in water troughs at EMAI after the November 1999 contamination. Black shading indicates a positive culture and the figures within indicate the number of replicates (n) that were culture positive

Water trough 70% shaded EMAI November (outside)

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	36	40	44	48	52	56
Water	2	2	2	2	2	2	2	2	2										
Sed.	2	2	2	2	2	2	1	1	1				1	1					
Wks to max GI		3-4	5	4-6	4-6	5-7	4-8	8	6-7				9	6					

Water trough 70%+70% shaded EMAI November (inside)

Week	n	0	2	4	6	9	10	12	16	20	24	28	32	36	40	44	48	52	56	67
Water	2	2	2	2	2	2	2	2	2	2				2						
Sed.	2	2	2	2	2	2	2	2	2	2	1	1			2	2	1			
Wks to max GI		3-4	3-4	4-7	4-7	7	7-8	7	5-6	8-9	8	5		8-11	6-8	9	7			

7.4 Discussion and conclusions

This is the first field experiment to evaluate the duration of survival of the sheep strain of *M. paratuberculosis* in water and the first study conducted in water troughs deliberately contaminated with faeces. In other studies the cattle strain has been used in small bottles or bowls of water (refer to Table I in the first section of this report for a review).

The troughs used in this study contained dam water and sediment. They were of the type commonly used as stock watering troughs. Visually the water appeared similar to that in a typical farm situation. One trough was located within a shade house while the other was on a verandah but close enough to the edge to receive direct sun on its sides. Both troughs had lids made of shade cloth.

Under these conditions the organism survived for 48 weeks which was much longer than in pellets/soil in the same environment.

The results are similar to those reported in the literature. Under laboratory conditions survival of the cattle strain in distilled water has been observed for 455 days. Survival in tap water and pond water in sealed bottles was > 9 months (Table I). These times were longer than those reported for survival of the organism in bovine faeces (Table I).

A D-value of approximately 2 months (69 days) was reported for cattle strain in distilled water (Table I). The D-value in water was not evaluated in this pilot study but can be inferred from the starting concentration in water of 10^6/L to be about 0.55 months based on a 6 log decline over 11 months. This is clearly a much greater rate of decline than that seen in distilled water in a controlled laboratory study.

Collectively the data suggest that water might be a greater risk than pasture and soil with respect to long term persistence of the organism on a farm after destocking. A literature review on the risks of water was undertaken during the final phase of this project in order to develop recommendations for further research.

7.4.1 Literature review and recommendations

Background

Paratuberculosis or Johne's disease is a chronic enteric infection of animals caused by *M. paratuberculosis,* a member of the *Mycobacterium avium* complex (MAC). This bacterium is believed to be an obligate parasite of animals, i.e. does not replicate in the environment. This is part of the taxonomic description of the organism[1]. *M. paratuberculosis* is sometimes isolated from clinical samples from animals in which there is no history of paratuberculosis and no evidence of disease in the herd/flock/region. This can be interpreted either as laboratory error or passive excretion after ingestion from an environmental source, either pasture or water. Based on the current definition of the species *M. paratuberculosis,* this finding indicates the presence of an infected animal (somewhere), either in farm livestock or free-living mammalian reservoirs such as rabbits[26,27], foxes[28], deer[29] or feral or straying domestic species.

The principal means of transmission of the organism is faecal-oral, a direct result of intestinal lesions and shedding of large numbers of organisms into the environment. The faecal route of transmission can be direct, eg by consumption of faecal material from pasture or teats, or indirect, eg by consumption of water contaminated by faeces. Transmammary and intrauterine transmission from infected dams also occurs.

Understanding of the means of transmission of the organism has led to design of logical control strategies to reduce the impact of the infection or eliminate it completely. For control of paratuberculosis the degree of environmental contamination can be reduced by culling clinical cases

and subclinical faecal shedders and reducing contact between adult animals which may be shedders and juveniles which are most susceptible to infection. For eradication of the infection it is thought that destocking and spelling of pasture for a period sufficient for die-off of the organism, followed by restocking with healthy animals will be effective[30].

Survival of *M. paratuberculosis* in water may pose a greater threat to OJD control and eradication programs than survival in terrestrial locations. Water may also be an efficient vehicle for transmission between farms. The magnitude of difference observed between the duration of survival in water and that in faeces/soil in the same general environment is sufficiently great to be of concern in relation to eradication and control of OJD.

There is anecdotal evidence from southern NSW that OJD may be spread between farms within a water catchment by means other than movements of sheep. Water-borne spread has been suggested as a possible means of transmission.

Evidence for non-mammalian hosts and environmental survival/replication of Mycobacterium avium *complex organisms*

Although *M. paratuberculosis* is believed to be an obligate parasite of animals it was cultured recently from infective third stage trichostrongylid nematode larvae that developed in faeces containing the organism and in faeces of sheep with paratuberculosis[31,32]. These were the first reports of the organism being associated with a free-living non-mammalian host. There is no evidence that nematodes play a role in environmental survival or transmission but it is a possibility.

Perhaps the isolation of *M. paratuberculosis* from nematodes is not surprising as nematodes are microbivorous and there is a precedent in that many bacterial species including human enteric pathogens have been isolated from free-living nematodes and other invertebrates in water supplies[33,34]. The range of aquatic animals that are microbivorous extends from the microscopic protists to the metazoans. The protists comprise the protozoan groups amoebae, ciliates and flagellates and are ubiquitous in aquatic environments. There are numerous metazoans including crustaceans, nematodes and trematodes, also ubiquitous. There is a similar range of microbivorous animals in soil and detritus and many genera are found in both soil and water. Insects also feed on or in faeces on land and in water. The protists have a significant impact on the bacterial population in water[35]. Feeding behaviour includes phagocytosis (eg amoebae) and filtration (eg ciliates) and may be selective. The surface properties of bacteria (eg cell wall antigens, charge) may be important in this respect[35].

There are precedents for microscopic environmental hosts for pathogenic bacteria. Protozoa are recognised environmental hosts for legionellae and could be involved in the epidemiology of some mycobacterial infections. *Legionella pneumophila* is the cause of legionnaires disease, an often fatal condition most often acquired through aerosols from improperly maintained air conditioning systems. The bacterium is engulfed by a variety of species of aquatic amoebae, replicates to high titre within vacuoles in the amoebae, persists there if the amoebae encyst, and in that state is protected from chemical disinfectants such as chlorine[36]. This also applies to *Listeria monocytogenes*[37] as well as a range of gram positive and negative bacteria[38,39]. These bacterial pathogens may have host preferences. Although *L. pneumophila* is known to infect six genera of protozoa[40] , other *Legionella* species are more specific. *L. longbeachae* infects soil amoebae found in south eastern Australia[38].

M. avium is emerging as a serious pathogen in immunocompromised humans, most often associated with HIV/AIDS. There are several reports confirming that amoebae ingest *M. avium*[41-43]. The bacterium resists being killed by slowing fusion of the phagosome with lysosomes as well as by producing extracellular factors. Intracellular replication was shown in one study[42] and could not be ruled out in another[43]. It spreads to infect other amoebae. Passage of *M. avium* within amoebae induces a change in phenotype through induction of previously quiescent genes, rendering the bacilli more pathogenic: infectivity for other amoebae, human macrophages, human intestinal epithelial cells and beige mice is significantly enhanced[42]. Similarly, an increase in virulence was noted when virulence-attenuated *M. avium* was passaged in peripheral blood mononuclear cells[44] and entry into macrophages was

facilitated by prior culture in macrophages[45]. Thus amoebae seem to provide stimuli resembling those of mammalian phagocytic cells. In contrast to *M. avium*, non-pathogenic mycobacterial species such as *M. smegmatis* did not survive phagocytosis by amoebae[42]. In this respect they are like *E. coli*, digestible and a potential food source. When exposed to deleterious environmental stimuli such as low nutrient conditions, amoebae form cysts, which are resistant to disinfectants. In encysting studies, *M. avium* was found within the cyst wall and remained viable in that location; upon release, the bacterium could be cultured[43]. The intracellular activities of protozoa and macrophages are very similar. Evolutionary pressure from amoebae in aquatic systems may be the driving force for the development of survival strategies by intracellular bacteria that result in pathogenicity in animals and man.

Evidence for an aquatic source of **Mycobacterium avium** *complex infection*

Non-tuberculous mycobacteria in general and those in the MAC in particular are highly adapted to aquatic ecosystems, both fresh and saline, and to moist soil and these are suggested sources of infection for man[46-49]. *M. avium* is ubiquitous in aquatic systems and is acquired by AIDS patients from municipal/domestic water supplies[42,46].

Aerosolisation of mycobacteria in surface water is one means suggested for tuberculin sensitisation of animals and man living near water[46,50] and disease in humans[48]. In the late 1960s a geographic bias in the distribution of sensitivity to mycobacterial PPD was noted in the south eastern United States and was later associated with the presence of MAC organisms in low-saline coastal waters[51]. Their origins were uncertain but could have included the draining rivers and soils in the catchments of these rivers. Replication of the organisms in water at temperatures >15.5°C and a preference for saline concentrations < 1.9g/100 ml were demonstrated[52]. The organisms were released into aerosols above a river and were present in rainwater[53]. Critical appraisal of these experiments[52] suggests that carryover of nutrients from culture media could explain replication in nutrient-poor water but the presence in aerosols is without question.

When mycobacteria are suspended in water, air bubbles rising through the water column concentrate the bacteria which are ejected into the atmosphere as the bubbles burst. The aerosolised mycobacteria then could be carried considerable distances by air movements. Mycobacteria become concentrated many hundred fold as bubbles form and rise in a water column. Bubbles 95um in diameter contained an average of 69 mycobacterial cells when they formed in a suspension containing 7×10^5 mycobacteria/ml[50]. In a study in a river system in the USA, daily droplet production was 0.32 ml/m^2 of water surface, which was calculated to release about 3×10^3 *M. intracellulare* cells/m^2 of water surface each day[54]. *M. intracellulare* was concentrated in bubbles to a greater extent than *M. scrofulaceum*[54] suggesting that water may play different roles in the epidemiology of infection of different mycobacterial species and subspecies. The survival of mycobacteria in aerosols is thought to be brief, merely a matter of hours[55]. However, this does not appear to be consistent with their survival on surfaces and in biological samples and methodologies for studying aerosols might therefore be questioned. Despite apparent short term survival in aerosols this is the route believed responsible for tuberculosis infection in man[55].

Although mycobacteria may be found free in the water column they are also important members of microbial biofilm communities. The species concerned include many non-tuberculous opportunistic pathogens. A biofilm is visible as slime-like growth on wet surfaces. Mycobacterial concentrations ranging from 10^3-10^4/cm^2 are common in biofilms[56]. Residence in a biofilm may be associated with increased resistance to noxious environmental conditions including disinfection[57].

Assessment of the likelihood of **M. paratuberculosis** *having an environmental life cycle and being transmissible in water and aerosols*

There is evidence from other studies of prolonged survival of *M. paratuberculosis* in water (Table 1 this report). This can occur in the absence of other living organisms, eg. in distilled/sterilised water. There is also anecdotal evidence of transmission within a catchment area, with water being the suggested route. It is possible that *M. paratuberculosis* may behave in a similar fashion to other members of the MAC and be concentrated in bubbles in a water column and ejected at the water-air

interface to form aerosols, which might travel laterally. The potential for aerosolised *M. paratuberculosis* to initiate infection by inhalation is unknown. The organism is infectious by most routes if given in high enough doses, but inhalation is probably not the natural route. However, aerosols may settle on pasture and be ingested.

The organism is now known to associate with free-living stages of sheep nematode parasites, reminding us that in nature other invertebrates will be in contact with it.

The paratuberculosis organism is a subspecies of *M. avium*. It is reasonable to hypothesise that *M. paratuberculosis* evolved from *M. avium* and that this might have occurred in an aquatic environment. *M. paratuberculosis* can be expected to have many characteristics in common with *M. avium*. The two species have similar cell wall structure and surface antigenicity, evidenced by specific studies of glycopeptidolipid TLC profiles[58] and antigens and the fact that serological tests do not clearly distinguish between infection with *M. paratuberculosis* and *M. avium* (numerous authors). These are key factors likely to influence phagocytosis by macrophages and also, potentially, by amoebae.

It is highly likely that *M. paratuberculosis* cells will encounter protists such as amoebae in the environment and that they will be susceptible to phagocytosis by amoebae. *M. paratuberculosis* may have a potential to survive and replicate within amoebae. If this is the case the duration of environmental survival of *M. paratuberculosis* might be closely linked to the population dynamics of protozoa.

7.4.2 Research recommendations

A multifaceted project was submitted to the NOJDP Technical Sub-Committee in January 2001, and a recommendation was made in May 2001 to proceed with most stages. The proposal consisted of the following:

A. Laboratory study

1. To develop sampling methods for water, including site of sampling in the water column (surface, or deep) and sediment and the need for replication
2. To reassess culture methods for water, including concentration techniques and decontamination routine.
3. To determine whether M. avium subsp. paratuberculosis is present in aerosols formed by rising bubbles and assess methods for collection and culture of aerosols
4. To assess the ability of representative protozoan species (ciliates and amoebae) to ingest or phagocytose M. avium subsp. paratuberculosis and for the organism to withstand this process
5. To determine whether the organism can replicate within protozoa
6. To assess the feasibility of disinfection of water including filtration, UV sterilisation, ozonation, heat and chemical treatments

B Field study

1. To continue sampling contaminated water troughs at Camden to determine the full potential of survival in water
2. To determine whether the organism is still detectable in water bodies and water sources after various periods of decontamination of properties enrolled in Trial 1.1
3. To culture water sources in areas where there is local geographic clustering of OJD infection and where water is thought to be the source. Sampling of water, sediments and aerosols over water sources would be undertaken in a range of still waters, small streams and larger streams or rivers.
4. To assess the feasibility of geographic modelling of the spread of OJD by water or aerosol, using geographic information system data and distribution maps of OJD affected farms (pilot study).

8.0 Part 5. Evaluation of modified culture preparation methods for environmental samples

8.1 Background

The results of culture of some samples in survival experiments conducted from November 1999 indicate relatively short term survival in pellets and soil. It is uncertain whether this is real or an artefact of laboratory methodology. The results in Part 1 suggested that the organism associated with particulate matter in soil and this reduced the sensitivity of its detection compared to detection in faeces. The experiments conducted in Part 1 involved cultures from soil only a short time after mixing faeces with soil so it is possible that the strength of attachment between the organism and soil particles was not fully developed. In addition, moisture levels in soil changed throughout the studies, ranging from very moist to very dry. This might also have affected the strength of association of the microbe with soil. If this was the case laboratory methods might need to be developed to increase the chance of dissociation prior to culture. The experiment described below is an attempt to do this.

8.2 Materials and methods

8.2.1 Pilot experiment

The standard method of culture involves mixing soil/faeces with saline and standing for 30 mins. During this step coarse material settles to the bottom and is excluded from the culture. It was hypothesised that this step might contribute to loss of sensitivity if the organism was associated with coarse particles. Variations to this method were tried with the aim of increasing dissociation. The variations were:

1. Saline, allow to settle overnight, room temperature (RT)
2. Saline with 0.1% v/v Tween 80, allow to settle overnight (RT)
3. Saline, allow to settle for 30 min (standard method) (RT)
4. Saline with 0.1% v/v Tween 80, allow to settle for 30 min (RT)
5. Saline, rotating mixer overnight, allow to settle for 30 min at 37°C
6. Saline with 0.1% v/v Tween 80, rotating mixer overnight, allow to settle for 30 min at 37°C

The remainder of the method was standard (i.e. the supernatant was collected and added to HPC, and the sediment from this was added to VAN, before inoculating BACTEC media. Incubation was for 20 weeks. Growth was confirmed by PCR-REA.

Twelve samples collected on 22.02.2000 and stored for 5 months at -80°C were used in this experiment on 20.07.2000. Samples were tested with methods 1-4, and 11 of these with methods 5-6. The samples and previous culture results are given in Table 5.1. Samples were chosen because they were likely to be positive based on location and time since contamination. Only 2 of the 12 samples were culture positive when first tested.

8.2.2 Extended experiment

A large panel of samples was tested using method variation 5. There were 229 samples comprising 169 previously-tested and 60 newly-collected.

The previously-tested samples were from the EMAI pasture box November 1999 contamination at T=20 weeks collected on 21.03.2000 and stored for 7 months at -80°C and T=32 weeks collected on 13.06.2000 and stored at -80°C for 4 months before this experiment commenced on 12.10.2000.

The newly-collected samples were collected at EMAI on 09.10.2000 and comprised 2 sets of 10 pellets (actually faecal matter, mostly non-pelleted due to time since contamination of boxes) and 2 replicates pools of 10 underlying soil cores collected from across 4 boxes as shown in Table 2 (total

60 samples). For boxes in 0% shade only, 1 replicate was collected as faecal material was very difficult to locate.

8.3 Results

8.3.1 Pilot experiment

The majority of samples were culture negative with all method variations (Table 5.1). The 2 previously culture positive samples returned a positive result with method 5, but not with method 3 (the original method). Sample 12 was positive with method 2, and sample 11 with method 5. As method 5 gave the same results as the original method and identified one additional sample it was reasoned that this method may be useful and should be tested further with a larger number of samples.

Table 5.1. Pilot experiment. Sample descriptions and culture results (weeks to peak GI) using variations to the original culture method

Sample	Description*	Shade %	Box	Time (wks)	Original culture result	Culture result with method variation					
EMAI pasture boxes November 1999 contamination						1	2	3	4	5	6
1	P1	0	2	6	-	-	-	-	-	-	-
2	P2	0	2	6	-	-	-	-	-	-	-
3	S1	70	3	16	-	-	-	-	-	-	-
4	S2	70	3	16	-	-	-	-	-	-	-
5	P1	70	3	16	-	-	-	-	-	-	-
6	P2	70	3	16	-	-	-	-	-	-	-
7	P1	100	7	16	6	-	7	-	-	8	-
8	P2	100	7	16	6	7	-	-	-	7	6
9	S1	100	7	20	-	-	-	-	-	-	-
10	S2	100	7	20	-	-	-	-	-	-	-
11	P1	100	7	20	-	-	-	-	-	6	-
12	P2	100	7	20	-	-	9	-	-	n.t.	n.t.

P - pellet sample, S - soil sample
n.t. - not tested

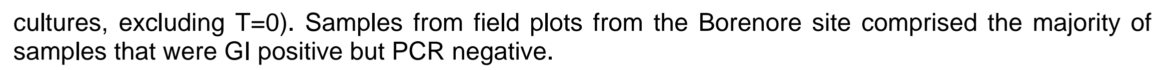
8.3.2 Extended experiment

Of the 169 previously-cultured samples, only three had different results when re-cultured using method variation 5 (Table 5.2). One previously negative sample was culture positive, while 2 previously positive samples were culture negative. Seventeen samples developed peak GI in 8 to 12 weeks but were PCR negative and smears of BACTEC medium did not contain acid fast rods.

Of the 60 newly-collected samples, 2 were culture positive using method variation 5 (Table 5.3). An additional 4 samples developed peak GI in 5-14 weeks but were PCR negative and smears of the BACTEC medium did not contain acid fast bacilli.

The prolonged incubation required for peak GI indicated that these samples contained very few viable organisms and the differences in results may have been due to distribution of the few organisms in the sample rather than to any genuine difference between the methods. That is, some aliquots of 2 g of sample did not contain any viable organisms.

There was a relatively high rate of growth of irrelevant organisms associated with use of method variation 5. Twenty-one of 229 samples (10.9%) were in this category. However, this was similar to the long-term average with the original method used for January and November 1998 contaminations (13.0% of 1822 cultures) and results for November and January 2000 contaminations (7.9% of 2408



cultures, excluding T=0). Samples from field plots from the Borenore site comprised the majority of samples that were GI positive but PCR negative.

Table 5.2. Extended experiment. Culture results for samples that were previously-cultured then stored at -80°C

Category	No. samples	Culture using original method		Method variation 5	
		Result	Weeks to peak GI (range)	Result	Weeks to peak GI (range)
Previously -ve, now -ve	165	neg		neg	
Previously -ve, now +ve*	1	neg		pos	9
Previously +ve, now +v	1	pos	8	pos	8
Previously +ve, now -ve*	2	pos	8-9 wks	neg	

*all 3 samples were sediments from the water trough experiment

Table 5.3. Extended experiment. Newly-collected sample descriptions and culture results (weeks to peak GI) using method variation 5

Description	Boxes	Sample	n	Method variation 5	
				No. culture positive	Weeks to peak GI (range)
November contamination T = 48 weeks					
100%	1-4	P	2	0	
		S	2	0	
	5-8	P	2	0	
		S	2	0	
	9-12	P	2	0	
		S	2	0	
70%	1-4	P	2	0	
		S	2	0	
	5-8	P	2	0	
		S	2	0	
	9-12	P	2	0	
		S	2	0	
0%	1-4	P	1	0	
		S	1	0	
	5-8	P	1	0	
		S	1	0	
	9-12	P	1	0	
		S	1	0	
January Contamination T=36 weeks					
100%	13-16	P	2	0	
		S	2	0	
	17-20	P	2	2	7-8
		S	2	0	
	21-24	P	2	0	
		S	2	0	
70%	13-16	P	2	0	
		S	2	0	
	17-20	P	2	0	
		S	2	0	
	21-24	P	2	0	
		S	2	0	
0%	13-16	P	1	0	
		S	1	0	
	17-20	P	1	0	
		S	1	0	
	21-24	P	1	0	
		S	1	0	

8.4 Discussion and conclusions

In the pilot experiment, samples 7 and 8, which were previously culture positive, were the only samples that yielded positive results with more than one method variation. However, the time taken to achieve peak GI was greater than that recorded originally, and results with a repetition of the original method were negative. As the samples had been well-mixed in an electric blender the most likely explanation for this is a reduction in the number of viable organisms in the samples associated with storage. However, two samples that were originally culture negative yielded positive results (with method variations 2 and 5), suggesting either that these two methods were more sensitive than the original method or that these samples contained so few organisms that not all aliquots of them contained viable organisms. The prolonged incubation periods tend to support the latter view. Assuming deterioration of samples, it appeared that methods 2 and 5 might be useful, with method 5 being more beneficial. The small sample size (12) precludes any certainty in this assessment. When evaluated on a larger scale, method variation 5 did not offer any advantage over the original method.

It was concluded that there would be no benefit in retesting the large number of samples collected during this project. Accepting that the analytical sensitivity of culture from environmental samples is sub-optimal, a breakthrough in culture techniques is still required to overcome the likely association of the organism with particulate matter in environmental samples. At present this particulate matter needs to be excluded from cultures otherwise a high rate of culture contamination occurs (unpublished observations).

9.0 Part 6. Evaluation of the loss of the organism from pellets during prolonged wetting

9.1 Background and aim

The results of culture of some samples in survival experiments conducted from November 1999 indicate relatively short term survival in pellets and soil. It was an unusually wet year and there is a possibility that rainfall disturbed the pellets on soil, possibly eluting the organism and washing it from the experimental plots/boxes. Thus culture results may not reflect the survival of the organism but rather merely its presence/absence. The cumulative rainfall at each site is given in Table 6.1.

Table 6.1. Cumulative rainfall at experimental sites

Weeks after contamination in November 1999	EMAI cumulative rainfall (mm)	Borenore cumulative rainfall (mm)
2	23	33
4	28	49
6	41	113
8	75	163
10	127	225

An experiment was conducted with the following aim: to determine whether exposure of pellets to simulated high rainfall events causes *M. paratuberculosis* to be completely eluted from the pellets, with culture of pellets then yielding negative results.

9.2 Materials and methods

A 300 g sample of the January 2000 faecal mixture used to contaminate plots was removed from storage at -80°C on 17.08.2000 and thawed overnight at 4°C. The sample was mixed then dispensed in weighed 5 g aliquots in a series of 35 ml polycarbonate tubes. Tubes were numbered 1 to 17, each in duplicate (A, B) (Table 6.2). The tubes were then frozen at -80°C until required. They were thawed as above on 21.08.2000 when the experiment commenced.

Tube 1 (A) was placed aside at room temperature (23°C) as a positive control at T=0, without wetting. Sterile water (12.5 ml) was added to each remaining tube, which resulted in pellets being covered to a depth of 25 mm. Tubes were allowed to stand for 1.5 hrs and then the water was decanted into a waste container, retaining the faecal material using a coarse sterile sieve. Tube 2 (A&B) was then capped and placed aside at room temperature. Water was added to the remaining tubes as above and the process repeated for a total of 4 wettings each day over 4 days until there were no tubes remaining.

Cultures were then undertaken on the faecal material in all tubes.

9.3 Results

Most pellets had disintegrated by the 5th wash and soak step. Positive culture results were obtained for all tubes (Table 6.2). Each sample developed peak growth index in 5 or 6 weeks.

Table 6.2. Allocation of tubes to treatments and results of culture

Tube	Amount rainfall simulated (mm)	No. washes	Day of last wash	Culture result
1A	0	0	Positive control. No wash	+
2 A, B	25	1	1	+
3 A, B	50	2	1	+
4 A, B	75	3	1	+
5 A, B	100	4	1	+
6 A, B	125	5	2	+
7 A, B	150	6	2	+
8 A, B	175	7	2	+
9 A, B	200	8	2	+
10 A, B	225	9	3	+
11 A, B	250	10	3	+
12 A, B	275	11	3	+
13 A, B	300	12	3	+
14 A, B	325	13	4	+
15 A, B	350	14	4	+
16 A, B	375	15	4	+
17 A, B	400	16	4	+

9.5 Discussion and conclusions

Simulated rainfall of 400 mm, involving intermittent inundation and soaking of pellets did not completely elute the organism from the faecal material, even though the faecal pellets broke down to an unstructured form. This indicates that the organism was quite closely associated with particulate matter in the faeces. It would therefore appear unlikely that heavy rainfall could explain the negative culture results from faecal matter in the field trials. However, due to time constraints it was not possible to include the effect of a long time interval in this trial. It is possible that repeated cycles of wetting and drying over several weeks to months affects the interaction of the organism with faecal matter, rendering it more easily dissociated from the faeces and more susceptible to elution from faeces.

10.0 Success in achieving objectives

Project TR.055
1. By September 1998, to determine the survival of *Mycobacterium paratuberculosis* under a representative range of field conditions

This objective was achieved

Project TR.055A
1. To continue monitoring contaminated sites on farms in NSW to determine the duration of survival of the organism
2. To study in more detail the survival of the organism in sheep pellets and in the immediate environment of sheep pellets

These objectives were achieved

Project OJD.003
1. To show that a full summer (1999-2000) is adequate for decontamination of OJD

The work was completed but the results indicated that this objective was not realistic. The organism survived for more than 4 months

2. To correlate survival with solar radiation levels over a full summer

This objective was achieved. Shade as a proxy for solar radiation was highly significant. Further statistical analysis will be undertaken.

3. To draw inferences upon extension of research to areas in northern, western and southern NSW

This objective was partially successful. The work was completed but unseasonal rainfall events may have compromised experiments in these areas. Data can be extrapolated from the main experimental sites.

11.0 Impact on Meat and Livestock Industry

The impact on industry now and in five years time will flow from the new knowledge arising from the work, the use made of this knowledge and the impact the recommendations may have on future research.

11.1 New knowledge arising from this project

The principle outcomes from this project relate to provision of information to support recommendations for pasture spelling to control or eliminate OJD. While much of what was found was consistent with knowledge available at the time the project was commissioned, some of the findings were unexpected and will stimulate further thinking about control and eradication of OJD. In particular:

1. Mycobacterial culture methods rely on chemical decontamination of samples, which destroys some mycobacteria, so absolute values for duration of survival in any kind of environmental sample requiring decontamination will underestimate the true period of survival. Furthermore, although the culture techniques used in this study were the best available they could detect only a small proportion of the viable organisms known to be present in soil samples due to inferred binding of the organism to soil particles. At least 90% of the viable organisms present in soil cannot be detected with current culture methods (Part 1). However, decay rates measured in soil samples or faeces are not affected by the inefficiency of culture provided that the culture method is equally (in)efficient for all samples.

2. Pasture contamination on OJD affected farms can be widespread and its location is generally not predictable. Low-lying areas may become a focus for contamination due to movement of faecal material. Under the conditions studied contamination was not detectable after one complete summer in most sites previously found to be infected (Part 2). This was an uncontrolled experiment where we had no information about the time when each site became contaminated or the starting levels of contamination and results therefore need to be interpreted with caution.

3. Moisture was not a prerequisite for long term survival but shade was a significant factor.

4. Under controlled conditions survival at low levels was observed for > 12 months after contamination in the summer period with amounts of faeces representative of those seen in extensively grazed paddocks in the central tablelands of NSW (contamination rates of 10^4 to 10^6 organisms per square centimetre) (Part 3). Apparent dormancy was observed, and this prolonged the duration of survival. The factors leading to dormancy and reversion to a viable state could not be determined with this experimental design because cultural and environmental observations were too infrequent or superficial. Dormancy appears to be a significant issue in relation to prolonged environmental survival and there are precedents from studies of other mycobacteria.

5. Survival duration and decay rates were measured or estimated commencing in summer. There was no statistically significant difference in survival attributable to the time of contamination (November compared to January) however, there was a trend for decay rates to be greater in November than January in exposed sites. It is assumed that decay rates for winter months would be less, but they were not measured.

6. The organism survives for long periods in faecal pellets, moves into the surface litter as pellets break down and probably also enters the soil profile. Thus it can be cultured readily from the surface layers of the soil. The organism also moves away from infected sites in run-off water (Part 3).

7. Pasture emerging through contaminated faeces becomes contaminated with relatively high concentrations of the organism. The factors favouring contamination of pasture were not studied, nor were the reasons for decay of the organism present on foliage, but these may include

exposure to solar radiation and the washing effect of rainfall. Methods for culture of foliage still need to be optimised (Part 3).

8. The organism was found to be associated with infective third stage larvae of intestinal nematode parasites that developed in the faeces of the sheep used in Part 3. This was published separately[31,32]. These larvae may be found on pasture and may contribute to infectivity of pasture.

9. The organism survives for prolonged periods in water. The duration of survival in water is longer than that in soil in the same environment (Part 4). Based on studies of other bacteria including the related organism *M. avium*, there is potential for interactions between *M. paratuberculosis* and single-celled aquatic animals (literature reviewed in Part 4).

10. Faecal shedding rates of the organism were determined using sheep with multibacillary OJD obtained for Part 3 of this study, amounted to 10^8 viable organism per gram or more than 10^{10} viable organisms per sheep per day and were reported independently[59]. Sheep in earlier stages of the disease process would shed fewer organisms. Faecal output ranged from about 500 grams per day to over 1000 g per day among sheep.

11. Given that the organism contaminates soil and that sheep consume significant amounts of soil each day, a process for estimating the decontamination interval for pasture to render it safe was proposed. Separate examples were given for eradication of OJD on an endemically infected property, dealing with a short term incursion by infected rams and pasture management to reduce losses due to OJD.

12. For eradication of OJD short (< 12 months) destocking intervals would be most unwise. The current recommendation of 15 months including two full summers is adequate with certain assumptions but it might not be adequate in all circumstances. Decontamination intervals following transient contamination of land can be shorter than those following long term contamination by an endemically infected flock. Decontamination intervals to reduce the impact of OJD by pasture spelling or management on endemically infected farms can be quite short because of the rapid decline phase. In general, the practices recommended for control of internal parasites will be beneficial for OJD control provided that adult sheep used to prepare pasture for young sheep are not heavy shedders of *M. paratuberculosis*

13. The NOJDP trial to evaluate eradication of OJD by destocking (Trial 1.1 in the NOJDP business plan) provides an opportunity to investigate persistence of the organism. In the event of failure to eradicate the disease a thorough property evaluation must be undertaken to ensure that three factors are considered:
    1. Reintroduction of infection with sheep used for restocking
    2. Lateral spread from the surrounding properties or catchment
    3. Persistence of the organism on the property after destocking, either in soil, water or resident vertebrate or invertebrate hosts.

14. Further research is required to develop better understanding of the biology of the organism and its interaction with sheep in order to refine the ideas developed during this study.

15. Additional insights into the behaviour of the organism will be gained when the information from this study is considered by the broader scientific community as it will stimulate research to confirm or refute the findings.

16. Additional applications for the new knowledge generated in this study will emerge when the report is considered by technical, regulatory and industry participants associated with the NOJDP.

12.0 Conclusions and recommendations

12.1 Conclusions

The sheep strain of *M. paratuberculosis* that is responsible for almost all cases of OJD in Australia was found to be extremely resistant in the environment. The results were consistent with those from several experiments conducted overseas using the cattle strain.

The duration of survival exceeded 12 months in faecal pellets in a shaded location but was significantly less in unshaded treatments. Sunlight, including factors such as UV, visible and infra-red radiation, is a very significant factor influencing survival of the organism.

Moisture levels and lime application did not appear to influence survival. Considering that the duration of survival in soil was underestimated because the culture method was imperfect, the data on duration of survival needs to be interpreted with caution.

The duration of survival in water was considerably greater than in soil or pellets in the same environment.

Decay rates for the organism were estimated and were found to be inversely proportional to the period of observation. There was a rapid decay phase lasting about 6 weeks during which the vast majority of viable bacteria declined. This was followed by a variable period of dormancy during which the organism could not be cultured, and sometimes a period of apparent replication during which its numbers increased. Finally there was a slow decline phase lasting many months.

There is biological potential for the organism to associate with and possibly replicate within aquatic and terrestrial invertebrates.

12.2 Recommendations

1. Decontamination intervals for eradication of OJD need to take account of:
    a. Decay rates. Conservative estimates of decay rate should be used
    b. The level of infection in the flock prior to destocking which can be measured objectively by PFC (prevalence of faecal shedders and level of shedding)
    c. The presence of environments likely to be conducive to survival and which might be fenced off
    d. The amount of soil ingested by sheep, which can be estimated based on type of soil, stocking rate, pasture type, rainfall and other factors.

A decontamination interval of 15 months is likely to sufficient in many but not all cases, depending on the assumptions used.

2. Decontamination intervals following transient contamination of land can be shorter than those following long term contamination by an endemically infected flock.

3. Decontamination intervals to reduce the impact of OJD by pasture spelling or management on endemically infected farms can be quite short because of the rapid decline phase. In general, the practices recommended for control of internal parasites will be beneficial for OJD control provided that adult sheep used to prepare pasture for young sheep are not heavy shedders of *M. paratuberculosis.*

4. Simple spreadsheet-based computer models need to be developed to facilitate estimation of decontamination intervals for individual situations using a stepwise approach based on that

outlined in this report. Further information may need to be gathered about rates of soil ingestion by grazing sheep in Australia, but much of this information may already exist in the literature. Probabilistic models that can account for incomplete knowledge can be developed using commercial software (eg @risk).

5. Knowledge about age susceptibility of sheep is required because shorter decontamination intervals might be possible if age-resistant sheep were used as restockers.

6. It is important to measure D-values for contaminated faeces and soil in the winter months because it is possible that season may be less significant than local or micro-environmental shade influences. This would reduce the component of economic hardship imposed by being required to commence destocking at the beginning of summer. This research should be conducted using the pasture box method developed in this study. Greater flexibility should be given as to when decontamination can start in the summer period.

7. In future experiments using pasture boxes it is important that contamination with faeces be undertaken after transport of boxes rather than before transport because of the potentially deleterious effects of pooling of water caused by vibrations during transport. It would also be desirable to protect boxes from heavy rain using removable covers.

8. Specific recommendations concerning research on the survival of the organism in water and its association with aquatic invertebrates have already been made to NOJDP and are included in Part 5 of this report.

9. In vitro studies of the survival of the organism within faecal pellets exposed to measured doses of UV radiation are required to confirm that incident UV radiation does not sterilise organisms within pellets. Similar in vitro studies of the effects of temperature flux also need to be undertaken.

10. Basic research on dormancy and environmental replication of *M. paratuberculosis* is needed to support the NOJDP. The methods needed to do this largely already exist. Factors to be examined include low oxygen levels as a stimulus for dormancy in vitro, presence or absence of RNA synthesis in non-cultivable organisms and the genetic mechanisms involved commencing with study of genes known to be involved in dormancy in other mycobacterial species. Gene expression and regulation in dormancy can be studied by a range of techniques including differential hybridisation (subtractive or microarray) and protein expression/proteomics. These aspects of the biology of *M. paratuberculosis* are fundamental and well-suited to study in a post-graduate student research program. Further biometrical analysis of data collected in the present study may assist by identifying other potential stimuli for dormancy such as temperature fluctuations or changes in incident radiation.

11. The findings of this study need to be discussed widely because it is impossible to consider all relevant technical issues nor to foresee all relevant present and future policy and farm management factors in a single report.

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Appendix I - samples for the on-farm study

Table I-1. Site descriptions and sample identification for Property 1.Sampling date 17.11.97

Site No.	Slope °	Aspect of slope °	Pasture	Site description	Faeces
Property 1. Vittoria. About 400 sheep only remain on farm. Paddock selected for sampling was recently grazed by the flock. Gum trees mostly yellow box or similar.					
1	0		Cover of dry barley grass.	Sheep camp on north-south running ridge-top beneath gum trees. Soil dry. Site on south side of tree.	Scattered faecal pellets.
2	0			Further along ridge beneath gum tree on a sheep route. Dry soil.	Occasional piles of pellets.
3	5	north east	Light dry barley grass	Further along ridge, adjacent to trees. Faeces in sample	Present
4	5	north	no vegetation	Main sheep camp in shade of gum tree on open ridge top	2 cm layer of manure covers entire area
5	8	north	light cover dry native grasses	Open area on ridge adjacent to sheep route. No shade.	
S 6	0			Open area on crest of ridge; sheep camp area	moderate amount of faeces but no accumulation
7	5	100°	cover of mixed grasses	In gully under tree below sheep camp on crest; damp soil	Some fresh faeces about
8	0		Well covered by fescue?	Level terrace on creek bank within erosion gully that faces due north	Nil
X1, X2, X3				Single samples from creek bed area, marked with pegs	
Water from creek				near site 8	
9	10	east	Light cover native grasses	In eastern flowing gully off main ridge	Nil
10				ditto, 20m downhill from site 9	Nil
11				Sheep camp on hill top; soil reasonably moist. No pellets in sample. Large tree 2 m east of site.	Large nos. pellets
12				1 m from tree, in open paddock in the base of a gully. Pellets in sample.	Many
13				Open site in the base of a gully, 10-15 m n.e. of site 12. Soil very dry. Pellet in the sample.	Fresh faeces in the site
14			Long grass	Rim of dam, 10 m s.e. of dam. Soil very dry.	Fresh faeces within the site
15				Under a lone tree, shaded area, 1.5 m on ne side. Faeces in the sample	Present
16				Crest of ridge, 6 m from eastern boundary. Soil dry. Open area. Faeces in the sample. 100 m south of site 15.	Present
17				Exposed area on hill top. Soil very dry. Pellet in the sample.	Present
18				Sheep camp. Down hill of and beneath a tree located 5 m west of site. Soil moist. Fresh pellet in the sample.	Large amount present
19				Under a tree on a slope. Soil moist	Little
20				20 m west of creek bed. Dry soil. Open area.	Present
X4-X5				10 cores from portable yards where 400 sheep were housed overnight last week. Mostly dry compressed pellets and dust.	
Y1				Sediment from dam in paddock 27	
Y2				Water from dam in paddock 27. Dam shared with paddock 21 which contained cattle	
Y3-Y5				Single samples of manure collected from beneath shearing shed	

Table I-2. Site descriptions and sample identification for Property 2. Sampling date 17.11.97.

Site No.	Slope °	Aspect of slope °	Pasture	Site description	
Property 2. Borenore (all samples from a single 20 acre paddock north facing paddock on which remaining 101 sheep have been run for several months). Gum trees mostly yellow box or similar.					
1	3	north	Barley grass and thistles	Sheep camp beneath acacia trees. Well shaded 70%+.	Scattered pats and pellets
2	5	north		Near site 1. Open to east and north but shaded from west.	
3	5			East side of large gum tree; sample contains faeces	
4	5			West side of large gum tree. Faeces in sample	
5	0			Open area near fence	
6				On a small soak with green pick. Otherwise similar to 5	
7				Within a small ditch near site 6	
8				Site shaded in late afternoon by small gum tree	
9				East side of tree	
10				Damp corner	
X1, X2				Single samples of manure from beneath shearing shed	
11				Within a group of trees 10m west of dam. Fully shaded. Soil moist. Fresh faeces in sample.	Present
12				At northern end of group of trees, shaded in afternoon. Dry soil. Faeces in sample.	Older faeces present.
13				East of dam in open area, on a slope running into dam. Soil dry. Faeces in sample.	Present
14				Directly north of dam, 3 m from fence at base of slope. Soil moist	Fresh faeces present
15				At end of gully where surface water leaves property and passes under the road. Partly shaded.	Nil
16				South of dam. One third of grid over moist soil; rest over very dry soil. Open area.	Old faeces present in small amounts
17				1 m south of fence near road. Open area. Very dry. No pellets in sample	A few fresh pellets present
18				10 m south of fence near road. Open exposed dry grassy area. No pellets in sample.	Old pellets present
19				2 m west of large tree. Half of grid site shaded at 4.45 pm. Open dry area.	Few present
20				5 m south of fence. Open exposed grassy area. Dry soil.	Small amounts old dry pellets present
Y1				Sediment from dam 1	
Y2				Water from dam 1	
Y3				Water from dam 2	
Y4				Sediment from dam 2	

Table I-3. Site descriptions and sample identification for Property 3. Sampling date 18.11.97.

Site No.	Slope °	Aspect of slope °	Pasture	Site description	
Property 3. Rockley. Sites 1-10 from paddock with 250 wethers on 67 acres with access to additional 40 acres through fallen fences. Sites 11-20 200 ewes on 60 acres. All sites in paddocks which had been constantly grazed by infected sheep.					
1	10	east	light cover of grasses	Western lee of stringybark	scattered pellets
2	10	north east	very light cover green grasses	Head of shallow gully in unshaded area	scattered pellets
3	7.5	100		further down gully from site 2	scattered pellets
4	10	40	sparse grass cover	faeces in sample	scattered pellets
5	11	100		near sheep camp. Faeces in sample	scattered faeces
6	7	north east		in shade (from west) on eastern side of large tree above the dam. Faeces in the sample	Scattered pellets and clumps
water from dam				collected 2.5 cm below water surface in area where sheep would be expected to drink.	
X1-X2				Single samples of mud from waters edge of dam	
7	3	north		grassed area adjacent to inlet gully of the dam	little present
8	0		Grass and herbs	within drainage gully to dam, at edge of dam. A moist area near sedges.	
9	5	east		Unshaded area in drainage gully below dam	
X3-X4				Single samples of soil from gully bed adjacent to site 9, marked with pegs	
10	4	east	Light grass cover	Open flat area at bottom of paddock	Scattered pellets
11	slight			Open area 1 m from old tree stump. Compacted soil (vehicle track?)	Nil
12			Grass	Open area 2 m south of large tree. Very dry. Faeces in the sample.	Fresh and dry faeces
13			Grass	Open exposed and very dry area in middle of a gully, possibly water would accumulate here.	Small amounts dry pellets
14			Less grass than sites 11-13	Shaded area within sheep camp with much accumulation of faeces, 2 m south west of large tree. Soft soil.	Heaps
15	sharp		Grass	Very dry, rocky, open exposed area sloping into a rocky gully with 5 kangaroos.	Nil
16			Grass	Open, dry, 1.5 m east of fence, shaded by large tree on other side of fence.	Very few pellets
17				open but breezy shaded area grazed immediately beforehand. Site between 3 trees, soft soil. Faeces in sample.	Large amounts dry and fresh pellets
18			Dry grass	Open exposed dry area, 3 m from a tree but unshaded. Dry faeces in sample.	Dry and fresh faeces present
19			Grass	Open, exposed dry area 3 m east of fence. Very hard dry soil.	Nil
20				Dry open area, unshaded, 0.5 m east of gate, 1 m north of fence.	Nil
Y1				Water from drinking trough	

Table I-4. Site descriptions and sample identification for Property 4. Sampling date 18.11.97.

Site No.	Slope °	Aspect of slope °	Pasture	Site description	
Property 4. Limekilns. All samples from Spring paddock.					
1	4	east	light native	Adjacent to fence on road, exposed ridge top, unshaded	old pellets
2	6	east	light barley grass and other sp.	Head of shallow gully in open unshaded area	scattered dry pellets
3	5	north east		Further down gully below site 2; normally soft ground, now hard, pugged by cattle hoof prints. On east side of gum tree shaded from west.	
4	5	east	nil	Sheep camp in light timber, shaded. Faeces in sample	Extensive build up old and fresh faeces
5	3	east	nil	East side of sheep camp, shaded from west.	Much old, some fresh
6	3	south	dense drying off green suggesting N drainage from sheep camp	Open bowl beneath camp in full sun	Scattered dry
7	3	east	scant	Open hillock beneath sheep camp in full sun	Scattered dry
8	7		scant	open hillside in full sun	Scattered dry
9	2	north	green pick grass and clover drying off	Open area on creek bank at bottom of paddock, unshaded	
X1-X2				single samples of soil collected from grassed area next to creek bed (dry) below site 9, marked with pegs	
10	5			open area similar and near to site 9	
X3-X4				Single surface soil samples, very dry and dusty from a gully draining out of a ditch in which all culls thrown after slaughter.	
11	0		grass	open exposed dry area 1 m east of fence, 2 m west of vehicle track. Soil very dry.	Nil
12	0			As for 11, 2.5 m south west of large tree, partially shaded	Dry pellets
13			grass	open, exposed area in a slight gully that might collect water, 1 m south of fence (cattle nearby)	Nil
14			grass	open exposed area 6 m north west of dam. Very dry hard soil. Cows nearby. Faeces in sample	Dry pellets
15	sharp		grass	open exposed area 3m north of deep gully. Very hard soil. Tree 15 m west of site. Faeces in sample.	Dry pellets
16	yes		dry grass	open dry area with tree 10 m south east, 10 m frm fence. Faeces in sample	dry pellets
17			Patchy grass	Shaded area 1 m west of tree. Dry soft soil. Faeces in sample	dry pellets
18			patchy grass	adjacent deep gully in open dry area with tree 20 m west, 5 m south of fence. Very dry soil	Nil
19	slight		patchy grass	open dry exposed area below hill, tree 6 m to south, fence 20 m to north, with ant nest at edge of grid. Dry hard stoney soil. Faeces in sample	dry pellets
20			green grass	Open exposed dry sunny site on north west side of large tree. Dry hard soil, 10 m west of fence	few pellets
Y1				Watery cattle faeces collected adjacent to dam	
Y2				Sediment from dam	
Y3				Water from dam	

Table I-5. Site descriptions and sample identification for Property 5. Sampling date 01.09.97.

Site No.	Slope °	Aspect of slope °	Pasture	Site description	
Property 5. Carcoar					
Clean area					
1	5	300	Mixed, green, 5 cm	Adjacent to an uphill neighbour Peg 2 m from fence	No pellets; scattered cow pats (not fresh)
2	0	open	Mixed, green, 5 cm	Open level area adjacent to shed, open to north but generally exposed Peg 17 m off north corner of shed	No pellets; scattered cow pats (not fresh)
3	5	350	Fresh green pick 3-5 cm	Sheltered beneath overhanging pine trees, but open to west/NW sun Peg 20 m south of gate, 2.5 m off fence	ditto but cattle present, fresh pats
4	4	360	Nil; bare soil	Immediately adjacent to lee of timber shelter fence, well below crest of hill	No pellets; scattered cow pats (not fresh)
5	7	60	Lush green pick	Camp area beneath deciduous trees Peg 112° 7m from 2nd northern most tree	No pellets; scattered cow pats (not fresh)
6	5	34	Mixed; debris area	Adjacent to Site 5 on western side of trees within old sheep carcase Peg 330° 8 m from western most tree	No pellets; scattered cow pats (not fresh)
7	3	0	Short green pasture	Open area on a sheep pad Peg 265° 20 m from western most tree	No pellets; scattered cow pats (not fresh); a few ?rabbit pellets scattered
8	<1	0	Well compacted, pasture very short	Below dam, almost in north running gully Peg 17 m north of fence post with the large white insulator	No sheep/goat pellets
9	1	212	Very short green pick	Adjacent to feed trough Peg 40° 4 m from trough	No sheep goat pellets; scattered cow pats
10	1	272	Very light, green pasture	Open area of pasture Peg 160° 40 m from trough, 3.5 m from fence	No pellets; scattered cow pats (not fresh)
Contaminated area					
11	10	80	Very short green (bowling green)	Western side of dam on pronounced easterly slope	Pellets everywhere
12	12	80	Very short green (bowling green)	20 m uphill of site 10; 1 m downhill of old sheep carcase	Pellets everywhere
13	4	34	Very short green pick	open, exposed elevated area Peg near Site 14	heavy pellets - 10 per 100 cm square
14	nr level		Very short green pick	open, exposed; nearby Site 13 Peg 120° 57 m from leaning tree	heavy pellets

15	2	0	Short lush green pasture plus herbs including nettles 3cm	Sheep camp; on north eastern side of and slightly shaded by leaning eucalypt Peg 58° 11 m from vertical part of trunk	very heavy pellets
16	0		ditto	Sheep camp; on south west side of tree. Peg 156° 6 m from vertical part of trunk	very heavy pellets
17	0		very lush green pasture	Open, almost on crest of low ridge; sheep camp Peg bearing 342° to leaning tree, 15 m off the fence (tree belt)	very very heavy pellets; innumerable per 100 cm square
18	1	26	short green pick plus standing dry pasture, sparse, 200 mm high	In shallow gully leading into dam, natural collection basin from surrounding pasture Peg 210° 55 m from eucalypt at head of dam	light pellets
19	1	314	Scant green pick; dry standing 100 mm high sparse pasture	open area Peg 15 m from gate, 2 m off fence	light pellets
20	1	314	ditto	open area adjacent to tree belt planting peg adjacent to corner post	light pellets
Miscellaneous samples					
21				Pool from within 10 cow pats adjacent to Site 1	
22				Pool from within 10 cow pats adjacent to Site 2	
23				Pool from within 10 fresh cow pats adjacent to Site 3	
24a				Pool of 10 soil cores from south western inlet area to dam, collected linear fashion rather than from a grid	
25				?rabbit pellets collected from the sheep pad adjacent to Site 7 (impossible for it to be sheep/goat; rabbits believed to be plentiful along the nearby creek)	
26				10 x pool of surface soil and sediment from waterline of dam adjacent to Site 11	
27				Putty-like faeces, believed to be from a single sheep, from near site 15	
28				Pooled cow faeces from Dexters grazing with goats in the paddock to the east of that containing Site 18	
29				50 faecal pellets from separate piles in sheep camp of paddock containing Sites 11-16; believed to represent 50 individuals, collected Sat 30.8.97 by B. Vickers and held at 4°C	
24b				at opposite (eastern) end of dam from 24a 01.06.98	
30				Near site 24 03.11.98	
31			100 mm thick green grass	3 m south west of site 24 03.11.98	
32			100 mm thick green grass	3m south west of site 31 03.11.98	
33				duck droppings from southern side of dam 03.11.98	
34			100 mm thick green grass	eastern side of dam 03.11.98	
35			100 mm thick green grass	east of site 34 03.11.98	
36				dam sediment from eastern side of dam 03.11.98	
37				dam sediment from southern side of dam 03.11.98	
38			thick grass	pellets (? rabbit) from paddock close to the dam 03.11.98	

Appendix II - collections of infected faeces

Table II-1. January 1998 contamination. Daily excretion of faeces and *M. paratuberculosis* by housed sheep at EMAI. Data are: Wgt, weight of faeces in grams; Smear result of examination of Ziehl Neelsen stained faecal smear; Cult, culture as week after inoculation when growth index of 999 first recorded.

Date	Animal 2			Animal 7			Animal 9			Animal 23			Animal 25			Animal 27			Animal 34		
	Wgt	Smear	Cult	Wgt	Smear	Cult	Wgt	Smear	Cult	Wgt	Smear	Cult	Wgt	Smear	Cult	Wgt	Smear	Cult	Wgt	Smear	Cult
13.01.98	903	+++	4	368	++	4	225	-	5	243	-	-	744	-	7	285	-	5	798	-	-
14.01.98	168*	+	4	280	+	4	170	-	5	94	-	-	567	+	-	171	-	5	11	-	-
15.01.98	976	+	4	595	+++	4	186	-	5	174	-	-	907	+	-	422	++	4	55	-	-
16.01.98	840	++	4	602	++	4	264	+	5	231	-	-	937	+	-	552	+	3	172	-	-
17.01.98	829	-	4	504	+	5	632	-	5	441	-	-	954	-	8	550	-	4	334	-	-
18.01.98	1329	+	4	828	-	4	515	-	5	695	-	-	1204	-	-	561	-	5	143	-	-
19.01.98	1531	-	3	1124	-	4	481	+	5	1219	-	-	1286	-	-	491	-	5	108	-	8
20.01.98	1480	+++	3	693	+++	3	447	-	5	1107	-	-	1026	+	-	483	-	5	766	-	-
21.10.98	2086	+++	3	311	++++	3	664	-	5	1488	-	6	1399	-	7	513	-	4	1218	-	-
22.01.98	1779	+++	3	330	++++	3	709	-	5	1517	-	-	1425	++	-	830	-	5	1870	-	7
23.01.98	1752	+++	3	314	+++	3	766	-	5	1648	-	-	1562	-	6	537	-	5	2018	-	8
24.01.98	1500	++	nt	150	+++	nt	750	+	nt	1350	-	nt	850	+	nt	600	+	nt	1300	-	nt
25.01.98	1900	+++	nt	350	+++	nt	950	+	nt	1400	-	nt	1250	-	nt	550	-	nt	1550	-	nt
26.01.98	1000	+++	nt	450	+++	nt	750	-	nt	1200	-	nt	900	-	nt	450	-	nt	1150	-	nt
27.01.98	850	+++	nt	250	+++	nt	550	+	nt	1050	-	nt	900	+	nt	500	-	nt	800	-	nt
Total	18923			7149			8059			13857			15911			7495			12293		
Gel test	3+			3+			3+			-ve			-ve			3+			-ve		
Pathology	Gross lesions present; multibacillary			Gross lesions present; multibacillary			Gross lesions present; multibacillary			No gross or histological lesions			No gross or histological lesions			Gross lesions present; multibacillary			No gross or histological lesions		

* faecal collection bag came adrfit and faeces were lost overnight; this amount was removed from rectum manually.
+ single acid fast bacilli in only one to three fields of the entire smear
++ occasional single or clumped acid fast bacilli
+++ single or clumped acid fast bacilli seen regularly, at least once in every second field
++++ numerous single or clumped acid fast bacilli seen in every field
nt not tested

Table II-2. November 1998 contamination. Daily excretion of faeces and *M. paratuberculosis* by housed sheep at EMAI. Data are: Wgt, weight of faeces in grams; Smear result of examination of Ziehl Neelsen stained faecal smear.

Date	Animal 246		Animal 247		Animal 248		Animal 250		Animal 253		Animal 257		Animal 266*		Animal 281	
	Wgt	Smear	Wgt	Smear	Wgt	Smear	Wgt	Smear	Wgt	Smear	Wgt	Smear	Wgt	Smear	Wgt	Smear
20.10.98	405	++	276	+	125	+++	309	+	286	+	328	+	225	-	-	
21.10.98	271	++	24	+	89	+++	166	++	184	-	68	+/-	75	-	-	
22.10.98	679	++	100	++	82	++++	86	++	294	+	66	-	167	-	66	-
23.10.98	732	++	260	+	Died		631	++	504	+	65	-	661	-	405	-
24.10.98	627		496	+			806		521	+	696		not collected		79	-
25.10.98	495		849	+			356		682	+	687		524	-	692	-
26.10.98	1161		121	+			427		659	+	813		176	-	482	-
27.10.98	Culled due to watery faeces		134	+			Culled due to watery faeces		1181	+	Culled due to watery faeces		Culled as smear -ve		838	-
28.10.98			308	+					900	++					842	-
29.10.98			655	+					856	++					760	-
30.10.98			331	+					743	++					-	-
31.10.98			350	+					250	++					-	-
01.11.98			450	+					250						-	-
02.11.98			200	+					650	+					-	-
03.11.98			158	-					958	+					871	-
04.11.98			390	+					1076	+					1076	-
05.11.98			529	+					677	+					1254	-
06.11.98			605	+					1025	+					944	-
Gel test	3+		3+		3+		2+		3+		1+		-ve		2+	
Pathology	Gross lesions present; multibacillary		Gross lesions present; multibacillary				Gross lesions present; multibacillary		Gross lesions present; multibacillary		Gross lesions present; paucibacillary AFB -ve		Gross lesions present; no histological lesions		Gross lesions present; multibacillary	

+ single acid fast bacilli in only one to three fields of the entire smear
++ occasional single or clumped acid fast bacilli
+++ single or clumped acid fast bacilli seen regularly, at least once in every second field
++++ numerous single or clumped acid fast bacilli seen in every field
nt not tested
Pathology findings in MN98/C785
*smear +ve when sampled on farm 6.10.98

Table II-3. November 1999 and January 2000 contamination. Daily excretion of faeces and *M. paratuberculosis* by housed sheep at EMAI. Data are: Wgt, weight of faeces in grams; Smear result of examination of Ziehl Neelsen stained faecal smear.

Sheep	AGID	Pathology	Faeces	26.10.99		27.10.99		28.10.99		29.10.99		30.10.99		31.10.99		01.11.99		02.11.99	
				wgt	smear	wgt	smear	wgt	smear	wgt	smear	wgt	smear	wgt	smear	wgt	smear	wgt	smear
3604	2+		pellet	540	-	772	-	1294	-	1285	-	1553	1+	1347	1+	467	2+	500	nt
3607	3+		liquid	1638	2+	2536	1+	842	3+	1027	3+	1209	3+	432	2+	506	3+	400	nt
3609	2+		soft	1286	-	725	2+	2271	-	1105	-	617	-	1898	-	1125	-	1650	nt
3611	3+		soft	46	3+	45	3+	240	2+	411	2+	833	-	1084	1+	778	-	1480	nt
3612	2+		soft	350	3+	617	2+	592	1+	956	1+	1192	-	1170	1+	832	1+	900	nt
3613	2+		soft	101	1+	853	1+	1438	1+	846	1+	252	1+	1300	-	312	1+	100	nt
				12.11.99		16.11.99		17.11.99		18.11.99		19.11.99							
3604	2+	Gross lesions present; multibacillary	pellet to liquid	1581	2+	735	3+	813	3+	798	3+	786	3+						
3607	3+	Gross lesions present; multibacillary	liquid	2009	1+	545	2+	1555	3+	1531	3+	1769	3+						
3609	2+	Gross lesions present; multibacillary	pellet to soft	1725	1+	1351	1+	1811	1+	1604	1+	1895	1+						
3611	3+	Gross lesions present; multibacillary	soft to liquid	1098	2+	555	3+	448	3+	588	3+	-	-						
3612	2+	Gross lesions present; multibacillary	pellet	657	-	836	1+	1197	-	913	-	1361	-						
3613	2+	Gross lesions present; multibacillary	pellet to soft	1550	1+	486	2+	1205	2+	1339	1+	1303	2+						

Appendix III - culture results from pasture plots, pasture boxes and water troughs

In the tables that follow the data are weeks required to attain growth index of 999, generally in each of two pools of 10 soil samples or 10 faecal pellets (in format pool 1, pool 2). All cultures were incubated for 20 weeks.

Table III-1. January 1998 contamination. Pasture plots at site 1 - Borenore.

Plot	Treatment	Time after contamination in months (date sampled)											
			0 28.01.98	1 03.03.98	2 31.03.98	3 04.05.98	4 01.06.98	5 29.06.98	6 03.08.98	7 08.09.98	8 13.10.98	9 10.11.98	10 7.12.98
1	Outside (slurry)	A	4, 3	8, 8	-, -	7, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	4, 4	10, 8	-, -	-, -	-, -	-, -	-, -	8, -	-, -	-, -	-, -
		C	4, 4	9, -	-, -	-, -	11 (270), -	-, -	-, -	-, -	-, -	-, -	-, -
2	Shade (slurry)	A	4, 4	-, -	3, 10	3, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	3, 4	-, -	-, -	- , 10	-, -	-, -	-, -	5, -	-, -	-, -	-, -
		C	4, 4	-, -	-, -	-, 12 (815)	-, -	-, -	-, 11	-, -	-, -	-, -	-, -
3	Shade + moisture (slurry)	A	4, 4	12, 6	-, -	9 (671), -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	4, 4	-, 12	-, 12	10 (215), -	-, -	-, -	8 (408), 10	-, -	-, -	-, -	-, -
		C	4, 4	8, -	9, 12 (256)	-, 12 (406)	-, -	-, -	-, -	10(553+/-), -	-, -	-, -	-, -
4	Shade + moisture + low lime (slurry)	A	4, 4	-, 8	3, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	4, 3	-, 8	-, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	4, 4	12, -	-, -	5 (835), 8	-, -	-, -	-, -	-, -	-, -	-, -	-, -
5	Shade + moisture + high lime (slurry)	A	4, 4	-, -	5, -	8, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	4, 4	12 (106), -	8, -	6 (361), 11 (338)	-, -	-, -	-, -	-, 9	-, -	-, -	-, -
		C	3, 4	-, 7	8, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -

6	Shade + moisture (pellets)	A	-----	-, -	6, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	4, 4	-, 5	-, -	-, -	-, -	-----	-----	-----	------	-, -	-, -
		B	-----	-, -	-, 11	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B-pellets	4, 4	-, -	8, 6	-, 3 (794)	-, -	-----	-----	-----	-----		
		C	-----	-, 10	-, -	-, -	-, 8 (105)	-, -	-, -	-, -	-, -	-, -	-, -
		C-pellets	4, 4	-, 5	-, -	7, -	-, -	-----	-----	-----	-----		
7 FD	Outside (pellets)	A	-----	-, -	-, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	4, 4	-, -	-, -	-, 18 (799)	-, -	-----	-----	-----	-----	-----	-----
		B	-----	-, 9	-, 6	-, -	-, 8 (242)	-, -	-, -	-, -	-, -	-, -	-, -
		B-pellets	4, 4	-, -	-, -	-, -	-, -	-----	-----	-----	-----	-----	-----
		C	-----	7, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C-pellets	4, 4	8, -	-, -	-, -	-, -	-----	------	-----	-----	-----	-----
	Dates		2.11.98 Week 0	9.11.98 Week 1	16.11.98 Week 2	23.11.98 Week 3	30.11.98 Week 4	7.12.98 Week 5	4.1.99 Week 9	8.2.99 Week 14	1.3.99 Week 17	29.3.99 Week 21	29.4.99 Week 25
8	Outside (pellets) extension plot	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	4, 4	7, 7	-, -	-, 8	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, 8	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B-pellets	4, 4	7, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C-pellets	4, 4	7, 7	6, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -

notes: discontinued sampling plots 1 - 7 and plot 8 after 22.06.1999

Table III-1 (continued). Results of culture of pasture plots at site 1 - Borenore.

Plot	Treatment	Replicate	Time after contamination in months (approx) (date sampled)						
			11 4.1.99	12 11.2.99	13 1.3.99	14 30.3.99	15 29.4.99	16 24.05.99	17 22.06.99
1	Outside (slurry)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
2	Shade (slurry)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
3	Shade + moisture (slurry)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
4	Shade + moisture + low lime (slurry)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
5	Shade + moisture + high lime (slurry)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
6	Shade + moisture (pellets)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -

7	Outside (pellets)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	Dates								
8	Outside (pellets) extension plot	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -

Table III-2. January 1998 contamination. Pasture plots at site 2 - Carcoar.

Plot	Treatment	Replicate	Time after contamination in months (approx) (date sampled)										
			0 28.01.98	1 03.03.98	2 31.03.98	3 04.05.98	4 01.06.98	5 29.06.98	6 03.08.98	7 08.09.98	8 13.10.98	9 10.11.98	10 7.12.98
1	Outside (slurry)	A	4, 4	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	4, 4	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	4, 4	11, -	-, -	-, -	8 (294), -	-, -	10 (316), -	-, -	-, -	-, -	-, -
2	Shade (slurry)	A	4, 4	-, 8	3, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	4, 4	-, -	-, -	-, -	8 (371),	-, -	8 (314), -	8 (217), -	-, -	-, -	-, -
		C	3, 3	-, -	10, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
3	Shade + moisture (slurry)	A	4, 4	8, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	4, 3	-, -	-, -	, 13 (849)	-, -	-, -	8(675),10	-, -	-, -	-, -	-, -
		C	3, 4	-, -	3, 8	-, -	-, -	-, -	8, -	-, -	-, -	-, -	-, -
4	Shade + moisture + low lime (slurry)	A	4, 4	-, -	-, -	-, -	-, -	-, -	-, 10	-, -	-, -	-, -	-, -
		B	4, 4	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	4, 3	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
5	Shade + moisture + high lime (slurry)	A	4, 4	8, -	-, -	-, -	-, -	-, -	9, -	6, -	-, -	-, -	-, -
		B	4, 4	-, -	5, 5	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	4, 4	-, -	-, 10 (815)	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
6	Shade + moisture (pellets)	A	-----	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	4, 4	-, -	-, -	-, -	-, -	-----	-----	-----	-----	-----	-----
		B	-----	-, -	-, 6	-, -	-, -	-, -	7, -	-, -	-, -	-, -	-, -
		B-pellets	4, 4	-, -	-, -	-, -	-, -	-----	------	-----	-----	-----	-----
		C	-----	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C-pellets	4, 4	-, -	-, -	-, -	-, -	-----	-----	-----	-----	-----	-----

7 BV	Outside (pellets)	A	-----	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	4, 4	-, -	-, -	-, -	-, -	------	------	-----	-----	-----	-----
		B	-----	-, 8	-, -	-, -	, 8 (322)	-, -	-, -	-, -	-, -	-, -	-, -
		B-pellets	4, 4	-, -	-, -	-, -	-, -	-----	------	-----	-----	-----	-----
		C	-----	-, -	9, -	-, -	-, -	-, -	-, -	-, 8	-, -	-, -	-, -
		C-pellets	4, 4	-, -	-, -	-, -	-, -	-----	-----	-----	-----	-----	-----
	Dates		2.11.98 Week 0	9.11.98 Week 1	16.11.98 Week 2	23.11.98 Week 3	30.11.98 Week 4	7.12.98 Week 5	4.1.99 Week 9	8.2.99 Week 14	1.3.99 Week 17	29.3.99 Week 21	29.4.99 Week 25
8	Outside (pellets) extension project plot	A	-, -	-, 10	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	4, 4	7, 7	6, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, 9	-, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B-pellets	4, 4	8, 8	-, 6	7, -	9, 9	9 (805), -	-, -	-, -	-, -	-, -	-, -
		C	-, -	7, 9	6, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C-pellets	4, 4	7, 7	6, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -

Control negative cultures: two series of 10 cores were taken before contamination on 28.01 from each replicate in plots 6 and 7.

Table III-2 (Continued). January 1998 contamination. Pasture plots at site 2 - Carcoar.

Plot	Treatment	Replicate	11 4.1.99	12 11.2.99	13 1.3.99	14 30.3.99	15 29.4.99	16 24.05.99	17 22.06.99
1	Outside (slurry)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
2	Shade (slurry)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
3	Shade + moisture (slurry)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
4	Shade + moisture + low lime (slurry)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
5	Shade + moisture + high lime (slurry)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
6	Shade + moisture (pellets)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -

7 BV	Outside (pellets)	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	Dates								
8	Outside (pellets) extension plot	A	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		A -pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		B-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C	-, -	-, -	-, -	-, -	-, -	-, -	-, -
		C-pellets	-, -	-, -	-, -	-, -	-, -	-, -	-, -

Control negative cultures: two series of 10 cores were taken before contamination on 28.01 from each replicate in plots 6 and 7.

Table III-3. January 1998 contamination. Pasture plots. Results of cultures from deep soil cores. Data are weeks required to attain growth index 999 in each of two pools of 10 deep soil samples.

Plot	Treatment	Replicate	9 months after contamination	
			Site 1-Borenore (Sampled 10.11.98)	Site 2-Carcoar (Sampled 17.11.98)
1	Outside (slurry)	A	-,-	-,-
		B	-,-	-,-
		C	-,-	-,-
2	Shade (Slurry)	A	-,-	-,-
		B	-,-	-,-
		C	-,-	-,-
3	Shade + moisture (slurry)	A	-,-	-,-
		B	-,-	-,-
		C	-,-	-,-
6	Shade + moisture (pellets)	A	-,-	-,-
		B	-,-	-,-
		C	-,-	-,-
7	Outside (Pellets)	A	-,-	-,-
		B	-,14*	-,-
		C	-,12*	-,-

*PCR products from these samples were not consistent with *M. paratuberculosis* in *Alw* I restriction digests

Table III-4. November 1998 contamination. Pasture boxes at EMAI. nt, not tested

Time after contamination			Sample					
Date	Box	Week	Pellets	Soil 2 cm	Soil 3.5 cm	Soil 6-8 cm	Run-off water (Box 10)	Grass
02.11.98	10	0	5, 4					
02.11.98	1	0	4, 5	4, 6	-----	-----	-----	-----
09.11.98	1	1	5, 5	5, 5	5, 5	5, 5	5	5
16.11.98	2	2	8, 8	9, 10	-, -	-, 9	11	8
23.11.98	2	3	8, 8	8, -	-, -	8, -	8	8
30.11.98	3	4	6, 6	-, -	7, -	-, -	-	7
07.12.98	3	5	7, 8	-, -	(255 wk9), -	-, -	-	-
14.12.98	4	6	8, 8	-, -	-, -	-, (654wk8)	-	-
21.12.98	4	7	9, -	-, -		-, -	-	-
04.01.99	5	9	- , 12	7, -		-, 8	-	-
11.01.99	5	10	11, -	-, -	-, -	-, -	-	-
25.01.99	6	12	-, -	-, -	-, -	-, -	-	-
08.02.99*	6	14	-, -	-, -	-, -	-, -	-	-
22.02.99	7	16	-, -	-, -	-, -	-, -	-	-
08.03.99	7	18	-, -	-, -	-, -	-, -	-	-
22.03.99	8	20	-, -	-, -	-, -	-, -	-	-
05.04.99	8	22	-, -	-, -	-, -	-, -	-	-
19.04.99	9	24	-, -	-, -	-, -	-, -	-	nt
27.05.99	9	29	-, -	-, -	-, -	-, -	-	nt
01.07.99	1	33	-, -	-, -	-, -	-, -	-	nt
14.02.01**	all	117	-ve all 8 samples	-ve all 16 samples			nt	nt

* sampling method for soil cores changed to prevent surface matter from contaminating the deep soil cores. From this date soil was collected as a single core, extruded and cut into sections. In earlier samplings, repeated samples at progressively greater depths were taken from the same site and contamination of deeper samples from surface litter cannot be ruled out.
** 2 soil cores and 1 faecal pellet sample from each of the 8 boxes

Table III-5. November 1999 and January 2000 contaminations. Pasture boxes at Borenore. For pellets, nt = not tested as pellets no longer visible

Boxes at Borenore 0% SHADE NOVEMBER 1999 CONTAMINATION

Sample	0	2	4	6	9	10	12	16	20	24	28	32	67
	3/11/1999	16/11/2000	30/11/1999	14/12/2000	4/01/2000	11/01/2000	24/01/2000	22/02/2000	21/03/2000	18/04/2000	16/05/2000	13/06/2000	14.02.2001
	1A	1B	1C	3A	3B	3C	5A	5B	5C	7A	7B	7C	1,2,3,4
pellets	3, 3	6, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	nt
soil	5, 5	6, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt
	2A	2B	2C	4A	4B	4C	6A	6B	6C	8A	8B	8C	5,6,7,8
pellets	3, 3	7, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	nt
soil	5, 5	6, 8	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt

Boxes at Borenore 70% SHADE NOVEMBER 1999 CONTAMINATION

Sample	0	2	4	6	9	10	12	16	20	24	28	32	67
	3/11/1999	16/11/2000	30/11/1999	14/12/2000	4/01/2000	11/01/2000	24/01/2000	22/02/2000	21/03/2000	18/04/2000	16/05/2000	13/06/2000	14.02.2001
	9A	9B	9C	11A	11B	11C	13A	13B	13C	15A	15B	15C	9,10,11,12
pellets	4, 7	4, 4	6, 6	6, 6	-, -	-, -	-, -	-, -	-, -	nt	nt	nt	nt
soil	5, 4	7, 6	7, 7	10, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	- -,- -,- -,- -
grass	nt	-	-	-	10	-	-	-	-	-	-	-	nt
	10A	10B	10C	12A	12B	12C	14A	14B	14C	16A	16B	16C	13,14,15,16
pellets	4, 3	5, 5	6, 5	7, 6	9, -	-, -	8, -	-, -	-, -	-, -	-, -	-, -	nt
soil	3, 6	7, 4	6, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	- -,- -,- -,- -
grass	nt	-	-	-	10	-	-	-	-	-	-	-	nt

Boxes at Borenore 0% SHADE JANUARY 2000 CONTAMINATION

Sample	0 24/01/2000	2 8/02/2000	4 22/02/2000	6 7/03/2000	8 21/03/2000	10 4/04/2000	12 18/04/2000	16 16/05/2000	20 13/06/2000	24 11/07/2000	28 8/08/2000	32 5/09/2000	55 14/02/2001
	17A	17B	17C	19A	19B	19C	21A	21B	21C	23A	23B	23C	17,18,19,20
pellets	4, 4	6, -	-, -	-, -	-, 10	-, -	-, -	-, 9	-, -	-, -	nt	nt	nt
soil	8, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt
	18A	18B	18C	20A	20B	20C	22A	22B	22C	24A	24B	24C	21,22,23,24
pellets	5, 6	7, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	nt	nt	nt
soil	5, 3	-, -	-, -	-, -	-, -	-, -	-, -	12, -	-, -	-, -	-, -	-, -	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt

Boxes at Borenore 70% SHADE JANUARY 2000 CONTAMINATION

Sample	0 24/01/2000	2 8/02/2000	4 22/02/2000	6 7/03/2000	9 21/03/2000	10 4/04/2000	12 18/04/2000	16 16/05/2000	20 13/06/2000	24 11/07/2000	28 8/08/2000	32 5/09/2000	55 14/02/2001
	25A	25B	25C	27A	27B	27C	29A	29B	29C	31A	31B	31C	25,26,27,28
pellets	4, 4	5, 5	6, 7	-, -	7, 6	-, 8	-, nt	nt	nt	nt	nt	nt	nt
soil	5, 6	-, 7	8, 7	-, -	-, -	-, -	-, 9	-, -	-, -	8, -	-, -	-, -	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt
	26A	26B	26C	28A	28B	28C	30A	30B	30C	32A	32B	32C	29,30,31,32
pellets	4, 4	5, 4	5, 5	-, 8	6, 6	6, 5	8, 8	nt	nt	nt	nt	nt	nt
soil	6, 6	6, 6	6,	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt

Table III-6. November 1999 and January 2000 contaminations. Pasture boxes and water troughs at EMAI.

Boxes at EMAI   0% SHADE   NOVEMBER 1999 CONTAMINATION

Sample	0	2	4	6	9	10	12	16	20	24	28	32	48	67
	3/11/1999	16/11/2000	30/11/1999	14/12/2000	4/01/2000	11/01/2000	24/01/2000	22/02/2000	21/03/2000	18/04/2000	16/05/2000	13/06/2000	09/10/2000	14/02/2001
	1A	1B	1C	2A	2B	2C	3A	3B	3C	4A	4B	4C	1,2,3,4	1,2,3,4
pellets	4, 4	-, -	-, -	-, -	-, -	-, -	-, 6	-, -	-, -	-, -	-, -	-, -	nt	nt
soil	7, 6	8, 10	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt	nt
	5A	5B	5C	6A	6B	6C	7A	7B	7C	8A	8B	8C		5,6,7,8
pellets	3, 5	-, -	-, -	-, -	-, -	-, -	-, 6	-, -	-, -	-, -	-, -	-, -	nt	nt
soil	6, 6	8	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt	nt
	9A	9B	9C	10A	10B	10C	11A	11B	11C	12A	12B	12C		9,10,11,12
pellets	4, 4	-, -	-, -	-, -	-, -	-, -	-, 6	-, -	-, -	-, -	-, -	-, -	nt	nt
soil	6, 5	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt	nt

Boxes at EMAI   70% SHADE   NOVEMBER 1999 CONTAMINATION

Sample	0	2	4	6	9	10	12	16	20	24	28	32	48	67
	3/11/1999	16/11/2000	30/11/1999	14/12/2000	4/01/2000	11/01/2000	24/01/2000	22/02/2000	21/03/2000	18/04/2000	16/05/2000	13/06/2000	09/10/2000	14/02/2001
	1A	1B	1C	2A	2B	2C	3A	3B	3C	4A	4B	4C	1,2,3,4	1,2,3,4
pellets	3, 6	8, 12	8, 7	6, 6	-, -	-, -	-, 7	-, -	-, -	-, -	-, -	-, -	-,-	nt
soil	6, 5	-, 7	-, -	8, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	8	-	-	-	-	-	-	-	-	-	nt	nt
	5A	5B	5C	6A	5C	6A	7A	7B	7B	7B	7B	7B	5,6,7,8	5,6,7,8
pellets	7, 6	8, 7	-, 6	6, 6	-, -	-, -	6, 7	-, -	-, -	-, -	-, -	-, -	-,-	nt
soil	6, 5	-, -	-, -	7, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	6	-	-	-	-	-	-	-	-	-	-	nt	nt
	9A	9B	9C	10A	9C	10A	11A	11B	11B	11B	11B	11B	9,10,11,12	9,10,11,12
pellets	5, 4	8, 8	6, -	7, 6	-, 8	-, -	-, 7	-, -	-, -	-, -	-, -	-, -	-,-	nt
soil	7, 6	8, -	8, -	-, 7	7, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	7	-	-	-	-	-	-	-	-	-	nt	nt

Boxes at EMAI **100% SHADE** **NOVEMBER 1999 CONTAMINATION**

Sample	0	2	4	6	9	10	12	16	20	24	28	32	48	67
	3/11/1999	16/11/2000	30/11/1999	14/12/2000	4/01/2000	11/01/2000	24/01/2000	22/02/2000	21/03/2000	18/04/2000	16/05/2000	13/06/2000	09/10/2000	14/02/2001
	1A	1B	1C	4A	4B	4C	7A	7B	7C	10A	10B	10C	1,2,3,4	1,2,3,4
pellets	4, 3	6, 6	6, 6	8, 6	7, 7	8, -	-, -	6, 6	-, -	10, -	-, 8	-, -	-,-	- -,- -,- -,- -
soil	5, 5	-, 8	7, 7	7, 10	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	7	8	-	10	-	-	-	-	-	-	-	nt	nt
	2A	2B	2C	5A	5B	5C	8A	8B	8C	11A	11B	11C	5,6,7,8	5,6,7,8
pellets	3, 3	7, 6	6, 6	6, 6	7, 7	-, -	-, -	6, 6	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
soil	6, 5	-, 7	6, 7	8, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	6	-	-	7	7	-	-	-	-	-	-	nt	nt
	3A	3B	3C	6A	6B	6C	9A	9B	9C	12A	12B	12C	9,10,11,12	9,10,11,12
pellets	5, 3	10, -	6, 6	6, 6	-, 7	9, 7	-, -	5, 6	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
soil	6, 5	6, 8	7, -	8, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	6	9	-	7	-	-	-	-	-	-	-	nt	nt

Boxes at EMAI **0% SHADE** **JANUARY 2000 CONTAMINATION**

Sample	0	2	4	6	8	10	12	16	20	24	28	32	36	55
	24/01/2000	8/02/2000	22/02/2000	7/03/2000	21/03/2000	4/04/2000	18/04/2000	16/05/2000	13/06/2000	11/07/2000	8/08/2000	5/09/2000	09/10/2000	14/02/2001
	13A	13B	13C	14A	14B	14C	15A	15B	15C	16A	16B	16C	13,14,15,16	13,14,15,16
pellets	4, 4	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	nt	nt
soil	8, 5	-, -	-, -	-, -	-, -	-, -	2, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt	nt
	17A	17B	17C	18A	18B	18C	19A	19B	19C	20A	20B	20C	17,18,19,20	17,18,19,20
pellets	4, 4	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	nt	nt
soil	5, 5	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt	nt
	21A	21B	21C	22A	22B	22C	23A	23B	23C	24A	24B	24C	21,22,23,24	21,22,23,24
pellets	4, 4	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	nt	nt
soil	5, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	-	-	-	-	-	-	-	-	-	-	nt	nt

Boxes at EMAI **70% SHADE** **JANUARY 2000 CONTAMINATION**

Sample	0	2	4	6	9	10	12	16	20	24	28	32	36	55
	24/01/2000	8/02/2000	22/02/2000	7/03/2000	21/03/2000	4/04/2000	18/04/2000	16/05/2000	13/06/2000	11/07/2000	8/08/2000	5/09/2000	09/10/2000	14/02/2001
	13A	13B	13C	14A	14B	14C	15A	15B	15C	16A	16B	16C	13,14,15,16	13,14,15,16
pellets	4, 4	6, 5	-, 9	-, 7	6, 7	-, -	-, -	-, 7	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
soil	-, 8	5, 7	-, -	-, -	-, 8	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	8	-	10	-	-	-	-	-	-	-	nt	nt
	17A	17B	17C	18A	18B	18C	19A	19B	19C	20A	20B	20C	17,18,19,20	17,18,19,20
pellets	4, 4	5, 5	-, -	-, -	7, -	-, -	-, 8	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
soil	5, 5	11, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	-	-	7	-	-	-	-	-	-	-	nt	nt
	21A	21B	21C	22A	22B	22C	23A	23B	23C	24A	24B	24C	21,22,23,24	21,22,23,24
pellets	6, 5	6, 6	-, 7	-, -	7, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
soil	3, 4	6, 7	9, 9	-, -	-, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	7	-	-	-	-	-	-	-	-	-	nt	nt

Boxes at EMAI **100% SHADE** **JANUARY 2000 CONTAMINATION**

Sample	0	2	4	6	9	10	12	16	20	24	28	32	36	55
	24/01/2000	8/02/2000	22/02/2000	7/03/2000	21/03/2000	4/04/2000	18/04/2000	16/05/2000	13/06/2000	11/07/2000	8/08/2000	5/09/2000	09/10/2000	14/02/2001
	13A	13B	13C	16A	16B	16C	19A	19B	19C	22A	22B	22C	13,14,15,16	13,14,15,16
pellets	4, 5	6, 6	7, 6	-, 8	-, -	-, -	-, -	-, -	-, -	6, 8	-, 7	7, -	-,-	- -,- -,- -,- -
soil	-, 6	6, 7	-, 9	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	-	-	8	-	10	6	-	8	-	-	nt	nt
	14A	14B	14C	17A	17B	17C	20A	20B	20C	23A	23B	23C	17,18,19,20	17,18,19,20
pellets	4, 4	6, 5	7, 6	-, 11	6, 5	-, -	-, -	-, -	-, -	-, -	-, 8	-, 6	8,7	- -,- -,- -,8 -
soil	5, 5	8, 6	7, -	-, -	-, 9	-, -	-, -	-, -	-, -	-, -	-, 9	-, -	-,-	- -,- -,- -,- -
grass	nt	-	-	7	-	-	-	-	-	-	-	-	nt	nt
	15A	15B	15C	18A	18B	18C	21A	21B	21C	24A	24B	24C	21,22,23,24	21,22,23,24
pellets	6, 5	6, 6	7, 7	8, 9	6, -	-, -	-, -	6, 6	-, -	7, -	-, -	-, -	-,-	- -,- -,- -,- -
soil	4, 4	7, 5	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-,-	- -,- -,- -,- -
grass	nt	-	6	-	14	-	-	6	-	-	-	-	nt	nt

Trough of water **70% SHADE** **NOVEMBER 1999 CONTAMINATION** outside shade house

Sample	0	2	4	6	9	10	12	16	20	24	28	32	36	40	44	48	52	56	67
	03/11/99	16/11/99	30/11/99	14/12/99	4/01/00	11/01/00	24/01/00	22/02/00	21/03/00	18/04/00	16/05/00	13/06/00	11/07/00	08/08/00	05/09/00	09/10/00	09/11/00	07/12/00	14/02/01
Water 1L	3, 3	5, 5	4, 4	4, 4	7, 7	7, 4	8, 8	6, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-. -	-, -
Sediment	4, 3	5, 5	6, 4	6, 6	6, 5	8, -	8, -	7, -	-, -	-, -	-, -	9, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -

Trough of water **70+70% SHADE** **NOVEMBER 1999 CONTAMINATION** inside shade house

Sample	0	2	4	6	9	10	12	16	20	24	28	32	36	40	44	48	52	56	67
	03/11/99	16/11/99	30/11/99	14/12/99	04/01/99	11/01/00	24/01/00	22/02/00	21/03/00	18/04/00	16/05/00	13/06/00	11/07/00	08/08/00	05/09/00	09/10/00	09/11/00	07/12/00	14/02/01
Water 1L	3, 3	3, 3	4, 4	4, 4	7, 7	7, 7	7, 7	6, 5	8, 9	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-. -	-, -
Sediment	4, 4	4, 4	7, 7	7, 7	7, 7	8, 8	7, 7	5, 6	8, 8	8, -	5, -	-, -	8, 11	8, 6	9, 9	7, -	-, -	-, -	-, -

Table III-7. November 1999 and January 2000 contaminations. Pasture plots at Borenore.

Plots at Borenore **0% SHADE** **NOVEMBER 1999 CONTAMINATION**

Sample	0	2	4	6	9	10	12	16	20	24	28	32	67
	3/11/1999	16/11/2000	30/11/1999	14/12/2000	4/01/2000	11/01/2000	24/01/2000	22/02/2000	21/03/2000	18/04/2000	16/05/2000	13/06/2000	14/02/2001
	10A	10A	10A	10A	10A	10A	10A	10A	10A	10A	10A	10A	10A
pellets	4, 4	-, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	nt	nt	nt	nt
soil	6, 5	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	10B	10B	10B	10B	10B	10B	10B	10B	10B	10B	10B	10B	10B
pellets	3, 3	8, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	nt	nt	nt	nt
soil	5, 6	7, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	10C	10C	10C	10C	10C	10C	10C	10C	10C	10C	10C	10C	10C
pellets	6, 6	-, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	nt	nt	nt	nt
soil	4, 3	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	10D	10D	10D	10D	10D	10D	10D	10D	10D	10D	10D	10D	10D
pellets	6, 4	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	nt	nt	nt	nt
soil	8, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -

Plots at Borenore **70% SHADE** **NOVEMBER 1999 CONTAMINATION**

Sample	0	2	4	6	9	10	12	16	20	24	28	32	67
	3/11/1999	16/11/2000	30/11/1999	14/12/2000	4/01/2000	11/01/2000	24/01/2000	22/02/2000	21/03/2000	18/04/2000	16/05/2000	13/06/2000	14/02/2001
	9A	9A	9A	9A	9A	9A	9A	9A	9A	9A	9A	9A	9A
pellets	4, 3	6, 6	-, -	9, 6	-, -	-, -	-, -	-, -	nt	nt	nt	nt	nt
soil	5, 5	-, 7	-, 6	-, -	-, -	-, -	-, 12	-, -	-, -	-, -	-, -	-, -	-, -
	9B	9B	9B	9B	9B	9B	9B	9B	9B	9B	9B	9B	9B
pellets	5, 4	7, 7	8, 7	6, 6	-, 8	-, -	-, -	-, -	nt	nt	nt	nt	nt
soil	3, 5	-, 8	-, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	9C	9C	9C	9C	9C	9C	9C	9C	9C	9C	9C	9C	9C
pellets	4, 3	6, 6	7, -	-, 6	-, -	-, -	-, -	-, -	nt	nt	nt	nt	nt
soil	5, 6	-, -	6, -	-, -	-, 12	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	9D	9D	9D	9D	9D	9D	9D	9D	9D	9D	9D	9D	9D
pellets	4, 4	7, 8	7, 7	-, -	-, -	-, -	-, -	-, -	nt	nt	nt	nt	nt
soil	5, 5	-, -	9, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -

Plots at Borenore **0% SHADE** **JANUARY 2000 CONTAMINATION**

Sample	0 24/01/2000	2 8/02/2000	4 22/02/2000	6 7/03/2000	9 21/03/2000	10 4/04/2000	12 18/04/2000	16 16/05/2000	20 13/06/2000	24 11/07/2000	28 8/08/2000	32 5/09/2000	55 14/02/2001
	12A	12A	12A	12A	12A	12A	12A	12A	12A	12A	12A	12A	12A
pellets	3, 3	-, -	-, -	-, 8	-, -	-, -	-, -	-, -	nt	nt	nt	nt	nt
soil	6, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	12B	12B	12B	12B	12B	12B	12B	12B	12B	12B	12B	12B	12B
pellets	4, 3	-, -	-, -	-, 4	-, -	-, -	-, -	-, -	nt	nt	nt	nt	nt
soil	5, 6	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	12C	12C	12C	12C	12C	12C	12C	12C	12C	12C	12C	12C	12C
pellets	3, 4	-, -	-, -	-, 9	-, -	-, -	-, -	-, -	nt	nt	nt	nt	nt
soil	6, 6	-, 5	-, -	-, 9	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	12D	12D	12D	12D	12D	12D	12D	12D	12D	12D	12D	12D	12D
pellets	4, 3	8, -	-, -	-, -	-, -	-, -	-, -	-, -	nt	nt	nt	nt	nt
soil	8, 6	-, -	-, -	-, -	-, -	-, 12	-, -	-, -	-, -	-, -	-, -	-, -	-, -

Plots at Borenore **70% SHADE** **JANUARY 2000 CONTAMINATION**

Sample	0 24/01/2000	2 8/02/2000	4 22/02/2000	6 7/03/2000	9 21/03/2000	10 4/04/2000	12 18/04/2000	16 16/05/2000	20 13/06/2000	24 11/07/2000	28 8/08/2000	32 5/09/2000	55 14/02/2001
	11A	11A	11A	11A	11A	11A	11A	11A	11A	11A	11A	11A	11A
pellets	3, 3	7, 6	-, -	-, -	-, 6	-, -	nt	nt	nt	nt	nt	nt	nt
soil	5, 5	-, -	-, 7	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	11B	11B	11B	11B	11B	11B	11B	11B	11B	11B	11B	11B	11B
pellets	4, 3	6, 6	-, 6	-, -	7, 7	-, -	nt	nt	nt	nt	nt	nt	nt
soil	5, -	-, 11	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	11C	11C	11C	11C	11C	11C	11C	11C	11C	11C	11C	11C	11C
pellets	4, 4	6, 6	-, -	-, -	-, 6	-, -	nt	nt	nt	nt	nt	nt	nt
soil	-, 5	-, 6	-, -	-, 12	11, -	-, 8	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	11D	11D	11D	11D	11D	11D	11D	11D	11D	11D	11D	11D	11D
pellets	5, 5	6, -	-, -	-, -	-, 6	-, -	nt	nt	nt	nt	nt	nt	nt
soil	6, 6	-, 10	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -

Table III-8. November 1999 contamination. Pasture boxes at various locations.

Wagga Wagga

Sample	0 3/11/1999	4 30/11/1999	9 4/01/2000	12 24/01/2000	16 22/02/2000	20 21/03/2000
	1A	1B	1C	3A	3B	3C
pellets	4, 4	-, 7	-, -	-, -	-, -	-, -
soil	7, 6	-, -	-, -	-, -	-, -	-, -
grass	*	-	-	-	-	-
	2A	2B	2C	4A	4B	4C
pellets	4, 4	-, -	-, -	-, -	-, -	-, -
soil	7, 6	-, -	-, -	-, -	-, -	-, -
grass	*	-	-	-	-	-

Broken Hill

Sample	0 3/11/1999	4 30/11/1999	9 4/01/2000	12 24/01/2000	16 22/02/2000	20 21/03/2000
	1A	1B	1C	3A	3B	3C
pellets	3, 3	6, 7	-, -	-, -	-, -	-, -
soil	6, 6	-, 6	-, -	-, -	-, -	-, -
grass	*	-	-	-	-	-
	2A	2B	2C	4A	4B	4C
pellets	5, 6	-, -	-, -	-, -	-, -	-, -
soil	6, 7	-, -	-, -	-, -	-, -	-, -
grass	*	-	-	-	-	-

Bourke

Sample	0 3/11/1999	4 30/11/1999	9 4/01/2000	12 24/01/2000	16 22/02/2000	20 21/03/2000
	1A	1B	1C	3A	3B	3C
pellets	4, 4	-, -	-, -	-, -	-, -	-, -
soil	7, 6	-, -	3, 8	-, -	-, -	-, -
grass	*	-	-	-	-	-
	2A	2B	2C	4A	4B	4C
pellets	4, 4	-, -	-, -	-, -	-, -	-, -
soil	7, 6	-, -	-, -	-, -	-, -	-, -
grass	*	-	-	-	-	-

Cobar

Sample	0 3/11/1999	4 30/11/1999	9 4/01/2000	12 24/01/2000	16 22/02/2000	20 21/03/2000
	1A	1B	1C	3A	3B	3C
pellets	3, 3	-, 8	-, -	-, -	-, -	-, -
soil	6, 5	-, -	-, -	-, -	-, -	-, -
grass	*	-	-	-	-	-
	2A	2B	2C	4A	4B	4C
pellets	3, 3	-, -	-, -	-, -	-, -	-, -
soil	5, 5	-, -	-, -	-, -	-, -	-, -
grass	*	-	-	-	-	-

Armidale

Sample	0 3/11/1999	4 30/11/1999	9 4/01/2000	12 24/01/2000	16 22/02/2000	20 21/03/2000
	1A	1B	1C	3A	3B	3C
pellets	5, 4	7, 7	-, -	-, -	-, -	-, -
soil	7, 7	7, 7	-, -	-, -	-, -	-, -
grass	*	-	-	-	-	-
	2A	2B	2C	4A	4B	4C
pellets	3, 4	-, 7	-, -	-, -	-, -	-, -
soil	6, 6	-, -	-, -	-, -	-, -	-, -
grass	*	-	-	-	-	-

Goulburn

Sample	0 3/11/1999	4 30/11/1999	9 4/01/2000	12 24/01/2000	16 22/02/2000	20 21/03/2000
	1A	1B	1C	3A	3B	3C
pellets	4, 5	-, -	-, -	-, -	-, -	-, -
soil	7, 5	-, -	-, -	-, -	-, -	-, -
grass	*	-	-	-	-	-
	2A	2B	2C	4A	4B	4C
pellets	3, 3	-, -	-, -	-, -	-, -	-, -
soil	-, 7	-, 10	-, -	-, -	-, -	-, -
grass	*	-	-	-	-	-

Appendix IV - appearance of plots and boxes

Table IV-1. January 1998 contamination. Appearance of plots at site 1 - Borenore

Date	Grass %	Broad leaf weed %	Clover %	Max height cm	% ground covered	Faeces visible
Plot 1 & 7 (outside)						
28.01.98	rye, green & dry			2-3	20	(mowed to 3 cm)
03.03.98	50	50	some	4	20	yes
31.03.98	dry			5	20	yes
04.05.98	70	20	10	2-3		yes
01.06.98	30	30	40	4-5	40	yes
29.06.98	50 (winter + rye)	25	25	4 - 8	100 (dense)	hard to find
03.08.98	50	50		6	100	
07.09.98	50	50		10	100	
06.01.99	50	20	30	5	100	
29.03.99	45-60	40-35	15-5	5-15	80	
28.04.99	45	50	5	2-4	100	
24.05.99	40-45	30	25-30	3-6	100	
26.06.99	34-45	33-35	20-33	3-4	95	
Plot 2 (inside no moisture)						
28.01.98	50 (rye)	25	25	10		(mowed to 3 cm)
03.03.98	50	50		10		yes
31.03.98	50 (drying off)	25	25	10		yes
04.05.98	60 (rye, paspalum, winter)	20	20	4-8		yes
01.06.98	80 (rye, couch)	10	10	6-10		hard to find
29.06.98	85	10	5	10	75	hard to find
03.08.98	80	10	10	10-12	80	
07.09.98	80	10	10	10	80	
06.01.99	70	10	20	50	100	
29.03.99	75	20	5	30	75	
28.04.99	80	10	10	3-6	80	
24.05.99	70	25	5	10-15	85	
26.06.99	80	10	10	3	80	
Plot 3, 4, 5, 6 (inside with moisture)						
28.01.98	50	25	25	10		(mowed to 3 cm)
03.03.98	50	50		10		yes
31.03.98	50	25	25	25		hard to find
04.05.98	80 (rye)	10	10	30		hard to find
01.06.98	60	20	20	20		hard to find
29.06.98	70 (rye, dense, collapsed and rotting mat)	20	10	16	50 (dense where present)	no
03.08.98	80	10	10	10	50	
07.09.98	80	10	10	8	50	
06.01.99	50	35	15	50	80	
29.03.99	45-55	30-45	2-15	30	80-85	
28.04.99	70-80	10-20	5-20	3-6	80	
24.05.99	50-60	20-40	10-30	10-15	80-85	
26.06.99	30-40	30-50	10-20	3-5	80-85	

Table IV-2. January 1998 contamination. Appearance of plots at site 2 - Carcoar

Date	Grass %	Broad leaf weed %	Clover %	Max height cm	% ground covered	Faeces visible
Plot 1 & 7 (outside)						
28.01.98	0	0	0	0	0	(bare ground)
03.03.98	0	0	0	0	0	yes
31.03.98	0	0	0	0	0	yes
04.05.98	50	5	45	1-4	moderate	yes
01.06.98	25	5	70	4	dense	yes
29.06.98	5	10 (Echium)	80	4-6	100	hard to find
03.08.98	10	30	60	6	100	
07.09.98	10	65	25	12	100	
06.01.99	75	25		15	95	
29.03.99	10	40	50	nr	90-95	
28.04.99	30-60	30-40	10-30	2-4	80-90	
24.05.99	30-45	45-50	20-35	3-6	90-95	
26.06.99	20	40	40	5	95	
Plot 2 (inside no moisture)						
28.01.98		100 (Echium)		4-6		(mowed to 3 cm)
03.03.98		100		4-6		yes
31.03.98	<5	95		10		yes
04.05.98	80 (couch, other)	20		4	75	yes
01.06.98	65	30	5	8		yes
29.06.98	20	60	20	16	60	
03.08.98	30	50	20	8	70	
07.09.98	50	40	10	15	70	
06.01.99	60	35	5	30	90	
29.03.99	30	65	5	nr	80	
28.04.99	70	30	0	3-6	60	
24.05.99	60	40	0	10-15	65	
26.06.99	50	40	10	10	70	
Plot 3, 4, 5, 6 (inside with moisture)						
28.01.98		100 (Echium)		4-6		(mowed to 3 cm)
03.03.98		100		4-6		yes
31.03.98	5	93		30		hard to find
04.05.98	30	70		40		hard to find
01.06.98	10	80	10	40	100 (dense mat)	hard to find
29.06.98	5	90	5	45	100 (large plants form a canopy over light cover at ground level)	no
03.08.98	5	95		8	50	
07.09.98	40	60		12	25	
06.01.99	60	20	20	40	90	
29.03.99	30-45	45-60	5-10	nr	80-85	
28.04.99	40-70	30-60	0	3-6	70-80	
24.05.99	50-60	40-50	0	10-15	75-90	
26.06.99	50-60	20-30	20	5	75-80	

Table IV-3. November 1998 contamination. Appearance of plot 8 at sites 1 and 2

Date	Grass %	Broad leaf weed %	Clover %	Max height cm	% ground covered	Faeces visible
Site 1. Borenore						
06.01.99	90	5	5	5	100	
29.03.99	80	20	0	5-15	95	
28.04.99	80	10	10	2-4	100	
24.05.99	80	20	0	3-6	100	
26.06.99	40	40	20	3	95	
Site 2. Carcoar						
06.01.99	35	65		20	90	
29.03.99	35	55	10	nr	85	
28.04.99	45	45	10	2-4	70	
24.05.99	45	45	10	3-6	80	
26.06.99	30	50	20	6	80	

Table IV-4. November 1999 and January 2000 contamination. Vegetation and comments about pasture boxes at EMAI

Date	November contamination boxes			January contamination boxes		
	Shade treatment			Shade treatment		
	0%	70%	100%	0%	70%	100%
11.11.1999	Even cover green grass to 75 mm displacing pellets - watered	Patchy cover green grass to 75 mm displacing pellets - reseeded and watered	Even cover green grass to 75 mm displacing pellets			
25.11.1999	Wilting & brown grass	Green grass with good cover	Lush green grass			
18.01.2000	Light cover mature grass, browning off - watered	Grass all browned off - watered	Grass brown and collapsed to form a surface mat - watered			
04.02.2000	Browned off - very hot days	Browned off - watered - very hot days	Browned off - watered - very hot days	Good germination - watered	Good germination - watered	Good germination - watered
14.02.2000	ditto	ditto	ditto	Browning off - watered	Green grass - watered	Yellowing and green grass- watered
21.02.2000	ditto	ditto	ditto	Browning off - watered	Green grass - watered	Yellowing and green grass - watered
08.03.2000	ditto	ditto	ditto	Yellowing and browning off - watered	Browning off - watered	Green grass
21.03.2000	Regenerating	ditto	ditto	Lush green grasses to 30 cm	Browning off	Browning off
05.04.2000	Sparse but tall green grass and broad leaf weed growth. Pellets still visible and soil moist	nil vegetation	nil vegetation	Lush dense grasses to 30 cm cut with shears to level with side of boxes. Fungal hyphae profuse at soil level which is totally shaded; pellets visible and soil very moist.	Very sparse green grass	Collapsed mat of brown grass. Pellets visible.
08.08.2000	Coarse vegetation 150 mm high. Pellets and faecal matter present.	All boxes dry and lacking standing vegetation. Dehydrated grass stems lying on surface. Pellets visible.		Good cover of fine grass 100 mm high. Pellets and faecal matter present.	All boxes dry and lacking standing vegetation. Dehydrated grass stems lying on surface. Pellets visible.	

Table IV-5. November 1999 and January 2000 contaminations. Vegetation and comments about pasture boxes at Borenore

Date	0% shade	70% shade
November contamination		
	boxes 1 - 8	boxes 9 - 16
11.11.1999	Variable germination but in general cover developing fairly evenly. 10 mm high	
19.11.1999	20 mm high	20 mm high
26.11.1999	40 mm high	60 mm high
01.12.1999	Grass drying out and less-well developed than 70% shade boxes	Grass developing well
03.12.1999	60 mm high	150 mm high. Ceased watering
10.12.1999	100 mm high. Ceased watering	
14.12.1999	Grass established to required height	
16.05.2000	Faecal material difficult to find	Faecal material no longer visible
15.02.2001	Tall vegetation cut. Pellets could not be found	
January contamination		
	boxes 17 - 24	boxes 25 - 32
28.01.2000	20 mm	20 mm high
04.02.2000	50 mm	70 mm high
11.02.2000	90 mm	120 mm high
18.02.2000	120 mm	150 mm high. Ceased watering.
25.02.2000	150 mm. Ceased watering.	
08.08.2000	Faecal material no longer visible	
15.02.2001	Tall vegetation cut. Pellets could not be found.	

Table IV-6. November 1999 contaminations. Vegetation and comments about pasture boxes at various sites

Date	November contamination boxes					
	Location					
	Armidale	Wagga Wagga	Bourke	Broken Hill	Cobar	Goulburn
3-5.11.1999	Faecal mixture covered in mud and pellets slightly depressed into the surface. Some surface pooling of water in some boxes. Faeces had shifted to one end in some boxes and was redistributed.					
11-15.11.1999	Uneven germination in some boxes at some locations. In general cover was variable to sparse. Heavy rain in Western division. Where present growth was to 75 mm.					
25.11.1999	Good even cover			Germinating grass lifting a crust of soil and faecal matter. Crust broken down manually and boxes watered.		
02.12.1999	Fairly even cover of green grass to 100 mm					
15.12.1999				Minimal vegetation. Pellets visible on surface.	Patchy grass cover with a hard pan forming on soil surface after pooling of water	
23.12.1999	Requested no further watering and to allow grass to brown off naturally if weather is dry.					
05.01.2000				Scant green grass with brown grass		
10.04.2000						Grass and broad leaf weeds to 150 mm

Table IV-7. November 1999 contamination. Vegetation and comments about pasture plots at Borenore

Date	Grass %	Broad leaf weed %	Clover %	Max height cm	% ground covered	Faeces visible
Plot 9 (70% shade)						
16.11.1999	60	30	10	3	40	
30.11.1999	30	30	9	**85**????	nr	
14.12.1999	40	20	40	20	85	
04.01.2000	35	30	30	15	80	
22.02.2000	80	20		20	75	hard to find
21.03.2000	75	20	5	15	75	
16.05.2000	75	15	10	5	50	
13.06.2000	50	45	5	8	50	
11.07.2000	nr	nr	nr	nr	nr	
08.08.2000	nr	nr	nr	nr	nr	
05.09.2000	80	10	10	12	60	
Plot 10 (0% shade)						
16.11.99	60	10	10	5	50	
30.11.1999	50	20	30	5	85	
14.12.1999	40	50	10	10	90	
04.01.2000	50	35	15	12	95	
22.02.2000	65	30	5	8	95	hard to find
21.03.2000	60	30	10	8	95	
16.05.2000	80	5	15	4	95	
13.06.2000	75	5	20	5	95	
11.07.2000	nr	nr	nr	nr	nr	
08.08.2000	nr	nr	nr	nr	nr	
05.09.2000	40	30	30	8	90	

Table IV-8. January 2000 contamination. Vegetation and comments about pasture plots at Borenore

Date	Grass %	Broad leaf weed %	Clover %	Max height cm	% ground covered	Faeces visible
Plot 11 (70% shade)						
16.11.99	60	30	10	5	40	
30.11.1999	45	40	15	16	50	
14.12.1999	40	30	25	15	65	
04.01.2000	45	45	10	10	70	
22.02.2000	55	30	15	15	85	
21.03.2000	70	20	10	15	75	
16.05.2000	70	25	5	5	65	
13.06.2000	40	55	5	8	50	
11.07.2000	65	30	5	4	60	
08.08.2000	50	45	5	5	50	
05.09.2000	50	40	10	10	50	
Plot 12 (0% shade)						
16.11.1999	70	20	10	3	85	
30.11.1999	60	25	15	5	85	
14.12.1999	60	30	10	8	85	
04.01.2000	60	30	10	10	90	
22.02.2000	65	30	5	10	95	
21.03.2000	55	30	15	10	95	
16.05.2000	75	10	15	4	90	
13.06.2000	75	5	20	5	95	
11.07.2000	75	15	10	5	95	
08.08.2000	50	30	20	6	95	
05.09.2000	45	30	25	8	90	

Appendix V - analysis of soil samples

Methods of soil analysis

Soil analyses for all samples except those used in the sensitivity trial (Part 1 this report) were performed by Analysis Systems, Incitec Ltd, Darcy Road, Port Kembla, NSW 2505 using the following methods:

Soil Colour	visual assessment of dry soil against Munsell colour chart.
Soil Texture	field texture technique of Northcote.
pH (1:5 water)	1 to 5 soil to water dilution, stirred, stand 1 hour and read while stirring, using combination electrode.
pH (1:5 $CaCl_2$)	1 to 5 soil to 0.01m Calcium Chloride, stirred, read using combination electrode
Buffer pH	Mehlich buffer - 1:1 soil water for 1/2 hour add buffer to 1:2 soil solution, stand 1 hour, read while stirring.
Organic Carbon	Walkey and Black, using H_2SO_4 and $K_2Cr_2O_7$ in 1:100dilution, colorimetric, read on UV vis spectrophotometer.
Nitrate Nitrogen	1:5 soil to water, intermittent stirring over 1 hour, centrifuged and nitrate measured colorimetrically in segmented flow analyser.
Su lfur (MCP)	1:5 soil to solution of 0.01M $Ca(H_2PO_4)_2$, vigorous shake for one hour, centrifuge, measured in segmented flow analyser or I.C.P
Sulfur (KCl-40)	3:20soil to solution of 0.25M KCl heated @ 40°C for 3 hours. Solution is filtered oxidised and heated @ 80°C for 16 hours. Measured turbidimetrically, or I.C.P
Phosphorus (Colwell)	1:100 solution of 0.5M $NaHCO_3$ end-over-end 16 hour shake, centrifuge, measured colorimetrically in segmented flow analyser
Phosphorus (Bray)	1.4:10 soil to solution of 0.03M NH_4F/0.024M HCl reciprocating shaker, for precisely 60 seconds, filtered immediately, measured calorimetrically in segmented flow analyser.
Phosphorus (Lactate)	1:50 soil to solution of 0.05M $C_6H_{10}CaO_6$ shaken by Orbital shaker for 90 minutes. Measured colorimetrically by segmented flow analyser.
Potassium calcium, magnesium, sodium	1:100 soil ratio of 0.0125M Barium Chloride, end-over-end 16 hour shake, centrifuge, measured on Atomic Absorption Spectrophotometer, or I.C.P
Aluminium	1:10 soil to solution of 1M KCl, 1 hour vigorous shake, centrifuged, measured on atomic Absorption Spectrophotometer, or I.C.P.
Chloride	1:5, soil to water intermittent stirring over 1 hour, centrifuged and chloride measured colorimetrically in segmented flow analyser.
Electrical Conductivity	1:5 soil to water, stirred, then intermittent stirring over 1 hour read by conductivity meter.
Copper, zinc, manganese, iron	1:10 soil to solution of DPTA, triethanolamine and $CaCl_2$ 1/2 hour vigorous shake, centrifuged, measured on Atomic spectrophotometer, or I.C.P
Boron	1:2, soil to solution of hot water, reflux for five minutes, centrifuged, measured colorimetrically in segmented flow analyser or I.C.P.

CALCULATIONS

Cation exchange capacity	sum of K, Ca, Na and Al (if present)
Calcium/Magnesium ratio	Ca/Mg
Aluminium saturation	Al x 100/ Cation exchange capacity
Sodium (ESP)	Na x 100/Cation exchange capacity

Table V-1. Results of analyses of soil samples from pasture plots and boxes

Site	Colour	Texture	pH (1:5 water)	pH (1:5 CaCl2)	Organic Carbon (%C)	Sulfate Sulfur (KCl40) mg/kg	Phosphorus Colwell mg/kg	Phosphorus Bray mg/kg	Potassium meq/100g
Sensitivity trial - moderate organic*		2-10% organic matter	6.4						
Sensitivity trial - high organic*		5-10% organic matter	7.1						
EMAI T=0 box trial 01.11.98	Dark Yellowish Brown	Very Fine Sandy Loam	6.1	5.1	0.4	8	12	3	0.06
EMAI box trial 29.09.99	Dark Yellowish Brown	Very Fine Sandy Loam	6.4	5.7	0.7	12	36	14	0.36
EMAI Nov 99 box trial first batch soil	Dark Yellowish Brown	Sandy loam	5.8	4.9	0.3	10	20	2	0.06
EMAI Nov 99 box trial last batch soil	Dark Yellowish Brown	Sandy loam	5.8	4.8	0.7	6	11	5	0.11
Borenore surface Plot 3 20.7.99	Brown	Clay Loam	6.4	5.8	2.4	35	41	11	0.34
Borenore surface Plot 4 20.7.99	Brown	Clay Loam	6.9	6.4	2.9	35	73	25	0.54
Borenore surface Plot 5 20.7.99	Brown	Clay Loam	7.4	6.8	2.5	25	54	15	0.44
Carcoar surface Plot 3 20.7.99	Brown	Clay Loam	6.7	6	2.7	7	40	11	1.04
Carcoar surface Plot 4 20.7.99	Brown	Clay Loam	6.9	6.3	2.8	8	47	11	1.01
Carcoar surface Plot 5 20.7.99	Brown	Clay Loam	7.4	6.8	2.5	8	42	10	0.79
Borenore standard Plot 2 16.9.99	Brown	Clay Loam	5.1	4.3	2.3	8	29	9	0.34
Borenore standard Plot 3 16.9.99	Brown	Clay Loam	5.9	5.1	2	19	29	7	0.32
Borenore standard Plot 4 16.9.99	Brown	Clay Loam	6.1	5.3	1.9	17	30	11	0.35
Borenore standard Plot 5 16.9.99	Brown	Clay Loam	6.6	5.9	2	17	34	12	0.36
Borenore standard Plot 6 16.9.99	Brown	Clay Loam	6.1	5.4	2	16	32	10	0.39
Carcoar standard Plot 2 16.9.99	Brown	Clay Loam	6.4	5.7	2	8	31	8	1.02
Carcoar standard Plot 3 16.9.99	Brown	Clay Loam	6.6	5.9	2.2	5	29	7	0.89
Carcoar standard Plot 4 16.9.99	Brown	Clay Loam	6.7	6	2	4	25	6	0.85
Carcoar standard Plot 5 16.9.99	Brown	Clay Loam	7.2	6.5	1.7	4	24	5	0.74
Carcoar standard Plot 6 16.9.99	Brown	Clay Loam	6.5	5.7	1.7	3	21	3	0.68

* results from Victorian Institute of Animal Science

Table V-1. Results of analyses of soil samples from pasture plots and boxes (continued)

Site	Calcium meq/100g	Magnesium meq/100g	Aluminium meq/100g	Sodium meq/100g	Chloride mg/kg	Elect. Cond dS/m	Nitrogen (Kjeldahl) (%)	Iron DTPA mg/kg
Sensitivity trial - moderate organic*	19			0.49				
Sensitivity trial - high organic*	22			2.5				
EMAI T=0 box trial 01.11.98	2	1.3	0.06	0.1	<5	0.02	<0.02	13
EMAI box trial 29.09.99	2.6	1.4	N/T	0.28	82	0.12	0.04	12
EMAI Nov 99 box trial first batch soil	1.1	1.1	0.2	0.08	7	0.02	0.02	16
EMAI Nov 99 box trial last batch soil	1.7	1.3	0.24	0.13	7	0.02	0.03	30
Borenore surface Plot 3 20.7.99	5.5	2.5	N/T	0.14	15	0.12	0.22	141
Borenore surface Plot 4 20.7.99	8	3.4	N/T	0.15	19	0.15	0.27	85
Borenore surface Plot 5 20.7.99	8.6	3.2	N/T	0.14	13	0.12	0.21	72
Carcoar surface Plot 3 20.7.99	9.8	1.6	N/T	0.16	8	0.08	0.31	52
Carcoar surface Plot 4 20.7.99	9.6	1.4	N/T	0.13	9	0.11	0.33	41
Carcoar surface Plot 5 20.7.99	10.3	1.3	N/T	0.11	9	0.1	0.27	23
Borenore standard Plot 2 16.9.99	2.8	0.5	0.59	<0.05	6	0.04	0.16	180
Borenore standard Plot 3 16.9.99	3.6	1.6	<0.05	0.08	10	0.06	0.16	124
Borenore standard Plot 4 16.9.99	4	1.8	N/T	0.07	11	0.05	0.17	98
Borenore standard Plot 5 16.9.99	5.6	2	N/T	0.08	10	0.06	0.18	75
Borenore standard Plot 6 16.9.99	4.5	2	N/T	0.07	9	0.06	0.19	82
Carcoar standard Plot 2 16.9.99	7.4	1	N/T	0.08	9	0.09	0.22	53
Carcoar standard Plot 3 16.9.99	7.4	1.1	N/T	0.11	7	0.06	0.21	61
Carcoar standard Plot 4 16.9.99	7.2	1	N/T	0.11	6	0.06	0.24	50
Carcoar standard Plot 5 16.9.99	7.8	0.9	N/T	0.09	<5	0.05	0.19	32
Carcoar standard Plot 6 16.9.99	5.7	0.9	N/T	0.1	<5	0.05	0.18	66

*results from Victorian Institute of Animal Science