ENVIRONMENT AGENCY

Ministry of Agriculture Fisheries and Food

# Cryptosporidium in Farmed and Wild Animals and the Implications for Water Contamination

COVENTRY UNIVERSITY

School of Natural and Environmental Sciences

## Research Report

February 1998

MAFF Ref No CSA 2783 PROJECT WA0515
ENVIRONMENT AGENCY Ref No I561 R&D TECHNICAL REPORT P146

# ACKNOWLEDGEMENTS

This research project was funded jointly by the Ministry of Agriculture, Fisheries and Food and the Environment Agency.

The authors express their gratitude for the guidance offered by the Steering Group including:

| | |
|---|---|
| Ms N Bailey | The Environment Agency |
| Dr P Costigan | The Ministry of Agriculture, Fisheries and Food |
| Dr D Cowan | The Central Science Laboratory |
| Dr I Davidson | The Ministry of Agriculture, Fisheries and Food |
| Dr M Forshaw | The Environment Agency |
| Mr R Quy | The Central Science Laboratory |
| Dr R Sweeting | The Environment Agency |
| Mr W Yeomans | The Environment Agency |

The authors also express their thanks to the staff of Warwickshire College for their continued support and co-operation over several years.

Finally, we gratefully acknowledge the important contributions to the work of two former members of the Coventry research team, Rachel Chalmers and Stephanie Bull, and all technical staff past and present.

Any opinions expressed within this report are those of the authors and do not necessarily represent the opinions of any of the organisations associated with this study.



**AUTHORS: A.P. STURDEE, A.T. BODLEY-TICKELL & S.E. KITCHEN**

**CRYPTOSPORIDIUM RESEARCH GROUP**
**School of Natural and Environmental Sciences**
**Coventry University**
**Priory Street**
**Coventry CV1 5FB**

# CONTENTS

# 1 SUMMARY

The distribution of the intestinal, protozoan parasite *Cryptosporidium parvum* has been investigated on a single 190 hectare site, the Warwickshire College Estate, located in the English Midlands, by Coventry University with assistance from the Central Science Laboratory (CSL) to assess rat populations.

The environmentally resistant form of *Cryptosporidium*, the oocyst, is shed in large numbers in animal droppings. It enters new hosts via the mouth and can cause cryptosporidiosis, a prolonged, severe diarrhoea. The Estate contains a stud and a commercial farm situated in an area of crop fields, conservation land, pasture and woodland. A stream which drains much of the Estate flows south-west along its north western margin. The aims of this study were (1) to produce a quantitative and comprehensive account of the occurrence of the parasite in livestock and wild mammals by analysis of faecal samples, (2) to explore routes and reservoirs of infection amongst the animal groups tested and (3) to identify potential pathways for the contamination of water courses. During the 3 year investigation, 3374 faecal samples were tested using a diagnostic monoclonal antibody to identify the parasite. 545 physical media samples, taken weekly or fortnightly from the stream, the farm drainage ditch, other outfalls and fields used for slurry spreading, were also analysed. A second location, Lodge Pond, 3.5 km from the Estate and free of livestock influence, was monitored routinely for the presence of oocysts in faecal samples from its rat population and in the pond water.

All livestock and wild mammal groups on the Estate contained a percentage of infected individuals (known as the prevalence) which shed *Cryptosporidium* oocysts. In descending order of average prevalence the values were: calves (48%), house mice (39%), wood mice (39%), bank voles (28%), rats (26%), lambs (19%), ewes (9%), bull beef (9%), horses (6%), and dairy cows (6%). There was rarely any clinical sign of infection, i.e. the vast majority of positive animals appeared perfectly healthy. The quantities of oocysts per gram of faeces tested were generally highest in those groups with the highest prevalence. Furthermore, the prevalence of *Cryptosporidium* in the the rats at Lodge Pond was high in the absence of contact with livestock or their waste. Rodents, therefore, are an important reservoir of potentially infective oocysts. Rodents were found all over the Estate, but particularly in hedgerows, woodland and farm buildings. There is evidence from the presence of infected house mice in the calving units and the timing of first infection of neonatal calves which is suggestive of the former infecting the latter. This is supported by the observation that no dam shedding oocysts at the time of giving birth produced a calf which was itself positive for *Cryptosporidium* on the occasion of first testing. Peak oocyst shedding occurred during autumn and winter, coinciding with the calving season and high *Cryptosporidium* prevalence amongst wild mammal populations.

Data for the average *Cryptosporidium* prevalences and oocyst concentrations, combined with information on population numbers, typical quantities of droppings and duration of infection has enabled calculation of the approximate numbers of oocysts generated by the animals of the Estate in a typical year. This figure is between $10^{11}$ and $10^{12}$ oocysts, of which perhaps 2% are attributable to wild mammals. A high proportion of oocysts (probably >60%) would be viable initially (that is, having the capacity to infect a new host). Enumeration of oocysts from stream samples and other sites, when combined with assessments of flow rates, indicated that about $10^{9}$ oocysts ($1/600^{th}$ of the Estate's total annual production) escape from the Estate into the South-west Warwickshire river system each year, of which perhaps a third are still viable. This is equivalent to $1/2000^{th}$ of all livestock oocysts and $1/200^{th}$ of those from wild mammals. About $10^{8}$ oocysts probably enter the stream annually from the farm drainage ditch which is therefore an important conduit. However, this ditch also received oocysts from beyond the Estate. Analysis of slurry at the time of spreading on the fields indicated that about $1/200^{th}$ of the annual oocyst production was still present in the slurry, but the oocysts were almost certainly of very low viability. On average, we estimate that just under a million viable oocysts from all sources leave the Estate in the stream every day of the year.

The Estate farm is a model of good management. The animals were healthy, well housed and their waste material processed according to current good practice. The assumptions made to generate the annual oocyst totals may be contentious in detail but collectively have produced probably conservative rather than exaggerated outcomes. This study has confirmed that *Cryptosporidium* is ubiquitous amongst mammals and established a benchmark for what may be the irreducible, minimum background level of the organism to be expected in the UK countryside.

# 2 INTRODUCTION

This report is the account of a project funded jointly by the Ministry of Agriculture, Fisheries and Food and the Environment Agency (MAFF Ref: WA0 1515, contract CSA 2783; Environment Agency Ref: I561) from March 1995 to February 1998, for work on the Warwickshire College Estate (the "Estate") by the Cryptosporidium Research Group of Coventry University. The purpose was to complete an intensive investigation of a single site to establish a benchmark for the occurrence of *Cryptosporidium* in a lowland agricultural area and to explore the movement of the organism into local surface waters.

*Cryptosporidium* is an intestinal protozoan parasite transmitted via the faecal-oral route. The species commonly infecting mammals is *Cryptosporidium parvum*, which is also responsible for the disease cryptosporidiosis in humans. The organism exists outside its host in a robust form known as an oocyst, which is 3.5-5.0 μm in diameter, and is resistant to environmental pressures. *C. parvum* is a major problem for water suppliers, since oocysts are also resistant to chlorination, and there have been a number of waterborne outbreaks of cryptosporidiosis (Smith and Rose, 1998).

## 2.1 Previous investigations

This project was established to extend a 2 year survey of the distribution of *Cryptosporidium* species in livestock and small wild mammals carried out on the Estate from 1992-1994 (the "pre-contract survey"). Over four thousand faecal samples were tested for the presence of oocysts and the data analysed to provide figures for the occurrence of the parasite in livestock and wild animals. The earlier investigation showed that *Cryptosporidium parvum* was widespread in the environment, being found routinely in the faeces of livestock and small rodents (Chalmers *et al.*, 1995). A summary of the results from the 1992-94 study is presented in Appendix 7.2 and discussed, where appropriate, in the context of the MAFF/Environment Agency project. Explanation of terms and abbreviations used in this report are in the Glossary (Appendix 7.5).

## 2.2 The Estate and its management

### 2.2.1 Estate description

The Estate is situated 17 miles south of Coventry at Moreton Morrell (National Grid Reference SP 30 55). The Estate provides agricultural, equestrian and environmentally based courses for approximately 2000 students annually. The 190 hectare main site contains teaching, administration and accommodation buildings, stables and stud, and a commercial mixed farm. The site slopes from 105 metres above sea level to the south east to 45 metres in the north west. The predominant soil type is heavy clay. The farm buildings are located near the bottom of the slope, well separated from the stables higher up. Fields, bounded by hedges, are used for grazing and growing fodder crops. There are areas of woodland, copses, ponds and streams, one of which marks the north western border of the site and takes water draining from the Estate (Appendix 7.1.1 site map, 7.1.2 aerial photographs, 7.1.3 surface geology). The livestock comprise cattle, sheep and horses of several types and a full range of ages. Numerous wild mammals are also found on the Estate. The average annual rainfall in recent years has been in the order of 600 mm with average daytime temperatures ranging from 1.2 - 16.4°C. Detailed weather records for the region have been obtained from Horticulture Research International at Wellesbourne, located 3 km from the Estate.

## 2.2.2 Farm husbandry

**Dairy herd**: 125 pedigree Holstein Friesian cows spend winter indoors and graze the fields at other times. They are milked twice a day. Calving occurs from September to February producing Holstein Friesians and Simmental crosses.
**Home-bred calves**: Calves are removed from the dams within 24 hours of birth and housed in individual pens. Groups of pens are arranged in two lines separated by a walkway. Calves in adjacent pens are able to have physical contact with each other. After weaning at 6 weeks they are grouped in yards of up to 14 animals. Bull calves are either sold off the farm at 2-4 weeks old (Holsteins) or enter the bull beef unit (Simmental crosses). Some pure bred heifers are retained to renew the dairy herd and the rest, plus the Simmental crosses, are sold off the site after weaning.
**Bought-in calves**: About 90 cross bred bull calves arrive at 2 weeks old, are housed individually and grouped after weaning. Some are sold on at 100 kg and others enter the bull beef unit.
**Bull beef**: About 80 animals housed in 5 yards according to age and sold for slaughter after a year.
**Simmental suckler herd**: Initiated in 1991 with the intention of stabilising at about 40 cows plus followers. It has not been sampled.
**Sheep**: 150 pedigree Lleyn ewes are maintained. Ewes are housed indoors 6 weeks before lambing in February and turned out to grass with their lambs in March. They are weaned in July to stock the flock or for sale off site.
**Horses**: Stables are located 500 metres from the farm buildings (see site map Appendix 7.1.1). About 75 horses are housed in 3 yards or graze the fields; additional animals are resident temporarily during courses and equestrian events. Student horses leave the site during vacations. Two stallions at stud service resident and visiting broodmares. Foals and mares are stabled together in a separate yard.
**Other livestock**: There is a game bird rearing unit near the farm buildings, an animal care teaching facility containing common and exotic animals and there are farm/stable cats and a sheep dog.

## 2.2.3 Disposal of animal waste

Farm yard manure (FYM) and that from the stables is transported to a midden on a concrete platform located by the farm buildings and is spread onto the fields periodically. Liquid seeping from the midden drains into an underground holding tank and is pumped into the slurry lagoon (Appendices 7.1.1 and 7.1.2). The lagoon receives four further piped inputs carrying viscous and liquid wastes from various areas of the farm, including the calving units and the stables. The lagoon is mechanically stirred from time to time during filling and just before spreading, which occurs via pumping of liquefied slurry along flexible tubing to fields on the Estate for distribution by spraying with a "rain gun". Clean waters (precipitation and some yard washings) run into surface and underground drains. A proportion of these waters may find their way into the farm drainage ditch which is a surface channel running from near the farm to an underground section (culvert) emptying into the stream ( see site map - Appendix 7.1.1).

## 2.2.4 Conservation, wildlife and pest control

Areas of woodland and hedgerows are conserved to provide cover for game birds, shelter for livestock and wildlife habitat. Tree planting and hedgerow regeneration are pursued actively. There are two ponds and a stream which drains the site to the north west (Appendix 7.1). Land drains reduce waterlogging in fields adjacent to the stream. Wild mammals inhabiting or passing through the Estate are listed in Appendix 7.1.4A. Vermin and pest control measures are implemented regularly at the stables and particularly in and around the farm buildings.

# 3 AIMS AND OBJECTIVES

The current study (from March 1995 to February 1998) was established to describe and quantify the distribution of the intestinal protozoan parasite *Cryptosporidium* in a community of farmed and wild animals within a discrete sample area and to relate this to locality, the possibility of cross infection between animals, and the potential pathways for contamination of water courses. Results were to be presented graphically and quantitatively including maps of oocyst distribution with flow charts illustrating probable contamination routes and relative oocyst numbers. Faecal specimens from livestock and wildlife and samples of various physical media would be analysed for the presence of oocysts of *Cryptosporidium*.

The programme was organised as four distinct objectives.

**A** To maintain and update the existing database (from the 1992-94 survey) of the occurrence of *Cryptosporidium* in livestock and wild mammals on the Estate in relation to *Cryptosporidium* species, host species; cohort, individual animal, location, date and weather and to explore the inter-relationships between these variables.

**B** The trapping, tracking and sampling of rats for estimation of population sizes, distributions, and presence of *Cryptosporidium* in a statistically significant sample on the Estate and to determine occurrence of *Cryptosporidium* in rats on a second site lacking livestock.

**C** To develop methodologies and expertise in sampling and testing physical media (stored animal wastes, substrates contaminated with faeces, static dirty water and drainage waters) for *Cryptosporidium* and relate this to the potential for contamination of water courses.

**D** To provide appropriate Quality Assurance on laboratory testing and analyses via standard scientific protocols plus liaison and sample exchanges with accredited independent UK laboratories involved with *Cryptosporidium*.

Objective B was accomplished in collaboration with the Central Science Laboratory (Sand Hutton, York) who had the expertise necessary to study rat populations. Laboratories involved in the quality assurance included those of the PHLS (Coventry), the Cryptosporidium Reference Unit (Rhyl), the Moredun Research Institute (Edinburgh), and for physical media samples the water quality control laboratory of South West Water (Exeter).

# 4 METHODS, RESULTS & COMMENTARY

## 4.1 Overview of methods and results

4337 samples of faeces and physical media were tested for *Cryptosporidium* between March 1995 and January 1998 (Table 1). These included samples from a second field site, called Lodge Pond (Appendix 7.1.5), about 5 kilometres from the Estate, introduced because it had a rat population and a pond but no slurry spreading or livestock in the vicinity. The 'other wild mammal' category also included samples from additional sites.

**Table 1: Summary of field and quality assurance samples tested for *Cryptosporidium* 1995-98.** Physical media includes slurry, waste liquors, dirty and clean waters. Faecal samples were analysed from both livestock and wild animals.

| Objective | Sample category | Samples tested | Number positive | % positive |
|---|---|---|---|---|
| A | Bull beef | 136 | 6 | 4 |
| A | Calves (home bred) | 1101 | 205 | 19 |
| A | Dairy cows | 347 | 16 | 5 |
| A | Ewes | 49 | 4 | 8 |
| A | Horses | 178 | 8 | 4 |
| A | Lambs | 77 | 13 | 17 |
| A | Buildings mice | 303 | 107 | 35 |
| A | Hedgerow mice/voles/shrews | 126 | 60 | 48 |
| A | Suggett Spinney (small mammals) | 511 | 154 | 30 |
| A | Other wild mammals | 176 | 13 | 7 |
| B | Rats (Estate) | 131 | 34 | 26 |
| B | Rats (Lodge Pond) | 240 | 66 | 28 |
| C | Physical media (Estate) | 494 | 284 | 58 |
| C | Physical media (Lodge Pond) | 51 | 33 | 65 |
| D | Quality assurance (faecal samples) | 349 | na | na |
| D | Quality assurance (physical media) | 69 | na | na |
| **TOTALS** | | **4337** | **1004** | na |

All faecal and physical media samples were processed according to the methods detailed in the protocols (Appendix 7.3) and tested for oocysts using a *Cryptosporidium*-specific monoclonal antibody and immunofluorescence microscopy (IFAT). Oocyst size was measured using modified Ziehl-Neelsen (MZN) staining and a calibrated microscope eyepiece. The presence of sporozoite nuclei was determined by 4',6-diamidino-2-phenylindole (DAPI) staining (Grimason *et al.*, 1994) to help confirm identifications. Some samples with sufficiently high numbers of oocysts were analysed for percentage viability by the DAPI/PI (propidium iodide) dye inclusion/exclusion method (Campbell *et al.*, 1992). Uniquely identified sample data were stored in a Microsoft Access database; Minitab (Minitab Inc., Pennsylvania, USA) and Microsoft Excel were used for analysis. Cartography software (Map Maker Pro, Mapmaker Ltd, Ard Carroch, Carradale, Mull of Kintyre, Argyll, Scotland, UK) was used to generate maps.

Model scenarios describing the annual yield of oocysts on the Estate and their fate are discussed in Section 5. Note that not all animal groups were tested for all three years to allow a shift of focus onto the physical media in the latter part of the project.

# 4.2 Objective A: Survey of livestock and wild mammals

## 4.2.1 Sampling schedule

The 1992-94 survey took faecal samples from every accessible animal category on the Estate and established *Cryptosporidium* occurrence (% samples positive) for the groups tested (Appendix 7.2).

The pattern of sampling for the 1995-98 contract survey is shown in Table 2 (rats are treated separately under Objective B). The strategy was firstly to confirm and extend data from the earlier survey by sampling representative categories of mainly adult animals, the bull beef, horses, ewes, lambs, dairy cows and non-trapped (other) wild mammals for one year. Secondly, to focus on calves, the category most susceptible to infection, and their dams (from the dairy herd) for 3 years to establish the pattern of infection during calving. Finally, since mice had been shown in the 1992-94 survey to have a high prevalence of *Cryptosporidium*, to continue study of the parasite in mice and other small mammals by trapping in and around the farm buildings and Suggett Spinney.

The 1995-98 survey data was analysed to provide both the occurrence (samples positive for *Cryptosporidium* divided by the number of samples tested) and prevalence (Margolis *et al.*, 1982) (number of animals positive for *Cryptosporidium* divided by the number of animals tested). Note that the percentage occurrence is often different from prevalence due to repeat sampling of animals during the same or later sampling periods.

**Table 2: Sampling timetable for animals and summary of prevalence calculations for 1995-97.** Shading indicates sampling during the contract year (March - February) although not necessarily for the whole period.

| | 95/96 | 96/97 | 97/98 | *Cryptosporidium* test results | | |
|---|---|---|---|---|---|---|
| Animal group | | | | indiv. animals tested | no. positive | prevalence (%) |
| Bull beef | | | | 69 | 6 | 9 |
| Horses | | | | 114 | 7 | 6 |
| Hedgerow mice/voles | | | | 120 | 56 | 47 |
| Other wild mammals | | | | na | na | na |
| Ewes | | | | 44 | 4 | 9 |
| Lambs | | | | 69 | 13 | 19 |
| Dairy cows | | | | 283 | 16 | 6 |
| Home bred calves | | | | 230 | 110 | 48 |
| Buildings mice | | | | 282 | 105 | 37 |
| Suggett Spinney small mammals | | | | 490 | 150 | 31 |

Animal groups directly associated with the calves were sampled for all three years to explore routes of infection. Suggett Spinney was used to establish minimum population numbers of small mammals for a discrete part of the farm conservation area. It was not possible to sample livestock randomly due to the need to integrate research activities with farm management practices. Sampling was therefore semi-random. The maximum possible number of animals was selected at every sampling event but was never equal to the whole group. The sampling of wild animals depended on trapping or collection of identifiable faeces from the ground. In the latter case, samples cannot be assigned to a particular individual thus prevalence cannot be calculated. All sampling of animals ceased late in 1997. The results in Section 4.2.3 (below) are presented in the same order as the animal categories appear in Table 2. This means that the groups which were sampled during a single project year only are described first, beginning with the bull beef in Section 4.2.3.1.

## 4.2.2 Methodology

Faecal material was obtained from livestock following excretion (either triggered by insertion of a swab or coincidental with sampling). For trapped animals (Longworth live-mammal traps, Penlon Ltd., Abingdon, Oxfordshire) faecal pellets were taken from the floor of the trap and the animal's ears marked

with miniature Hauptner tags (Brookwick Ward Ltd., Fife, Scotland, UK) for identification on recapture. All samples were attributed to uniquely identified (via ear tags or artificial or natural pelt markings) individuals whose location and available personal details (such as age, gender, breed, species, state of health) at the time of sampling were recorded. The one exception was the 'other wild mammal' category, for which droppings could be identified as to species but could not be attributed to individual animals. The initial laboratory processing of faecal samples varied depending on the nature of the material, some requiring filter screens to remove large fibres, others requiring fat removal or rehydration. The main procedure was the same for all faecal samples and was calibrated to enable calculation of oocyst numbers per gram of starting material as indicated in the protocols of Appendix 7.4.2. The Chi squared test was employed to check for significant differences when comparing group, monthly, seasonal and annual data as appropriate. A *P*-value of $\leq 0.05$ was required for significance.

## 4.2.3 Results

### 4.2.3.1 Bull beef

The bull beef herd was tested quarterly during 1995/96. Of the 136 samples tested from 69 individuals during the year, 6 were positive giving an occurrence of 4% (Table 1), and prevalence of 9% (Table 2). The occurrence during the 1992-94 pre-contract survey was 1%, indicating no significant change, confirming low prevalence among fattening calves (Quilez *et al.*, 1996).The 1995/96 contract data showed no seasonality ($P > 0.05$).

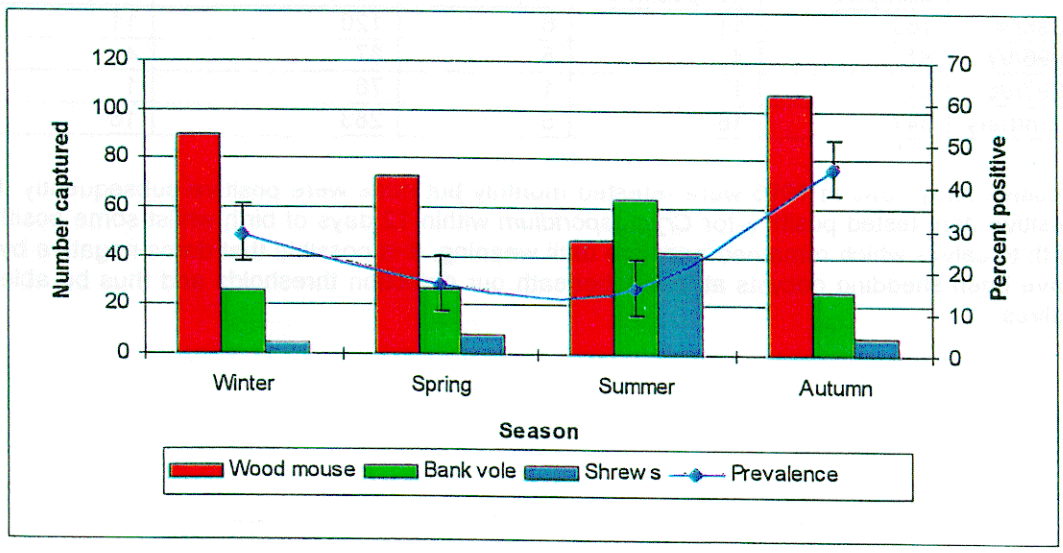
### 4.2.3.2 Horses

Adult horses were tested quarterly during 1995/96. Occurrence was only 4% (8/178 positive, Table 1), with a prevalence of 6% (7 individuals positive, Table 2). A much larger number of samples was tested during the 1992-94 survey which also gave a low occurrence (6% for adults, 3% for foals, Appendix 7.2). These low values are consistent with other studies, which did not find any positive specimens in groups of yearlings and adult mares (Johnson *et al.*, 1997; Xiao and Herd, 1994).

### 4.2.3.3 Hedgerow mice/voles/shrews

Trapping occurred on four occasions in 1995/96 realising 126 small mammals and a sample occurrence of 48% (Table 1). Six of the 126 were recaptures leading to an overall prevalence of 47% (56/120, Table 2). Of these, 40/77 (52%) wood mice, 9/23 (39%) bank voles and 7/20 (35%) shrews were found to be shedding oocysts. Seasonal prevalence tested on the overall mammal totals was significantly different ($P < 0.01$). During the pre-contract survey of 1992-94 sampling was also done quarterly. The 1992-94 data has been analysed to provide seasonal prevalence (Chalmers *et al.*, 1997) and has been combined with the 1995/96 data (Figure 1).

**Figure 1: Seasonal variation in prevalence of *Cryptosporidium* for small mammals caught in hedgerows and woodland margins of the Estate 1992-96.** (Bars = ± standard error).



Although the overall totals caught in each season were broadly similar, the proportion of each species fluctuated. Prevalence was significantly higher in autumn compared to spring and summer; nearly half the trapped animals were shedding oocysts in the autumn.

### 4.2.3.4 Other wild mammals

The total of 176 faecal samples from a variety of wild animals excluding rodents, were tested for *Cryptosporidium* (Table 1 and Appendix 7.1.4). Forty six of the 176 samples came from the Estate producing 4 positive results (9% occurrence). The additional material was collected from several other sites in England to provide a broader picture of the parasite in wild mammals. Overall, 13 samples were positive, an occurrence of 7%. This figure is low compared with that for rodents and may indicate that, amongst wild animals (although the sample was small), rodents are the preferred host for *Cryptosporidium*. Badgers and foxes showed highest occurrence among this group and this may result from their predation of small mammals such as wood mice, voles and shrews.

### 4.2.3.5 Ewes and their lambs

A total of 49 samples from ewes and 77 from lambs were taken in the spring of 1996 with 8% of ewe samples and 17% from the lambs testing positive for *Cryptosporidium* (Table 1). This translates into prevalences of 9% and 19% for ewes and lambs respectively (Table 2). Between 1992 and 1994, 425 faecal samples from ewes and 387 from lambs were tested, generating occurrences of 6% and 9% (Appendix 7.2). Differences between the years tested were not significant ($P > 0.05$).

The prevalence amongst lambs in a Canadian study (23%) was similar to that found in our 1996 survey (19%), but the former reported a higher prevalence (27%) amongst sheep (Olson *et al.*, 1997). Xiao *et al.* (1993) found that 100% of newborn lambs became infected with *Cryptosporidium*, but that the infection only lasted 3-4 days. Although weekly sampling could have missed some infections it is unlikely to have missed 80% of them.

The low figures observed for these groups in our study suggest that *Cryptosporidium* infection in sheep is not a significant problem on this farm. This may be as a result of the animals being put out to grass from an early age thus reducing the risk of infection by contact with infected neighbours and rodents living around the buildings.

### 4.2.3.6 Dairy cows

The dairy herd was monitored for *Cryptosporidium* at the time of calving, tests being conducted within 24 hours of the birth. Overall, only 16/347 samples were positive, an occurrence of 5% (Table 1). The three year prevalence figure (Table 2) is analysed further to show the yearly prevalences in Table 3 below.

**Table 3: Occurrence and prevalence of *Cryptosporidium* determined by faecal testing of dairy cows on the Estate 1995-98.**

| | OCCURRENCE | | | PREVALENCE | | |
|---|---|---|---|---|---|---|
| | Samples | no. positive | % positive | Indiv. animals | no. positive | % positive |
| 1995/96 | 169 | 11 | 6 | 120 | 11 | 9 |
| 1996/97 | 87 | 4 | 5 | 87 | 4 | 5 |
| 1997/98 | 91 | 1 | 1 | 76 | 1 | 1 |
| Summary | 347 | 16 | 5 | 283 | 16 | 6 |

Positive dairy cows in 1996 were retested monthly but none were positive subsequently. No calf from a positive dam tested positive for *Cryptosporidium* within 12 days of birth whilst some positive dams gave birth to calves which remained negative until weaning. It is possible that dams negative by our test could have been shedding oocysts at levels beneath our detection thresholds and thus be able to infect their calves.

### 4.2.3.7 Home bred calves

Special attention was paid to the home bred calves. The 1992-94 pre-contract project had shown that a high proportion of calf samples on the Estate were positive for *Cryptosporidium* (Appendix 7.2). Investigations during 1995 - 98 confirmed that 48% of individual calves were positive on at least one occasion. Of 1101 faecal samples tested for *Cryptosporidium,* 19% were positive.

**Table 4: Occurrence and prevalence of *Cryptosporidium* determined by faecal testing of home bred calves on the Estate 1995-98.**

| | OCCURRENCE | | | PREVALENCE | | |
|---|---|---|---|---|---|---|
| | Samples | no. positive | % positive | Indiv. animals | no. positive | % positive |
| 1995/96 | 560 | 83 | 15 | 92 | 36 | 39 |
| 1996/97 | 220 | 38 | 17 | 66 | 26 | 39 |
| 1997/98 | 321 | 84 | 26 | 72 | 48 | 67 |
| Summary | 1101 | 205 | 19 | 230 | 110 | 48 |

Prevalence increased significantly in 1997 compared to 1995 and 1996 (P = 0.001).

These high annual prevalences raised the question of the source and timing of infection with *Cryptosporidium.* Calves were removed from the dams within 24 hours of birth and penned individually, although animals in adjacent pens could lick each other through the bars. In 1997, the aim was to sample each calf as soon as possible after birth and then to concentrate on sampling some yards twice weekly to gain information on the timing and spread of infection amongst the calves.

There are four potential routes of infection in calves (Figure 2) which have been considered.

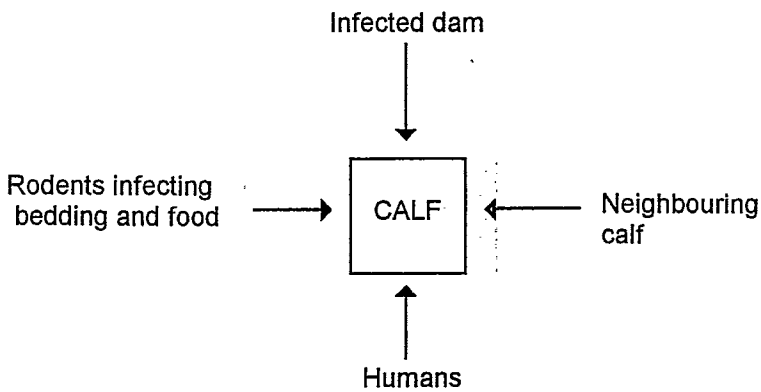



**Figure 2: Possible routes of infection for neonatal home bred calves on the Estate.**

Young animals lick and suck indiscriminately, particularly when isolated. They are in close proximity to their neighbours, usually on both sides, and are handled frequently by farm workers and other personnel. Research team members took care to use fresh gloves, boot covers and equipment when sampling to minimise our potential role in the spread of infection.

The pattern (timing and location) of infection in individual yards is of interest as this may indicate whether infection was passed between neighbouring calves and offer clues concerning the initial source(s) of infection. Tables 5A, 5B and 5C show oocyst shedding patterns in single yards during the 1997/98 calving season. The yards were full for much of the sampling period. Each pen contains the same animal for the duration of sampling. The shaded areas indicate positive results. Note that the experimentally determined oocyst shedding period for calves is 1-12 days (Fayer *et al.*, 1990). On occasion, individuals could not be sampled for various reasons, including the presence of students who were being trained, a calf judged to be too ill or aggressive, or sometimes the animal would not provide a sample.

**Table 5A: Infection pattern for calves in Yard 1 of the Estate, 1997/98.** Pens 1-7 and 8-14 were separated by a 2 metre wide walkway. Shaded boxes represent calves positive for *Cryptosporidium* and contain the number of oocysts counted on the test slide. n = negative, ns = not sampled.

| Sampling dates | Pen number | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| ID no. | 729 | 723 | 722 | 721 | 720 | 719 | 718 | 715 | 716 | 724 | 725 | 726 | 727 | 728 |
| DOB | 31/10 | 29/10 | 27/10 | 26/10 | 26/10 | 23/10 | 21/10 | 20/10 | 20/10 | 31/10 | 31/10 | 1/11 | 31/10 | 2/11 |
| Sex | m | m | m | m | f | f | f | f | f | f | f | f | m | f |
| Age (d) at first test | 3 | 5 | 14 | 8 | 22 | 5 | 14 | 3 | 8 | 14 | 3 | 6 | 3 | 5 |
| Age (d) at infection | 7 | 12 | 14 | 15 | | 15 | | 8 | 8 | 14 | 10 | 9 | 14 | 15 |
| 24/10 | | | | | | | | n | | | | | | |
| 28/10 | | | | | | n | | 1 | 560 | | | | | |
| 31/10 | | | | | | ns | | ns | ns | | | | | |
| 3/11 | n | n | | n | | ns | | ns | 41 | | n | | n | n |
| 7/11 | 8 | ns | | ns | | 2 | | ns | n | | ns | n | n | n |
| 10/11 | ns | 91 | 526 | 894 | | 2 | | ns | n | | 2500 | 2000 | ns | ns |
| 14/11 | ns | n | n | 63 | | n | | n | n | 3 | 97 | 2500 | 494 | ns |
| 17/11 | n | n | n | n | n | n | n | ns | n | n | n | 3 | ns | 500 |
| 21/11 | n | n | n | 2 | n | n | n | n | ns | n | n | n | n | n |
| 24/11 | n | n | | n | | n | | n | n | n | n | n | n | n |

**Table 5B: Infection pattern for calves in Yard 2 of the Estate, 1997/98.** Details as for Table 5A.

| Sampling dates | Pen number | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| ID no | 708 | 714 | 713 | 712 | 711 | 710 | 709 | 701 | 702 | 703 | 704 | 705 | 706 | 707 |
| DOB | 20/10 | 19/10 | 19/10 | 17/10 | 13/10 | 12/10 | 6/10 | 5/10 | 10/10 | 12/10 | 12/10 | 12/10 | 15/10 | 19/10 |
| Sex | m | f | f | f | f | f | f | m | m | m | m | m | m | m |
| Age (d) at first test | 4 | 5 | 5 | 14 | 11 | 19 | 14 | 5 | 10 | 8 | 19 | 12 | 9 | 15 |
| Age (d) at infection | 8 | 12 | 15 | 14 | 11 | 19 | 14 | 12 | 10 | 8 | 19 | 12 | 16 | 15 |
| 10/10 | | | | | | | | n | | | | | | |
| 14/10 | | | | | | | | n | | | | | | |
| 17/10 | | | | | | | | 124 | | | | | | |
| 20/10 | | | | | | | 2 | 500 | 4 | 1 | | | | |
| 24/10 | n | n | n | | 500 | | 386 | ns | 500 | n | | 500 | n | |
| 28/10 | 4 | ns | ns | | ns | | ns | ns | ns | ns | | ns | ns | |
| 31/10 | 2 | 500 | ns | 223 | 236 | 7 | n | n | n | 26 | 19 | 4 | 368 | |
| 3/11 | ns | n | 500 | ns | n | n | n | n | n | 6 | 1 | n | ns | 271 |
| 7/11 | n | n | n | n | ns | ns | n | n | n | n | n | ns | n | n |
| 10/11 | n | ns | ns | ns | n | ns | n | n | n | n | ns | n | ns | ns |
| 14/11 | n | n | n | n | | ns | n | n | n | n | n | n | n | n |
| 17/11 | | n | 2 | n | | n | | | n | n | n | | n | n |
| 21/11 | | | | 500 | | | | | | | | | | |

**Table 5C: Infection pattern for calves in Yard 4 of the Estate, 1997/98.** Details as for Table 5A.

| Sampling dates | Pen number | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| ID no. | 680 | 682 | 679 | 678 | 676 | 675 | 674 | 684 | 683 | 685 | 694 | 695 | 696 | 698 |
| DOB | 18/9 | 19/9 | 17/9 | 15/9 | 13/9 | 11/9 | 11/9 | 19/9 | 19/9 | 22/9 | 25/9 | 23/9 | 26/9 | 3/10 |
| Sex | f | f | f | f | f | f | f | f | f | f | f | f | f | f |
| Age (d) at first test | 12 | 11 | 6 | 4 | 3 | 8 | 15 | 4 | 4 | 4 | 9 | 3 | 12 | 21 |
| Age (d) at infection | 15 | 28 | 41 | 4 | 27 | 8 | 15 | | | 18 | 12 | 31 | 28 | 21 |
| 16/9 | | | | | n | | | | | | | | | |
| 19/9 | | | | 8 | ns | 2 | | | | | | | | |
| 23/9 | | | n | n | ns | n | | n | n | | | | | |
| 26/9 | | | n | n | n | n | 2 | n | ns | n | | n | | |
| 30/9 | n | n | n | n | n | n | n | n | ns | n | | ns | | |
| 3/10 | 128 | n | ns | n | ns | n | n | n | ns | n | n | n | | |
| 7/10 | 431 | n | ns | ns | ns | n | n | n | ns | n | 1 | n | n | |
| 10/10 | 24 | n | n | n | 5 | n | ns | n | n | 2 | ns | n | n | |
| 14/10 | ns | n | n | n | ns | n | n | n | n | ns | ns | ns | n | |
| 17/10 | n | 1 | n | n | n | 9 | n | n | n | ns | ns | n | n | |
| 20/10 | ns | ns | ns | ns | ns | ns | ns | n | n | n | ns | n | ns | |
| 24/10 | ns | ns | n | 44 | ns | ns | 17 | ns | ns | 872 | ns | 98 | 500 | 211 |
| 28/10 | n | n | 2 | 422 | 3 | n | | n | n | n | 500 | n | 500 | 346 |

Only a small minority of infected calves in yards 1 and 2 were single "isolates" spatially at the time of infection (e.g. A pen 6; B pens 5, 7 and 12); the majority of infected animals occupied adjacent pens. This suggests that a high proportion of the infection could have been transferred between calves, but leaves unanswered the source of the original infection(s). Yard 4 (Table 5C) shows several isolated calves which were positive within a fortnight of birth (e.g. C pens 4, 6, 7 and 12) and others which tested positive when older (e.g. C pens 1, 2, 5). The former could have been infected from their dams, but none of the dams showed a positive test at the time of calving. The latter, being much older at the time of first detected infection, were more likely to have encountered oocysts from another source, perhaps from rodent-infected bedding or feed. The patterns illustrated in Table 5 were typical of other yards analysed in the same way.

The age at which calves were first tested fluctuated over the years due to changes in the timing of access to the animals. The average age for first test was 7 days, 10 days and 8 days in 1995, 1996 and 1997 respectively, although in 1995 and 1997 many animals were tested before 1 week of age. The average age when calves were first positive was significantly lower in 1995 and 1997 compared to 1996 ($P$ = 0.0003) and this may be due to the earlier testing of the calves. The average age of first detectable infection was 15, 24 and 17 days in 1995, 1996 and 1997 respectively. The pre-patent period for *C. parvum* is 2 - 7 days (Fayer *et al.*, 1990) suggesting a non-maternal route of infection.

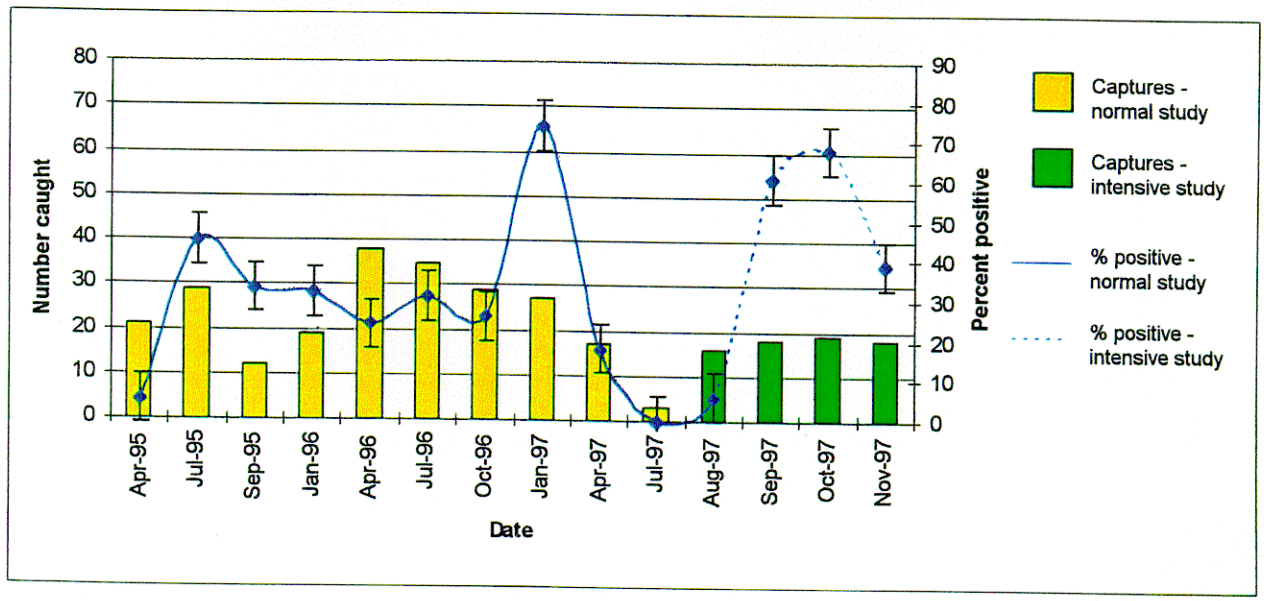
### 4.2.3.8 Buildings mice

Mice (predominantly house mice, but with a small number of wood mice and bank voles caught outside) were trapped in the farm yard and buildings during 1995/96 and 1996/97 but exclusively in and around the calving and calf pens during the latter part of 1997. The summary figures in Table 6 show a sample occurrence of 35% and prevalence of 37%. The proportion of infected animals captured has apparently increased over the three year study. However, Figure 3 shows that there are large fluctuations both in the numbers of animals caught and in the prevalence of *Cryptosporidium*.

**Table 6: Annual occurrence and prevalence of *Cryptosporidium* determined by faecal testing of small mammals captured in and around Estate farm buildings 1995-97.**

| | OCCURRENCE | | | PREVALENCE | | |
|---|---|---|---|---|---|---|
| | Samples | no. positive | % positive | Indiv. animals | no. positive | % positive |
| 1995/96 | 81 | 24 | 30 | 80 | 24 | 30 |
| 1996/97 | 131 | 48 | 37 | 130 | 48 | 37 |
| 1997/98 | 91 | 35 | 38 | 88 | 34 | 39 |
| Summary | 303 | 107 | 35 | 282 | 105 | 37 |

**Figure 3: Fluctuations in captures and prevalence for *Cryptosporidium* of small mammals in and around Estate farm buildings 1995-97.** (Bars = +/- standard error).




Nevertheless, it is clear that autumn and winter prevalence was particularly high in the latter two years of the study. Significantly, the autumn/winter small mammal peak prevalence coincides with calving. Mice seem to move widely within the buildings, defecating in bedding and food used by calves. Note that variation in numbers of animals caught is probably not seasonal; it is far more likely to have been related to farm management practices such as depletion and movement of grain stores, harbourage and the effects of poisoning.

### 4.2.3.9 Population estimates of small mammals in Suggett Spinney

Although the pre-contract survey of 1992-94 sampled small mammals for *Cryptosporidium* no attempt was made to estimate population sizes, simply to determine if rodents, in particular, carried the parasite. However, quantifying numbers of oocysts released into the environment requires at least an indication of population sizes for the animals sampled. Such information is not revealed by simple trapping. It requires a trapping regime performed to a rigorous protocol. Accordingly, Suggett Spinney, a small, discrete area of young woodland near the source of stream 2 and about 400 metres from the farm buildings, was chosen for a one year study (1995/96). The aim was to generate more detailed knowledge of small mammal populations, albeit on a single site, which could act as a crude guide to consideration of numbers elsewhere on the Estate. When the population study finished, trapping was continued at 3-monthly intervals to monitor *Cryptosporidium* prevalence for a second year.

Traps were arranged in a 6 x 6 grid with an interstation distance of 10 metres (x-axis) and 5 metres (y-axis). Three Longworth live mammal traps were placed at each station providing an excess of traps to maximise capture opportunities. Trapping was pursued for four consecutive nights after three nights pre-baiting and was done for one week in every six weeks (the maximum permitted interval). It is unlikely that all the animals in a population will be captured (Gurnell and Flowerdew, 1994), but the regime does allow an estimate of population size known as the "minimum number alive" (MNA) (Krebs, 1966).

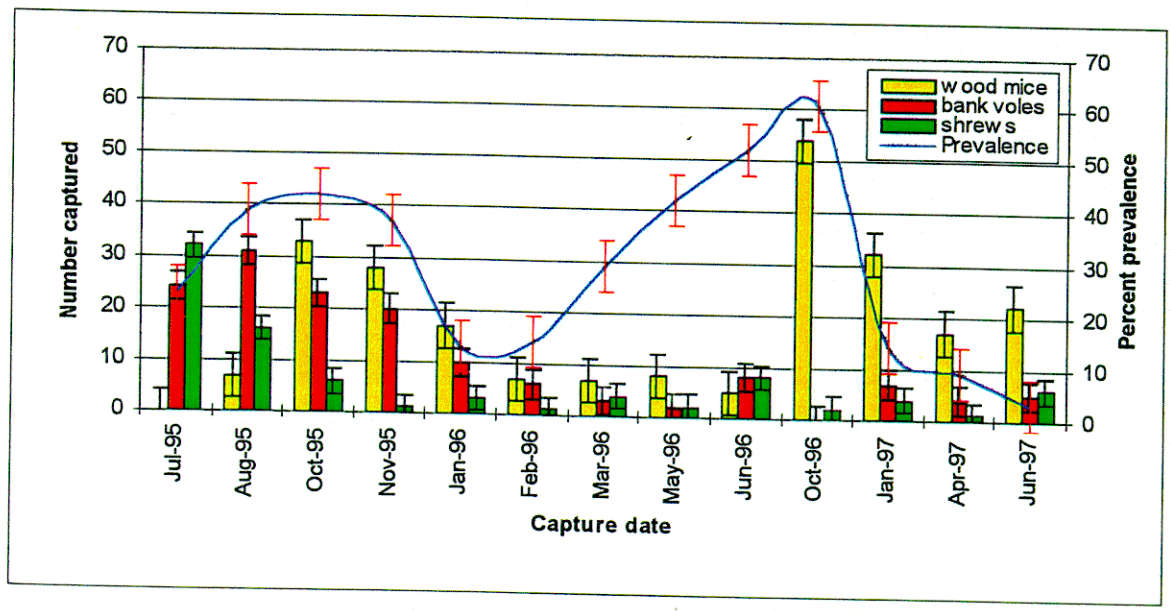
Tables 1 and 2 show the total numbers caught and the overall level of *Cryptosporidium* for the small mammals of the Spinney from 1995-97. The prevalence (31%) was very similar to the occurrence (30%) due to the small number of recaptures during each trapping session. Table 7 shows the estimates of the numbers of each species trapped during the population study of 1995-96 and the prevalence on each occasion.

**Table 7: Estimates of small mammal populations in Suggett Spinney on the Estate July 1995-June 1996.** Percentages of animals positive for *Cryptosporidium* in brackets.

| Trapping session | Wood mice | Bank voles | Shrews | TOTALS |
|---|---|---|---|---|
| 1. July 1995 | 0 (0%) | 24 (21%) | 32 (22%) | 56 (23%) |
| 2. Aug 1995 | 7 (14%) | 31 (32%) | 16 (67%) | 54 (39%) |
| 3. Oct 1995 | 33 (85%) | 23 (30%) | 6 (17%) | 62 (42%) |
| 4. Nov 1995 | 28 (46%) | 20 (25%) | 1 (0%) | 49 (37%) |
| 5. Jan 1996 | 17 (19%) | 10 (10%) | 3 (0%) | 30 (13%) |
| 6. Feb 1996 | 7 (14%) | 6 (17%) | 1 (0%) | 14 (14%) |
| 7. Mar 1996 | 7 (29%) | 3 (0%) | 4 (50%) | 14 (29%) |
| 8. May 1996 | 8 (50%) | 2 (0%) | 2 (50%) | 12 (42%) |
| 9. June 1996 | 5 (40%) | 8 (62%) | 8 (50%) | 21 (52%) |
| **TOTALS** | **112 (39%)** | **127 (27%)** | **73 (34%)** | **312** |
| **Average total population size = 35** | | | | |
| **Average % of animals shedding oocysts = 32** | | | | |

There were large fluctuations both in the numbers caught per species and in the totals trapped at different times of the year. This illustrates the difficulties inherent in obtaining reliable population data and the problems posed for interpretation and extrapolation. Nevertheless, it is recognised that the MNA method provides a good indication of changes in numbers of species seasonally and the relative proportions of the species present. The decline in total numbers caught from the autumn peak (best seen in Figure 4) was expected because of population decline during the winter and perhaps a reduction in foraging movements during bad weather, which would lessen the chances of animals being trapped.

**Figure 4: Populations and prevalences of *C. parvum* for small mammals in Suggett Spinney on the Estate from 1995-97.** Note that data from June 1996 onwards was not part of the population study. (Bars = ± standard error).




The MNA method underestimates numbers by 10-20% (Krebs, 1966), thus it can be said with some confidence that the 1800 square metres of Suggett Spinney used in this study supported a year round mixed population of small mammals averaging a minimum of 35 individuals during 1995-96, 32% of which proved positive for *Cryptosporidium*.

The population survey requiring a 6 week trapping interval was completed in June 1996, after which trapping and faecal sampling was continued at 3 monthly intervals to monitor the percentage of animals shedding oocysts. These data are combined with the population data in Figure 4. The less rigorous trapping regime used during 1996/97 seems to confirm a high autumn peak in numbers for wood mice

but strangely very few bank voles and shrews. The prevalence of *Cryptosporidium* in the autumn mice was very high at 60%, which dropped to 10% by January and was still low in June 1997.

## 4.3 Objective B: *Cryptosporidium* in rat populations

This objective formed part of a collaboration with the Central Science Laboratory (CSL), York. Population estimates, radio tracking and trapping were undertaken to discover the numbers and movements of rats, and their levels of *Cryptosporidium,* for a livestock farm (the Estate) and at an arable site (Lodge Pond). Faecal samples from trapped animals were tested for *Cryptosporidium.* Only the results of the *Cryptosporidium* tests are given here. The results of the rat population study have been reported separately by CSL (MAFF Report Project Code. WA0514, "Assessment of the role of farm populations of Norway rats (*Rattus norvegicus*) as vectors of *Cryptosporidium* infection", 1997).

Table 1 shows that almost twice the number of rat samples were obtained from Lodge Farm compared to the Estate, reflecting the surprisingly small number of rats living on the Estate. The reasons were thought to be the high level of disturbance due to the presence of students, relatively little available food and harbourage, and regular poisoning. Despite the disparity in numbers, the average proportion shedding oocysts at the two sites during the two year study was essentially the same (26.1%, Estate and 25.9%, Lodge), although the average figures hide substantial fluctuations (Table 8).

**Table 8: *Cryptosporidium* test results for rats trapped on the Estate and near Lodge Pond, 1995-97.** Data are number of rats tested with monthly prevalence in brackets. nd = rat trapping not done.

| Sample month | 1995/96 | | 1996/97 | |
|---|---|---|---|---|
| | Estate | Lodge Pond | Estate | Lodge Pond |
| March | 4 (50%) | 20 (15%) | 1 (0%) | 16 (44%) |
| April | 5 (40%) | 15 (7%) | 8 (37%) | 22 (23%) |
| May | nd | 2 (0%) | 11 (27%) | 16 (25%) |
| June | 17 (59%) | 7 (57%) | nd | nd |
| July | nd | nd | 13 (23%) | 2 (50%) |
| August | 2 (0%) | nd | 4 (0%) | 34 (47%) |
| September | nd | 6 (17%) | nd | nd |
| October | 10 (0%) | 4 (100%) | 8 (0%) | 14 (14%) |
| November | 11 (18%) | 15 (27%) | 14 (14%) | 11 (18%) |
| December | nd | nd | 11 (18%) | 14 (7%) |
| January | 4 (25%) | 1 (100%) | 7 (57%) | 9 (11%) |
| February | 1 (0%) | 6 (17%) | nd | 20 (15%) |
| Total (average) | 54 (31.5%) | 76 (25.0%) | 77 (22.1%) | 158 (26.6%) |

During 1995/96, 31.5% of the 54 Estate rat samples proved positive for *Cryptosporidium* compared to 22.1% of 77 samples in 1996/97. There is no obvious explanation for the drop which was revealed despite more animals being tested over the year. Occurrence in rats at Lodge Pond was similar over the two years which suggests that the presence of livestock infected with *Cryptosporidium* is not a prerequisite for the maintenance of the parasite in rats.

# 4.4 Objective C: Sampling and testing physical media

## 4.4.1 Sampling site selection

The term "physical media" covers the spectrum from semi-solid farm wastes, liquid slurry and liquors to dirty and clean waters. All animal waste on the Estate is concentrated in the slurry lagoon and midden near the cluster of farm buildings seen on the map in Appendix 7.1.1. After an extensive survey of the Estate involving the creation of maps, study of the surface geology (Appendix 7.1.3), charting land drainage patterns, aerial photography (Appendix 7.1.2, 7.1.2A) and monitoring continuity of flow of potential locations, seven sites were selected on the Estate for routine sampling of physical media.

A further two sites were sampled at Lodge Pond, which is in an area of arable land, where chemical fertilisers were used and the surrounding fields were not treated with slurry. The pond receives outfalls from field drains and a vehicle proving ground 1 km distant. The pond is thus free of livestock influence but has a rat colony nearby. Rats were observed swimming in the pond and there were numerous signs of other wildlife using the area.

The nine sample sites are described in Table 9 and identified on the maps of Figure 5. Routine sampling began in September 1996 and continued until January 1998 for the Estate sites and September 1997 for Lodge Pond. Sampling of the three fortnightly sites on the Estate was increased to weekly in the final six months to improve the level of monitoring during the 1997/98 calving period.

**Table 9: Sites used for the sampling of physical media on the Estate and Lodge Pond.**

| SITE CODE | LOCATION | SAMPLING FREQUENCY |
|---|---|---|
| C | Stream 2, upstream of confluence with stream 1 | Weekly |
| F | Stream 2, downstream of outfall G | Weekly |
| G | Outfall of farm drainage ditch into stream 2 | Weekly |
| H | Stream 2, upstream of outfall G | Weekly |
| I | Farmyard outfall | Fortnightly then weekly |
| J | Farmyard outfall | Fortnightly then weekly |
| K | Initial outfall into farm drainage ditch | Fortnightly then weekly |
| L2 | North side of Lodge Pond | Fortnightly |
| L6 | South side of Lodge Pond | Fortnightly |

The sites were chosen to provide information on the movement of oocysts away from the locus of the farm and into the small stream (Stream 2) running along the boundary of the Estate. Stream 1 was not selected for sampling since most of its catchment lay on a neighbouring estate. In addition to these nine sites, slurry was sampled at the point of distribution by collecting the semi-liquid material during the field spraying of summer and autumn 1996 and 1997.

The drainage ditch is the main conduit between the farm buildings and the stream. Outfall K which is the beginning of the drainage ditch is an underground pipe of 0.5 metre bore thought to carry surface runoff from a road 2 km away. Rain water and farm yard washings enter the drainage ditch via outfalls I and J. The drainage ditch empties into stream 2 at outfall G. The stream was sampled just above outfall G, at point H, and below outfall G at points F and C. A site beyond the confluence of the two streams was not sampled because of dilution with water from stream 1. Oocysts in animal droppings shed onto fields by both livestock and wildlife may find their way into stream 2 after rain; the same will apply to slurry spread on these fields. Thus samples from point C at the south eastern border represent the accumulation of everything leaving the Estate, with the exception of that from field 2.

## 4.4.2 Methodology

### 4.4.2.1 Sampling and treatment of physical media

Grab sampling (10 litre) rather than a filtration method was chosen because the latter would not cope with the generally dirty physical media. For concentration of oocysts three methods were considered: cartridge filtration (Anon., 1989), membrane filtration (Ongerth and Stibbs, 1987), and calcium carbonate flocculation (Vesey *et al.*, 1993). The latter was selected (see Appendix 7.3.5) because of its reported good recovery rates with low variability, but it has the disadvantage of reducing oocyst viability

(Campbell *et al.*, 1994). However, it was felt that very few 10 litre sized grab samples would contain sufficient oocysts to enable viability assessment, although duplicate samples were taken on an *ad hoc* basis for this purpose. A saturated saline flotation technique (adapted from Weber *et al.*, 1992) was used to further concentrate oocysts and, unless the final pellet was very large, the whole of it was stained with the anti-*Cryptosporidium* monoclonal antibody and DAPI for identification or the DAPI/PI combination for viability as appropriate (Appendix 7.3.10). Slurry samples were processed as described in Appendix 7.3.8. Recovery rate experiments are described under Objective D.

Statistically speaking, the distribution of *Cryptosporidium* oocysts in physical media such as surface waters is not Normal. This is illustrated in Appendix 7.5, showing boxplots of the survey data sets, compared with a boxplot of Normally distributed data. It is obvious that the latter is symmetrical about the median, whilst the survey data is highly skewed. Thus the means will overestimate the central tendency, although they do represent the totality of the data points. Median values offer a better representation in statistical terms, but do not indicate the number and size of the outlying values, which are important in environmental analyses. Consequently, both mean and median values are presented in the tables and figures below, to give as complete a picture as possible and to enable comparison with other similar surveys (which often give only mean values). Note that in bar charts of median values, missing bars indicate medians of zero. Differences were examined for significance by the Mann-Whitney U Test, whilst the Spearman Rank test was used to look for correlations between oocysts and other parameters.

### 4.4.2.2 Other parameters monitored

The temperature and pH of the media were recorded at sampling, and further small samples taken for assessing suspended solids and the general level of toxicity. The latter was analysed by the Enhanced ChemiLuminescent OXidation reaction (ECLOX™) test performed by Karen Sawcer of Birmingham University. This measures the toxicity of a sample to a chemiluminescent reaction, and is a useful screening technique rather than a method for identifying a particular toxin. A brief account of the protocol is given in Appendix 7.3.7. The weather and the timing and location of significant farming events such as calving, lambing and slurry spreading, were monitored to assess their impact on oocyst levels in surface waters (Figure 8).

**Figure 5A: Map identifying sites for routine sampling of physical media on the Estate.**

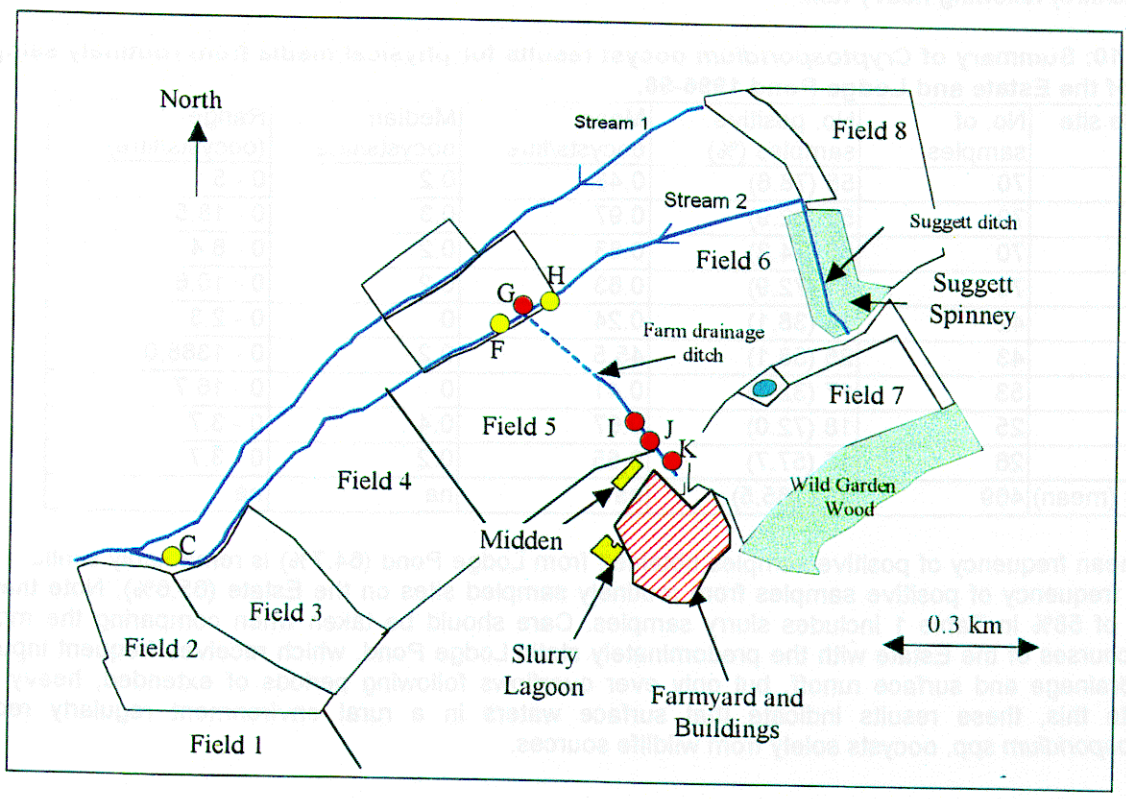
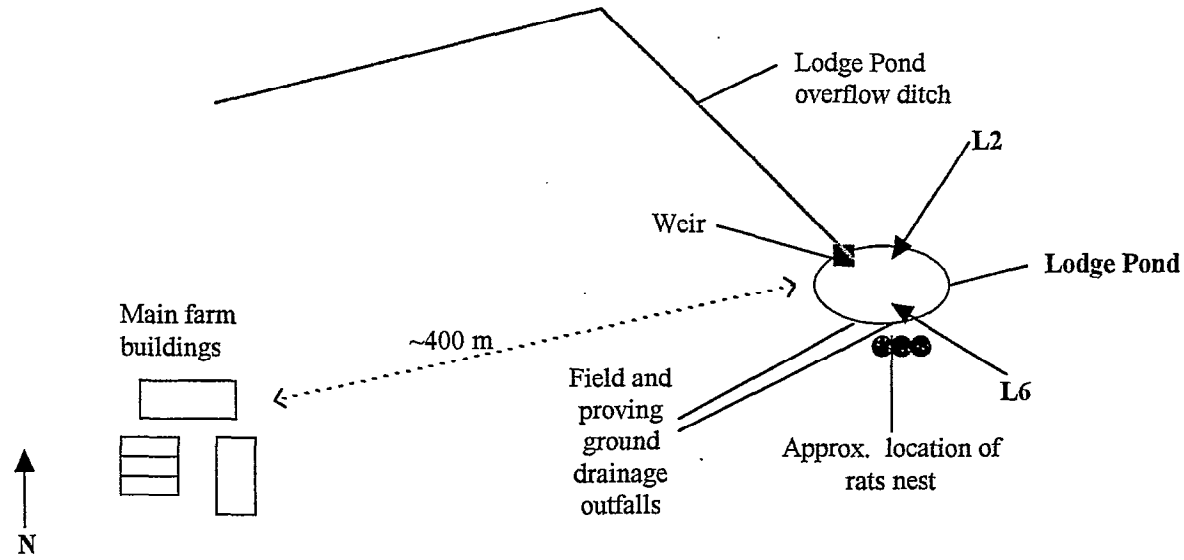


**Figure 5B: Schematic map of Lodge Pond.** (Not to scale - Lodge Pond dimensions: 26 metres x 38 metres).




## 4.4.3 Results

### 4.4.3.1 *Cryptosporidium*

64.7% of 469 physical media samples from the Estate and Lodge Pond were positive for *Cryptosporidium* during the seventeen month study (Tables 1 and 10) . Three of the four most frequently positive sites were in stream 2 (C, F and H). The last of these, H, is surprising since it lies upstream of the drainage ditch outfall which transports material from the farm yard outfalls I and J. Outfall J had one extraordinary result (1,388 oocysts/litre). The raw sample smelt strongly of calf faeces which suggested there had been a transient leakage of concentrated animal waste. The lowest frequency of positive samples was for site K which probably takes surface runoff exclusively from nearby roads, but despite this, it proved positive on 32% of occasions tested and produced one very high oocyst count (16.7 oocysts/litre) following heavy rain.

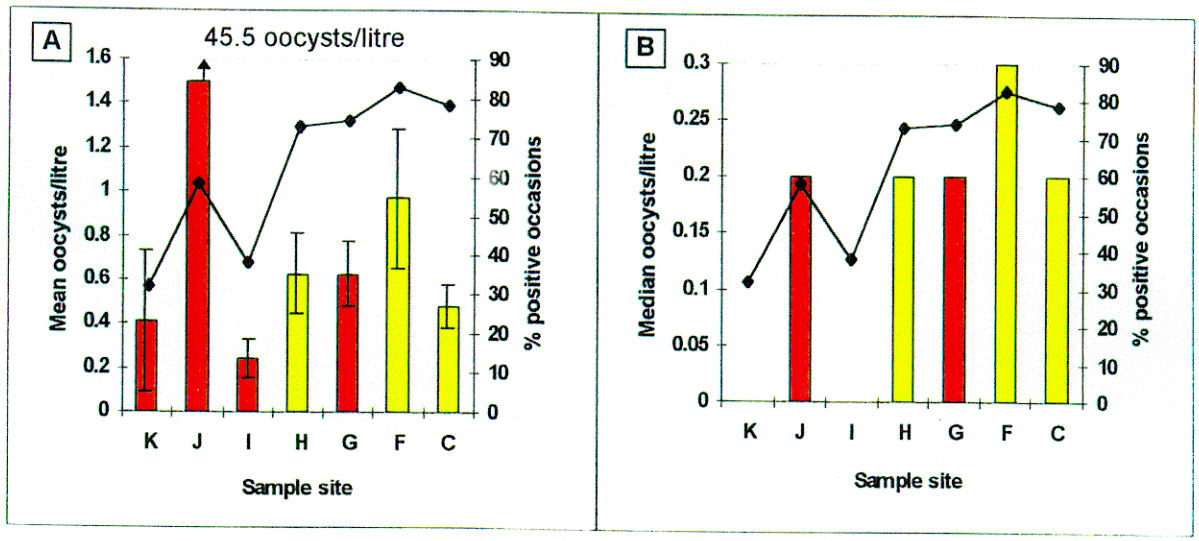
**Table 10: Summary of *Cryptosporidium* oocyst results for physical media from routinely sampled sites of the Estate and Lodge Pond 1996-98.**

| Sample site | No. of samples | No. positive samples (%) | Mean oocysts/litre | Median oocysts/litre | Range (oocysts/litre) |
|---|---|---|---|---|---|
| C | 70 | 55 (78.6) | 0.48 | 0.2 | 0 - 5.1 |
| F | 70 | 58 (82.9) | 0.97 | 0.3 | 0 - 18.5 |
| G | 70 | 52 (74.3) | 0.63 | 0.2 | 0 - 8.4 |
| H | 70 | 51 (72.9) | 0.63 | 0.2 | 0 - 10.6 |
| I | 42 | 16 (38.1) | 0.24 | 0 | 0 - 2.3 |
| J | 43 | 25 (58.1) | 45.5 | 0.2 | 0 - 1388.0 |
| K | 53 | 17 (32.1) | 0.41 | 0 | 0 - 16.7 |
| L2 | 25 | 18 (72.0) | 0.67 | 0.4 | 0 - 3.7 |
| L6 | 26 | 15 (57.7) | 0.55 | 0.2 | 0 - 3.7 |
| Totals (mean) | 469 | 307 (65.5) | na | na | na |

The mean frequency of positive samples obtained from Lodge Pond (64.7%) is remarkably similar to the mean frequency of positive samples from routinely sampled sites on the Estate (65.6%). Note that the figure of 58% in Table 1 includes slurry samples. Care should be taken when comparing the moving watercourses of the Estate with the predominately static Lodge Pond, which receives frequent inputs of field drainage and surface runoff, but only ever overflows following periods of extended, heavy rain. Despite this, these results indicate that surface waters in a rural environment regularly receive *Cryptosporidium* spp. oocysts solely from wildlife sources.

The mean oocyst results for the Estate reveal that, although the oocyst levels for the inputs into the drainage ditch were often high, the number of occasions on which they were positive is low compared with the drainage ditch (G) itself and the three stream sites. Whether above or below G, the stream sites were positive more than 70% of the times tested.

**Figure 6: Oocyst concentrations (A - mean, B - median) and percentage positive values for physical media sample sites on the Estate 1996-98.** (Bars = +/- standard error). The mean column for J is truncated and the standard error of the mean omitted. ■ Non-stream sites, ■ Stream sites, —— % positive occasions.




Particularly interesting is H, upstream of the drainage ditch outfall and nearest to the stream's source by Suggett Spinney. The Spinney is home to numerous rodents and other wildlife and the stream banks and nearby hedges also harbour wildlife. The adjacent fields (6 and 8) were not grazed, although field 6 received slurry and farmyard manure in the autumn. The field to the south east (Field 7) was grazed rarely. Thus, site H results seem to represent the background *Cryptosporidium* levels attributable to the sum of wildlife inputs plus some runoff from livestock faeces deposited on adjacent fields.

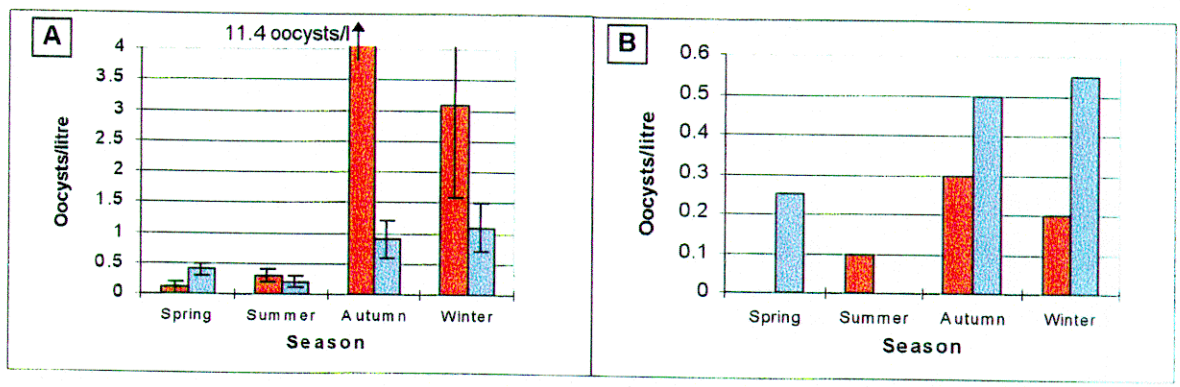
The weekly oocyst concentrations presented as monthly averages recorded for the Estate and Lodge Pond are shown for each site in Table 11. Figures for a farm studied by Kemp *et al.* (1995) have been included and indicate that the range of values is comparable to that for the Estate. Table 11 shows clearly that, for the Estate, oocyst levels were low for most of the year but with sporadic high values clustered in the autumn months. This is also true of Lodge Pond where months with higher averages coincided with elevated levels at one of the Estate sites, which is likely to be related to rainfall washing material from the banks into the pond. Intriguingly, two of the high oocyst months for Lodge Pond were also months when site K at the Estate had higher levels, significant because site K, which carries road runoff, should transport only oocysts originating from wildlife. These observations support the view that increased rainfall leads to increased levels of *Cryptosporidium* in watercourses and that a significant proportion of the oocysts may originate from wildlife sources.

**Table 11: Comparison of monthly averages for oocyst concentrations in physical media for the Estate and Lodge Pond during 1996-98 and similar data aligned by month for another farm (Kemp *et al.*, 1995).** Data are oocysts/litre. n = negative. ns = not sampled. Yellow = stream sites; red = farm sites; green = low oocyst concentration (0.1-1.49 oocysts/l); blue = medium oocyst concentration (1.5-3.9 oocysts/l); magenta = high oocyst concentration (>4.0 oocysts/litre).

| Sites | Estate | | | | | | | Lodge Pond | | Kemp *et al.* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Month | K | J | I | G | H | F | C | L2 | L6 | 1 | 2 | 3 |
| Sep | n | n | n | 1 | 0.1 | 0.4 | 0.6 | 0 | 0.3 | | | |
| Oct | 0.1 | n | 0.9 | 2.7 | 0.7 | 1.6 | 1.9 | ns | 0.1 | | | |
| Nov | 0.1 | 2.8 | 1 | 0.8 | 0.9 | 0.4 | 0.7 | 0.7 | 0.7 | | | |
| Dec | 3.2 | 1.2 | 0.6 | 0.4 | 0.1 | 0.2 | 0.3 | 0.9 | 2.2 | | | |
| Jan | 0.1 | 1.3 | n | 0.3 | 0.1 | 0.2 | 0.2 | 0.1 | 0.3 | | | |
| Feb | 8.7 | 1 | 0.4 | 0.8 | 0.7 | 0.7 | 0.4 | 2.1 | 1.4 | 0.51 | 0.08 | ns |
| Mar | n | n | 0.2 | 0.08 | 0.03 | 0.2 | 0.1 | 0.4 | 0.4 | 13.31 | 0.45 | 5.39 |
| Apr | 0.07 | 0.05 | n | 0.06 | 0.1 | 0.1 | 0.02 | 0.3 | 0.3 | 2.31 | 1.75 | 2.1 |
| May | 0.05 | 2.4 | n | 0.2 | 0.08 | 0.08 | 0.05 | 0.7 | 0.1 | 1.85 | 0.88 | 0.81 |
| Jun | 0.03 | 1.3 | n | 0.4 | 0.1 | 0.2 | 0.08 | 0.1 | n | 1.54 | 0.93 | 2.65 |
| Jul | 0.02 | 0.1 | n | 0.1 | 0.4 | 0.4 | 0.3 | 1.1 | n | 0.49 | 0.08 | 0.68 |
| Aug | 0.05 | n | 0.1 | 0.3 | 0.7 | 1.3 | 0.2 | 0.1 | n | | | |
| Sep | 0.04 | 2.4 | n | 0.5 | 0.4 | 0.5 | 0.6 | 1.8 | 1.6 | | | |
| Oct | 0.03 | 347.3 | 0.2 | 0.1 | 2 | 4.9 | 0.7 | | | | | |
| Nov | 0.1 | 62.2 | 0.1 | 1.3 | 0.9 | 0.9 | 0.8 | | | | | |
| Dec | 0.03 | 80.4 | 0.8 | 1.8 | 0.8 | 1.7 | 0.8 | | | | | |

Average monthly oocyst concentrations at the Kemp *et al.* (1995) farm were greatest from March to June (coinciding with their calving season and peak incidence of clinical cryptosporidiosis), especially for site 1 which received drainage from farm buildings and dairy wash runoff; this is the site most directly comparable to site J of the Estate, which had the highest levels of oocyst contamination during the autumn calving of 1997. Sites 2 and 3 of Kemp *et al.* (1995) were monitoring drainage from fields receiving cattle slurry and FYM and show generally slightly lower levels than site 1. It should be noted that the farm studied by Kemp *et al.* (1995) had a high prevalence of *C. parvum* amongst livestock, whereas, with the exception of the young calves, the prevalence on the Estate was low amongst livestock.

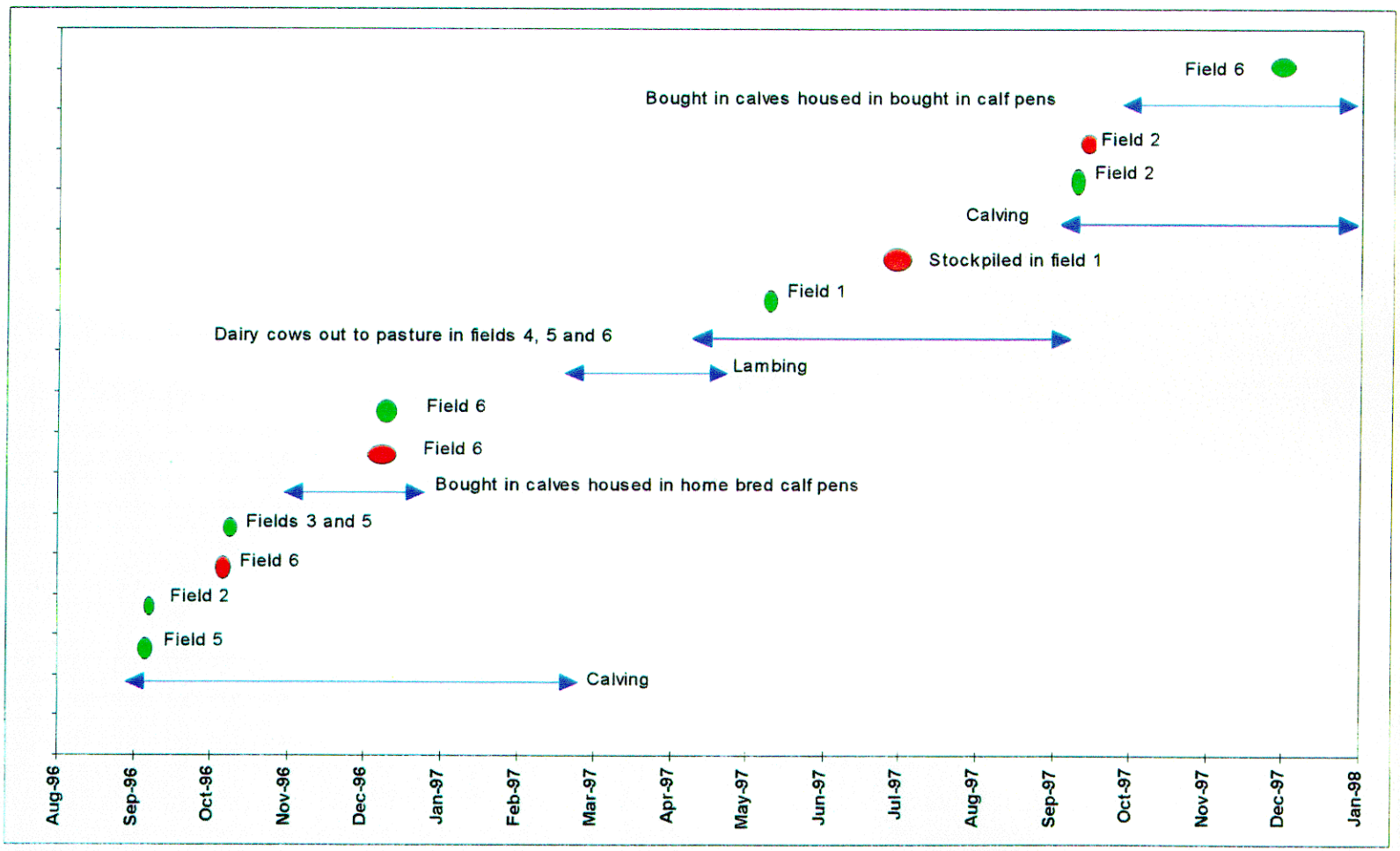
**Figure 7: Combined seasonal oocyst levels (A - mean; B - median) at the Estate and Lodge Sept 1996 - Aug 1997.** (Bars = +/- standard error). All Estate sites  Both Lodge sites




The seasonal pattern of oocyst levels peaked during autumn on the Estate and winter at Lodge Pond (Figure 7). Autumn and winter were significantly different to spring and summer at the Estate ($P$ <0.05), whilst at Lodge Pond only summer was significantly lower than autumn and winter ($P$ <0.05). Significant differences between the two sites only occur during spring ($P$ <0.05), which may reflect accumulation of oocysts due to the static nature of Lodge Pond, although it is also likely that wildlife using the Pond are

acting as a year-round reservoir for the organism (Chalmers *et al.*, 1995). Rats are likely to be of particular significance, due to their close association with the pond and high *Cryptosporidium* prevalence of about 30% (Figure 5B, Table 8).

**Figure 8: Significant agricultural events on the Estate from August 1996 to January 1998. Green (slurry) and red (FYM) spots represent short duration distribution events and the arrows long duration events.**



### 4.4.3.2 Other parameters

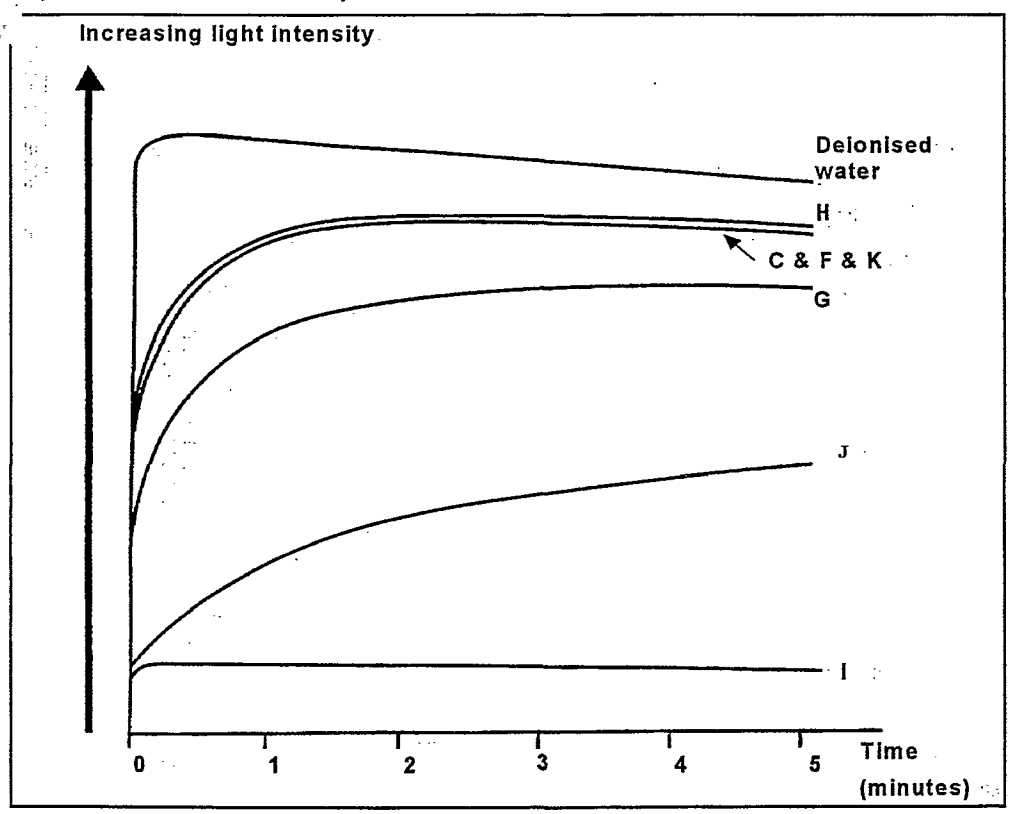
The temperature of the water varied from 1 to 22°C (mean 11.5°C), although on some occasions the farm outfalls were frozen, preventing sample collection. The mean pH was 8.1, and ranged from 5.4 to 10.6, although these outlying values were rare. Suspended solids and the toxicity level assessed by the ECLOX™ method were determined for the seven sites on the Estate to seek correlation between the toxicity of the media and occurrence of *Cryptosporidium*.

**Table 12: Comparison of *Cryptosporidium* oocyst levels with suspended solids and toxicity for seven sites on the Estate from Sept 1996 to Jan 1998 (ECLOX Sept 1996 to Sept 1997).** Data are means (+/- standard error).
EU = ECLOX units. 0-50, low toxicity; 51-99, medium toxicity; >100, high toxicity.

| Sample location | Temperature (°C) | pH | Suspended Solids (mg/l) | ECLOX Units (EU) | *Cryptosporidium* (oocysts/l) |
|---|---|---|---|---|---|
| C | 10.2 (0.5) | 8.2 (0.1) | 20.9 (3.6) | 11.6 (1.4) | 0.48 (0.1) |
| F | 11.2 (0.4) | 8.1 (0.1) | 25.6 (6.0) | 14.6 (2.2) | 0.97 (0.3) |
| G | 11.3 (0.5) | 8.1 (0.1) | 30.4 (7.8) | 48.2 (11.0) | 0.63 (0.1) |
| H | 11.1 (0.4) | 8.1 (0.1) | 14.2 (1.6) | 7.8 (1.1) | 0.63 (0.2) |
| I | 13.2 (0.7) | 7.9 (0.1) | 44.2 (9.4) | 325.0 (138) | 0.24 (0.1) |
| J | 12.4 (0.7) | 7.6 (0.1) | 362 (122) | 335.4 (85.3) | 45.5 (33.1) |
| K | 12.8 (0.5) | 8.0 (0.2) | 54.8 (15.6) | 21.8 (5.8) | 0.41 (0.3) |

The ECLOX response curves of Figure 9 derive from samples collected on 26/8/97 and are typical of those seen in this study. The weather data for the collection day indicated no unusual climatic influences (air temperature 10.2 - 23.0°C; rainfall 0.7 mm).

**Figure 9: Typical ECLOX response curves for physical media sites on the Estate (Sawcer *et al.*, [illegible]d).** Curve labels show sample site.



Sites C, F, H (stream samples) and frequently site K were relatively uncontaminated, indicated by a small reduction in light intensity and small EU's (< 50), although K occasionally exhibited high EU scores. Sites I and J were heavily contaminated, indicated by high EU levels (Table 12) and severe reduction in light intensity (Figure 9). Although sites I and J had similar EU results they gave very different response curves, indicating a possible chemical difference. Spearman Rank analysis of the combined data from sites I, J and K (farm drainage ditch inputs) against site G (drainage ditch outfall into Stream 2) showed a significant correlation between EU ($P$ <0.05) but not for suspended solids and oocysts ($P$ >0.05), although high levels of suspended solids at K correlated with high levels at G ($P$ <0.05). Therefore the only parameter which reflected clearly the combined inputs of the two farmyard outfalls and road runoff (K) into the drainage ditch measured at site G was EU. This is probably because suspended solids and oocysts are more likely to settle out, whereas EU is a measure of inherent aqueous contamination.

There was no correlation between oocyst concentrations and EU ($P$ >0.05), and the results illustrate the danger of using surrogate indicators for *Cryptosporidium.* Site C, for example, had an average EU of 11.6, and was considered relatively clean, but testing for *Cryptosporidium* revealed the presence of oocysts on 78.6% of sampling occasions. There were also no correlations between the other parameters measured and oocyst concentrations ($P$ >0.05)

## 4.4.4 Slurry results

76 slurry samples were tested according to the protocol described in Appendix 7.3.8. Samples were collected on trays placed in the path of the rain gun during distribution. Ten samples (13.2%) were positive for *Cryptosporidium.* The mean oocyst concentration averaged across the 76 samples was 0.08 oocysts/cm$^3$ of slurry examined which equates to 80 oocysts per litre. However, the processing losses during preparation of slurry for microscopical enumeration of oocysts were high. The use of spiked samples indicated a recovery efficiency of only 5%; 95% of oocysts in the slurry disappearing during the separation and concentration procedures. This is much higher than for less viscous physical media. The figure of 80 oocysts/litre must be multiplied by at least 20 to approach the true figure which may be in the region of 1600 oocysts/litre of slurry. The full slurry lagoon holds approximately 2,100 cubic metres, equal to $2.1 \times 10^6$ litres. The final figure for oocysts in the slurry lagoon is therefore likely to be close to $3.4 \times 10^9$ oocysts, most of which will be spread on the fields during the autumn. It was not possible to determine the viability of these oocysts since the numbers seen in the final samples were very small.

## 4.5 Objective D: Quality control and assurance

### 4.5.1. Procedures

The work of this project required collection of sufficient field samples for later statistical or other analyses from animal populations of various sizes, some known, but varying through the year and from year to year (livestock) and some unknown, but certainly variable (wild animals) and from physical media. Wherever possible, the sample number was set to detect a minimum *Cryptosporidium* prevalence of 5% For example, a population of 70 animals would require 40 samples to reliably detect 5% prevalence (Cannon and Rowe, 1982). Standard procedures for collection, recording and testing of samples were employed (examples of database recording forms are shown in Appendix 7.4). These procedures enabled samples to be traced through the system if required and with the use of positive (high and low) and negative control samples constituted the internal quality monitoring mechanism. For quality assurance purposes, processed samples of faeces and physical media were sent periodically to one of the laboratories listed in Section 3. There were few disparities between the internal and external assessments. Differences were discussed, probable reasons established and, on one occasion, further material sent for analysis.

### 4.5.2. Protocols

Standard protocols based mainly on published methods were established at the outset and used routinely. Faeces varied considerably: fatty material from neonates, watery diarrhoea of scouring calves, fibrous horse stools and semi-dry rodent pellets. Pre-treatments or additional treatments were devised as necessary to enable the variety of faecal types to enter the standard protocol. The same pre-treatment tactic was employed for samples of physical media which also varied in consistency from thick slurry to almost clean water. Protocols for the treatment and testing of all samples are detailed in Appendix 7.3.

### 4.5.3. Limits of detection for oocysts

#### 4.5.3.1. Recovery of oocysts from faeces

To calculate the number of oocysts per gram of raw faecal sample it is necessary to know the dilution factors and processing losses inherent in the procedure. The technique applied here (a modified formol-ether sedimentation method adapted for use with a faecal parasite concentrator, abbreviated to mFES/FPC) starts with 0.5 g of faeces and examines $1/100^{th}$ of the final pellet on a microscope slide. Thus, the theoretical limit of detection for the technique is $2 \times 10^2$ oocysts per gram of original faeces. However, all methods of processing raw material, whether faecal or physical media (including clean waters), cause loss of the target particle, in this case the 4-5 μm oocysts of *Cryptosporidium*.

To assess oocyst recovery from animal wastes a composite "model" faeces was prepared, incorporating elements met most frequently (fat, partially digested plant material and other debris) and used to create a dilution series containing known numbers of oocysts (generously provided by Steve Wright of the Moredun Research Institute, Edinburgh). Samples were processed by the standard protocol, mFES/FPC, then stained using IFAT and the oocysts counted (Appendices 7.3.2 and 7.3.3). The experimental design outlined in Figure 7.4.2 shows the extensive replication required to produce a statistically significant outcome. The "count range" column of Table 13 reveals that oocysts seeded at $5 \times 10^2$ and $1 \times 10^3$ do not produce values consistently above zero i.e. if a single count were performed there could be no oocysts detected despite the fact that the expected numbers of oocysts were 10 and 5. However, the next level ($5 \times 10^3$), always gave counts above 2 oocysts although with a mean value of 10, five times lower than the expected value for 20 μl.

**Table 13. Recovery and detection of Cryptosporidium oocysts from faecal material.** Means are based on 36 replicate counts (described in Appendix 7.4).

| Seeding level per g | Expected number in 20 µl | Mean count | Mean % recovery | Count range | 95% CL | CV % |
|---|---|---|---|---|---|---|
| $1x10^5$ | 1000 | 257 | 26% | 217-287 | 251-263 | 3.6 |
| $5x10^4$ | 500 | 118 | 24% | 81-147 | 111-124 | 8.1 |
| $1x10^4$ | 100 | 31 | 31% | 18-45 | 28-33 | 11.4 |
| $5x10^3$ | 50 | 10 | 21% | 3-16 | 8-11 | 21 |
| $1x10^3$ | 10 | 2 | 20% | 0-6 | 1-2 | 44.1 |
| $5x10^2$ | 5 | <1 | 14% | 0-3 | 0-1 | 37 |

The 95% confidence limits (CL) and one way ANOVA (data not shown) revealed no significant differences between results within seeding levels and indicate that the method was reliably reproducible at the range of levels of oocysts in the model faeces. The coefficient of variation (CV) shows that the smaller the number of oocysts the larger the variation. Oocyst recovery rates from this model faecal base were a third to a half lower than for oocysts prepared in a non-faecal aqueous suspension investigated in parallel experiments (data not reported here). Obviously the presence of faecal material prevented a fuller recovery of oocysts, but these experiments have revealed the detection limits to be expected when processing raw faeces.

This experiment aimed to reproduce, as far as possible, the conditions pertaining to routine testing of faecal samples. It revealed that the actual limit of detection of oocysts is between $1 \times 10^3$ and $5 \times 10^3$ oocysts per gram of raw faeces; it is very likely that a seeding level of $2\text{-}3 \times 10^3$ oocysts would have produced counts consistently above zero in the above experiments. It therefore seems reasonable to regard a conservative value of $3 \times 10^3$ oocysts per gram of raw faeces as the routine, actual limit of detection, but noting that it may vary upwards, or downwards, due to the heterogeneity of faecal specimens.

This method shows a considerable improvement on results with direct smears of faeces (data not reported here) for which the detection limit is in the region of one million oocysts per gram. The calibration experiment also determined that one oocyst on a microscope slide represents approximately 3000 oocysts per gram of raw faeces. However, faeces containing 3000 oocysts per gram could equally well generate slides scoring 4 or 5, or even more oocysts on occasion. Thus, a range of slide values representing 3000 oocysts must be specified. Based on the results above, Table 14 shows the likely numbers of oocysts per gram of raw faeces for selected ranges of oocyst counts.

**Table 14: Correlation of oocyst counts from IFAT slides with expected oocysts/g raw faeces.**

| Oocyst range seen on IFAT slide | Expected oocysts/g raw faeces |
|---|---|
| 1 - 6 | 3,000 |
| 7 - 17 | 5,000 |
| 18 - 40 | 10,000 |
| 41 - 80 | 25,000 |
| 81 - 150 | 50,000 |
| 151 - 300 | 100,000 |
| 301 - 500 | 200,000 |
| >500 | 500,000 |

These correlations have been used to generate the oocysts/g raw faeces for each animal category tested. Tables 15A and 15B summarise the statistics for oocyst counts, indicating the range of values found and the probable average count in raw faeces for each animal category. The data in the final column has been used as the basis for calculations of the total annual oocyst production by the animals of the Estate presented later in the report (Table 19).

**Table 15A: Statistics for oocyst counts from faecal samples of animals on the Estate 1995-98.**

| Animal group | Number of positive samples | Range of slide counts | Median | Mean | Average oocysts/gram raw faeces |
|---|---|---|---|---|---|
| Bull beef | 6 | 2 | 2 | 2 | 3,000 |
| Calves | 205 | 1-3000 | 9.5 | 177 | 100,000 |
| Dairy cows | 16 | 1-5 | 2 | 2 | 3,000 |
| Ewes | 4 | 2-3 | 2 | 2 | 3,000 |
| Lambs | 13 | 2-77 | 4 | 10 | 5,000 |
| Horses | 8 | 1-3 | 2 | 2 | 3,000 |
| Rats | 62 | 1-93 | 5 | 10 | 5,000 |
| House mice | 90 | 1-895 | 7.5 | 81 | 50,000 |
| Wood mice | 139 | 2-595 | 24 | 86 | 50,000 |
| Bank voles | 53 | 1-1123 | 11.5 | 65 | 25,000 |
| Shrews | 38 | 1-116 | 5 | 18 | 10,000 |

**Table 15B: Graded oocyst counts detected in positive faecal specimens from the Estate 1995-97.**
Low = ≤20 oocysts per 20 µl concentrate inspected, Medium = 20 - 50 oocysts and High = >50 oocysts. The percentage for each category is in brackets.

| Animal type | No. positives | Low count | Moderate count | High count | Uncounted |
|---|---|---|---|---|---|
| Bull beef | 6 | 5 (83%) | 0 | 0 | 1(16%) |
| Dairy cows | 16 | 16(100%) | 0 | 0 | 0 |
| Home bred calves | 206 | 119(58%) | 15(7%) | 65(32%) | 7(3%) |
| Horses | 8 | 8(100%) | 0 | 0 | 0 |
| Lambs | 13 | 12(92%) | 0 | 1(8%) | 0 |
| Rats | 62 | 57(92%) | 4(6%) | 1(2%) | 0 |
| Sheep | 4 | 4(100%) | 0 | 0 | 0 |
| House mice | 90 | 61(68%) | 11(12%) | 18(20%) | 0 |
| Wood mice | 139 | 64(46%) | 24(17%) | 51(37%) | 0 |
| Bank voles | 53 | 30(57%) | 11(21%) | 12(23%) | 1(2%) |
| Shrews | 38 | 30(79%) | 2(5%) | 6(16%) | 0 |

Table 15B divides counts of oocysts seen on the slides into three categories to enable comparison with the similar table for the 1992-94 survey in Appendix 7.2 (Table 7.2.2). Although there are apparently differences in detail, the pattern of counts for the three categories is broadly similar for the two surveys; adult livestock have counts exclusively in the low category, whilst young livestock and rodents have counts in the two higher categories.

## 4.5.3.2. Recovery of oocysts from physical media

The recovery rate for the flocculation technique was determined by testing 10 litre reverse osmosis (RO) or tap water samples seeded with 1 ml aliquots of RO water containing known numbers of oocysts. These were purified from positive calf faeces according to the method of Campbell *et al.* (1992) (Appendix 7.3.9). Oocysts were used fresh or allowed to age, since differences in recoveries have been reported (Smith and Hayes, 1997). Following the standard flocculation procedure, oocysts were stained by IFAT and DAPI (Appendix 7.3.5).

**Table 16: Recovery of *Cryptosporidium* oocysts from seeded waters by the flocculation technique.**

| Type of oocysts | Type of water | Oocyst seeding level | Number of oocysts recovered (% recovered) |
|---|---|---|---|
| Fresh | RO | 895 | 250 (28) |
| Fresh | RO | 895 | 177 (20) |
| Fresh | RO | 895 | 188 (21) |
| Fresh | RO | 895 | 214 (24) |
| Fresh | RO | 895 | 74 (8) |
| | | | **Mean recovery = 20.2%** |
| | | | |
| Fresh | Tap | 895 | 281 (31) |
| Fresh | Tap | 895 | 325 (36) |
| Fresh | Tap | 448 | 59 (13) |
| | | | **Mean recovery = 26.7%** |
| | | | |
| Aged | Tap | 99 | 0 |
| Aged | Tap | 99 | 10 (10) |
| Aged | Tap | 990 | 0 |
| Aged | Tap | 990 | 10 (1) |
| | | | **Mean recovery = 2.8%** |

Table 16 reveals that average recoveries of fresh oocysts from RO water and tap water were similar, but recovery of aged oocysts was poor, as has been reported elsewhere (Smith and Hayes, 1997). In the original description of the flocculation method, the recovery rate was quoted as being greater than 68% for all samples tested (Vesey *et al.*, 1993). More recent work has shown that the flocculation technique, like other methods for concentrating *Cryptosporidium* from water, gives extremely variable recoveries, even when carried out in the same laboratory using the same equipment and local water (Smith and Fricker, 1997), which is confirmed again here.

Field samples of physical media naturally will contain oocysts of various ages, affecting the percentage recovery. It is also the case that physical media other than clean waters may generate different, probably lower, recoveries. Thus a conservative estimate for the level of recovery during routine use of the flocculation technique would be 20%, indicating that actual quantities of oocysts in fresh samples would be at least five times higher.

## 4.5.4 Testing oocysts for viability

There are several methods for determining oocyst viability. The most suitable for routine use depends on the inclusion/exclusion of the fluorogenic vital dyes DAPI and PI (Campbell *et al.*, 1992, Appendix 7.3.10).

### 4.5.4.1 Viability of oocysts purified from animal faeces

Routine testing of oocyst viability was not feasible or possible for the large number of samples involved. However, to provide an indication of viability on exit from the host, faeces from calves showing high oocyst levels were examined (Table 17).

**Table 17: Viability of *Cryptosporidium* oocysts from calves tested by the DAPI/PI dye inclusion/exclusion technique.**

| Calf ID | % oocysts viable at assay (DAPI+PI-) | % oocysts potentially viable (DAPI-PI-) |
|---|---|---|
| BI 592 | 70.8 | 7.8 |
| BI Pen 12 | 67.5 | 4.9 |
| mm8752 | 54.6 | 9.3 |
| mm8753 | 62.1 | 2.2 |
| mm9274 | 57.3 | 5.0 |
| mm9288 | 60.8 | 5.6 |
| mm9290 | 63.8 | 4.4 |
| mm9300 | 49.7 | 6.0 |
| mm9367 | 68.5 | 9.9 |
| mm9372 | 47.7 | 32.1 |
| mm9416 | 65.0 | 11.0 |
| mm9419 | 47.6 | 12.2 |
| mm9420 | 52.3 | 9.7 |
| Averages | **59.1 %** | **8.75 %** |

Viability ranged from 48 - 71%, with an average of 59%. Potentially viable oocysts are those which exclude PI but have no visible sporozoite nuclei. If these are added to the first column, the overall average viability is 68%. This figure matches values reported from the literature (Campbell *et al.*, 1992; Bukhari *et al.*, 1995). The figure is not 100%, probably because there is some loss of viability during even short storage periods and during the testing procedure; further, it is known that the viability of oocysts varies during the shedding cycle (Bukhari and Smith, 1997). The production of oocysts will be subject to the same vicissitudes and attritions as any living system: unless all conditions are perfect, performance will be sub-optimal. Thus we should not expect 100% viable oocysts even in fresh faeces.

### 4.5.4.2 Viability of oocysts purified from physical media

Viability tests of physical media were not usually possible because, although the 10 litre grab samples frequently contained oocysts, the whole of the sample was employed in the IFAT test to detect the generally low oocyst numbers. On all but one occasion when high numbers were found, the source had reverted to low values on re-sampling. The result for that one occasion is shown in Table 18. W423 was the very high oocyst count sample from site J (Table 9), which drains part of the farmyard and outfalls into the drainage ditch. 54% were viable. It should be noted that the flocculation technique used to concentrate oocysts prior to testing reportedly causes substantial loss of viability (Campbell *et al.*, 1994). To check this, two samples containing oocysts of known viability obtained from calf faeces were subjected to the flocculation technique and then tested for viability a second time (Table 18).

**Table 18: Viability of *Cryptosporidium* oocysts before and after concentration by flocculation.**

| Sample code | Mean % viability before flocculation | Mean % viability after flocculation | % reduction in viability |
|---|---|---|---|
| PO 2 | 67.5 | 17.5 | 50.0 |
| PO 8 | 30.2 | 24.6 | 5.6 |
| **W423** | na | **53.7 %** | na |

A reduction in viability is confirmed, but the reasons for the variation in it are obscure. The average reduction was 28%. It is likely that the oocysts from outfall J (in sample W423) were of higher viability than 54%.

# 5 DISCUSSION AND SUMMARY OF RESULTS

This study has focused almost exclusively on a single site. The overall aim has been to create a benchmark for the distribution of *Cryptosporidium* in a typical area of agricultural land in lowland Britain. It has surveyed the mixture of livestock and wild animals living on the Estate of the Warwickshire College for the presence of *Cryptosporidium* during three consecutive seasons. Although not all animal groups have been examined for the whole period, a comprehensive picture of the production of parasite oocysts has emerged. The 190 hectare Estate is part of a large educational institution and is managed in an exemplary way particularly since it is used for teaching purposes. The farm is clean, with little unsightly rubbish on view, storage areas are neatly organised, machinery is in sound repair, animals are fit and healthy, housed in good conditions, and the procedures for the treatment and disposal of animal wastes accord with published guidelines. The Estate has had no history of clinical cryptosporidiosis.

This project also explored the movement of parasite oocysts into the stream draining the gently sloping site. We have attempted to gauge the proportion of the Estate's annual output of oocysts which enter the stream. The quantity of *Cryptosporidium* detected should be broadly representative of that part of lowland Britain which contains a patchwork of mixed and arable farming. However, the levels of oocysts found in the livestock populations and in the surface waters of this Estate may be low compared with other sites which are not managed as exemplars for teaching purposes.

## 5.1 A model for calculating the annual production of oocysts

The calculation of annual oocyst yield for the Estate described in Table 19 is presented as a scenario based on data from this study combined with that from the pre-contract survey of 1992-94, and a number of 'best guess' assumptions.

**Table 19: A model for calculating the annual oocyst yield for the Estate.**

| Animal group | Oocysts / g faeces | *Faeces/24h (g) | Oocysts/day per individual | Days shedding | No. animals shedding | Popn. size | Prevalence of Crypto.** | Oocysts per year | % of total |
|---|---|---|---|---|---|---|---|---|---|
| **Home bred calves** | 100000 | 2000 | 200000000 | 12 | 48 | 80 | 0.4 | 1.152E+11 | 19.0 |
| **Bought in calves** | 100000 | 2000 | 200000000 | 12 | 10 | 75 | 0.11 | 24000000000 | 4.0 |
| **Foals** | 14000 | 1000 | 14000000 | 12 | 2 | 20 | 0.05 | 336000000 | 0.1 |
| **Lambs** | 5000 | 100 | 500000 | 12 | 30 | 310 | 0.12 | 180000000 | 0.03 |
| **Dairy cows** | 3000 | 35000 | 105000000 | | | 110 | 0.05 | 2.10788E+11 | 34.7 |
| **Bullbeef** | 3000 | 25000 | 75000000 | | | 80 | 0.02 | 43800000000 | 7.2 |
| **Heifers** | 3000 | 20000 | 60000000 | | | 30 | 0.035 | 22995000000 | 3.8 |
| **Horses** | 3000 | 30000 | 90000000 | | | 76 | 0.06 | 1.49796E+11 | 24.7 |
| **Ewes** | 3000 | 1500 | 4500000 | | | 195 | 0.09 | 28825875000 | 4.8 |
| | | | | | | | | | |
| **House mice** | 50000 | 1.6 | 80000 | | | 100 | 0.37 | 1080400000 | 0.2 |
| **Wood mice/voles** | 50000 | 1.6 | 80000 | | | 500 | 0.47 | 6862000000 | 1.1 |
| **Rats** | 5000 | 16 | 80000 | | | 25 | 0.3 | 219000000 | 0.04 |
| **Other wild mammals** | 10000 | 100 | 1000000 | | | 100 | 0.07 | 2555000000 | 0.4 |
| | | | | | | | | **Total** | **100.0** |

* data from Chalmers, 1996 (page 217)
** percentages expressed in decimal notation

| | | |
|---|---|---|
| Sub-total: oocysts generated by wild mammals = | 1.07E+10 | **($1.1 \times 10^{10}$)** |
| Sub-total: oocysts generated by livestock = | 5.96 E+11 | **($6.0 \times 10^{11}$)** |
| TOTAL OOCYSTS GENERATED IN A YEAR = | 6.1E+11 | **($6.1 \times 10^{11}$)** |
| Percentage oocysts generated by wild mammals = | 1.8 | |
| Assume 0.5% wild mammal oocysts reach the stream VIABLE = | 5.36E+7 | **($5.4 \times 10^{7}$)** |
| Assume 0.05% livestock oocysts reach the stream VIABLE = | 3.0E+8 | **($3.0 \times 10^{8}$)** |
| Total VIABLE oocysts reaching stream in a year = | 3.52E+8 | **($3.5 \times 10^{8}$)** |
| Percentage viable oocysts from wild mammals reaching stream = | 18.0 | |

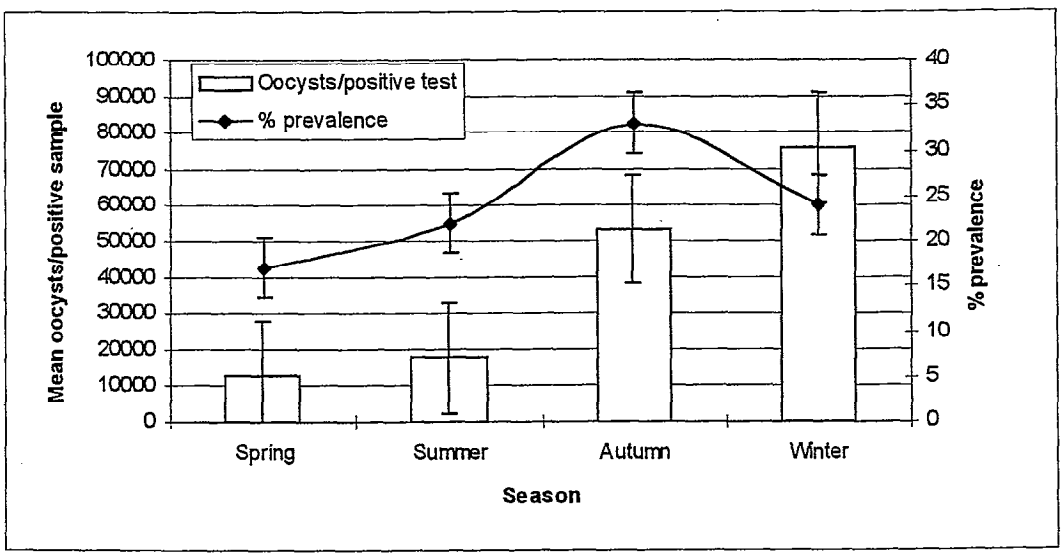
The average oocyst numbers per gram of fresh faeces are from Table 15A. Typical quantities of faeces produced per 24 hours were taken from the literature (Meehan, 1984; Chalmers, 1996). In the absence of published figures the quantity of droppings has been related to body size. Larger wild mammals were treated as a single group and an average weight of droppings per day set at 100 grams. Typical yearly populations of livestock were based on farm records. Population estimates for rats on the Estate were determined experimentally by the Central Science Laboratory. For other small mammals, the minimum number alive estimates determined for Suggett Spinney were used as a guide to the population on the Estate as a whole. Larger wild mammals were again treated as a single group and an estimate made for the number based on discussions with game keepers and Estate conservation staff. The average prevalences of *Cryptosporidium* in all the animal groups tested are those of Table 2 or based on the pre-contract data of Appendix 7.2. Young animals such as calves may only shed oocysts for up to 12 days (Fayer *et al.*, 1990) which seems to be confirmed by our data in Table 5, hence 12 days has been used in Table 19 for young livestock. The prevalence (expressed in decimal notation for calculation purposes) has been used to compute oocyst production for other animal categories on the basis that, whenever they were examined during the year there was, on average, that percentage of animals shedding oocysts.

The major contributors of oocysts in this model are cows (34%), horses (25%) and calves (19%). Calves have been assumed to be infected only once, shedding oocysts for 12 days. Some of them may become infected later, but as members of different groups such as bull beef, heifers or dairy cows. Although not many dairy cows and horses were infected they do produce large quantities of droppings. Conversely, the impact of lambs is small because quantities of faeces are small, and on this Estate the prevalence was not high. By far the greater proportion of oocysts are generated by livestock; less than 2% come from wild animals.

## 5.2 Seasonality of oocyst production and distribution

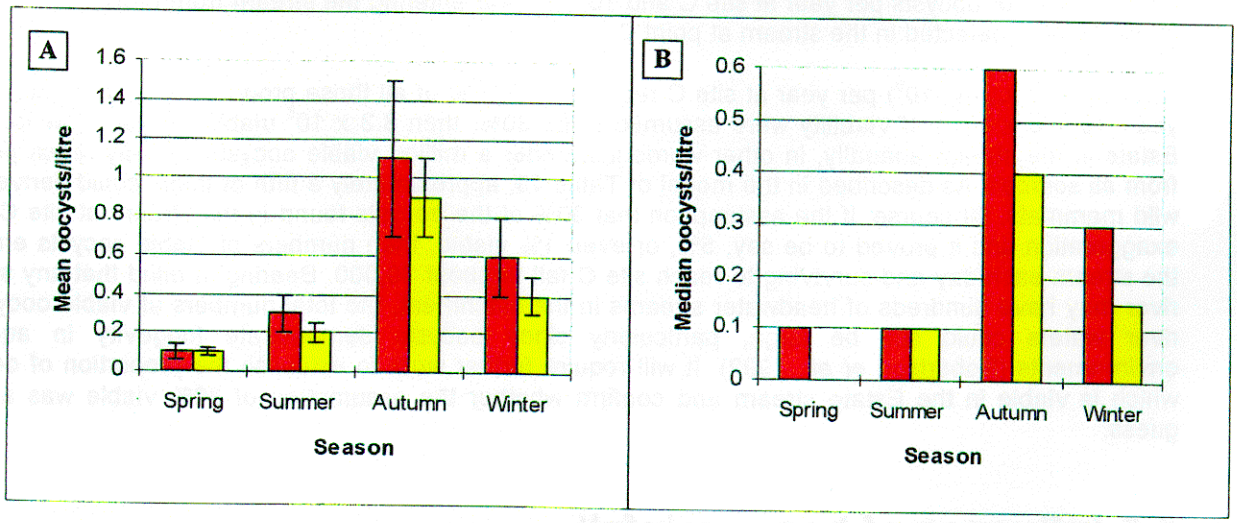
The yearly peak of oocyst production for the Estate seems to be in the autumn and winter during calving and when the wild mammal populations are highest (although, as already mentioned, their contribution to oocyst numbers is small). The evidence for this comes from determining the overall seasonal prevalence, combining data from all animals tested, and the mean oocyst counts per positive sample (Figure 10). Both values were significantly higher in autumn (September to November) than in spring and summer, although prevalence may have reduced during the winter.

**Figure 10: Seasonal prevalence on the Estate of all animal groups combined and average number of oocysts shed per positive sample.** (Bars = +/- standard error).



A similar rise in oocyst levels was evident from the study of physical media. Figures 11 A and B show the oocyst levels arranged seasonally for sampling sites C (stream 1, close to where it leaves the Estate) and G (outfall into stream 1 from the farm drainage ditch). Both sites peak in the autumn, both show a similar pattern, and the pattern for the year parallels that seen for the prevalence in Figure 10.

**Figure 11: Seasonal oocyst levels (A - mean; B - median) at the Estate Sept 1996 - Aug 1997.** (Bars = +/- standard error). ■ Site G ■ Site C.




## 5.3 Loss of oocyst viability

An unknown fraction of oocysts produced by livestock will be deposited inside buildings or the farmyard. Most of these oocysts will eventually pass to the midden or slurry lagoon since animal waste from the farm and stables is concentrated in these places. The analysis of slurry at the time of distribution has indicated that as many as $3.4 \times 10^9$ oocysts were released into the environment during the annual spreading onto the fields. However, if farm waste is managed properly, most oocysts should be destroyed by chemical changes, particularly resulting from the high concentration of ammonia (Ruxton, 1995), and predation by other organisms in the slurry. Thus most oocysts remaining in slurry should be non-viable by the time of distribution onto fields.

Livestock droppings deposited directly onto the fields will lie there, subject to dehydration, sunlight, heating, cooling and biological and chemical degradation processes. There is a high chance that oocyst numbers in field droppings will be much reduced in a relatively short time and the viability of the remainder low.

## 5.4 Numbers and viability of oocysts reaching the stream

The viability of oocysts on emergence from calves was tested several times, averaging 60%. Whatever the initial viability, it will drop in the days following emergence; thus numbers of viable oocysts entering the stream should be a small fraction of those generated on the Estate. The viability of oocysts found in surface waters could not be assessed in this study for reasons described in section 4.5.4.2, except in one case, from outfall J, when the viability was 54%. It is not known what fraction of the oocysts produced on the Estate find their way into the stream, but obviously some do. In the model of Table 19 we have assumed that a very small percentage of viable oocysts reach the stream; 0.05% (i.e. 1/2000$^{th}$) of livestock oocysts and 0.5% (i.e. 1/200$^{th}$) of wildlife oocysts (higher, because many are likely to be deposited very close to water). Thus we calculate that about 350 million viable oocysts enter the stream annually. This represents approximately one million a day, perhaps a fifth of them from wildlife.

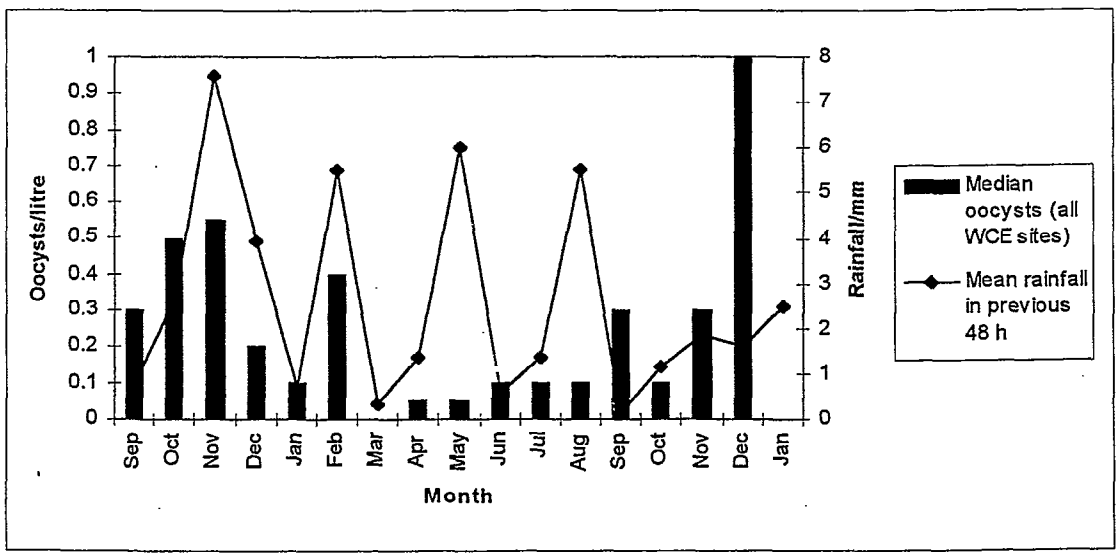
Enumeration of oocysts from samples taken from site C (where the stream leaves the Estate) has provided an average figure for the oocyst concentration in water at that point over a 17 month period. This value is 0.48 oocysts/litre (median = 0.2 oocysts/litre). The flow rate at site C has been calculated to be 8.2 litres per second (for comparison, outfall G, which can be measured directly, is 0.6 litres per second with an average concentration of 0.63 oocysts/litre). These data allow calculation of the annual quantity of oocysts flowing through site C and from outfall G. To allow for losses during processing, conservatively estimated to be at least times five (Section 4.5.3.2), a realistic factor of 7.5 has been introduced to derive actual oocyst numbers in raw waters. These figures, rounded to the nearest whole number, are $10^9$ oocysts per year at site C and $10^8$ per year entering the stream from outfall G, one tenth of the number detected in the stream at point C.

One billion oocysts ($10^9$) per year at site C represents 0.17% of all those produced on the Estate in one year ($10^9$ / 6 x $10^{11}$). If viability were assumed to be 30%, then 3.3 x $10^8$ viable oocysts flow from the Estate in the stream annually, in other words just under a million viable oocysts per day reach point C from all sources. As described in the model of Table 19, approximately a fifth of these could derive from wild mammals. Of course, if the assumption that 30% of the oocysts found in the stream at site C is an exaggeration and it proved to be say, 5%, or even 1% viable, then numbers of viable oocysts entering the stream each day and surviving to reach site C fall to about 30,000. Bearing in mind that any sizable river may have hundreds of headwater streams in the catchment, the total numbers of viable oocysts in river waters could still be large, particularly since oocysts demonstrate longevity in aqueous environments (Robertson *et al.*, 1992). It will require further work to establish the proportion of oocysts which is viable in the Estate stream and confirm whether the assumption of 30% viable was a good guess.

## 5.5 Influence of heavy rainfall

Heavy rainfall will flush material, including viable and non-viable oocysts, into water courses. With the possible exception of occasional links between oocyst concentrations in surface waters with rainfall (Figure 12) we have not been able to show correlation between oocysts in the stream with other factors such as slurry spreading and grazing in fields adjacent to water courses. The finding of occasional links between oocyst concentrations and rainfall concurs with the observation of Poulton *et al.* (1991): "...cryptosporidia often enter the aquatic environment through discrete contamination events of short duration. Rainfall is coincidental to only some of these incidents". Rainfall during spring was significantly lower than during the summer ($P$ <0.01), which may also have contributed to the significantly lower oocyst concentrations during the spring (section 4.4.3.1)

**Figure 12: Median oocyst concentration (all Estate sample sites) against mean rainfall in the 48 h preceding sampling Sept 1996 - Sept 1997.**



Oocysts flushed into the stream from fields during heavy rain will be joined by those from the farm drainage ditch (G) which combines inputs from outfalls K (carrying road runoff), I and J (off the farm yard). Thus the flushing effects of rainfall are visited not just on the stream's immediate surroundings but also on the ground being drained by the ditch, which is both extensive and some distance away (particularly the catchment of outfall K). Thus although Figure 12 does point to increases in oocyst concentrations in surface waters following heavy rain, the contributions from specific parts of the catchment (such as the adjacent fields) cannot be distinguished from the total. Further, despite the apparently high number of oocysts in slurry, $3.4 \times 10^9$, this represents only approximately $1/200^{th}$ of all those we calculate are generated on the farm each year. It is likely that only a very small proportion of these $3.4 \times 10^9$ oocysts will be flushed into the stream which renders it even more unlikely that any specific effects would be detectable.

## 5.6 The role of wild mammals

The importance of wild animals lies not in the total quantities of oocysts they generate, which is small, but in the places they leave them. Wild animals frequently live near water and excrete in or near it. This was especially evident at Lodge Pond where rats were seen swimming and the banks were littered with droppings of various wild animals. Rodents, which inhabit farm buildings excrete in those buildings, often in or near food and livestock bedding. Thus livestock are more likely to become infected by feeding near hedgerows, woodland margins, stream and pond banks, indeed any location visited frequently by rodents; young animals housed in buildings must be particularly vulnerable.

Neonatal livestock can obviously be infected by their parents. However, infection was detected rarely in adult livestock in this study, although it is possible that a single parent could infect her neonate who then passed it to other young animals in the group. Our study of the calves showed that the first evidence of infection (shedding oocysts) occurred from 4 to 28 days after birth and that no calves positive on first testing were born to dams which were positive at the time of calving. The most likely explanation is that some animals acquired *Cryptosporidium* from bedding or food contaminated with fresh, highly infective oocysts deposited by rodents; thereafter infection could be passed readily from pen to pen which is compatible with the patterns seen in Table 5.

## 5.7 Locations on the Estate with high levels of oocysts

Oocysts were detected frequently, often at high levels, in animal faeces at a number of locations on the Estate. The farm buildings, hedgerows and woodland margins are important sources of potential infection. Amongst the wild mammals, the high scoring rodents (mice, voles and rats) form perhaps the most important wildlife reservoir of *Cryptosporidium*, both in terms of infecting livestock and contaminating watercourses.

## 5.8 Summary of the main results

The *Cryptosporidium* test data for all animal cohorts/species sampled during 1995-98 have been amalgamated in Figure 13, ranking them in descending order of prevalence, and Figure 14, which compares the 1995-98 occurrence with that during the pre-contract survey of 1992-94. It is evident from Figure 13 that adult livestock have the lowest number of animals shedding oocysts and that the calves are the highest category. The next highest group is predominantly the adult and near adult rodents, which were caught in the live mammal traps. The high *Cryptosporidium* prevalence for all the rodent species sampled emphasises their importance as a reservoir of infection. Note that values for house mice, wood mice and bank voles do not match those of Table 2, which contains values for the mixture of species found at particular locations.

The occurrences (Figure 14) for some categories differ considerably between the two surveys; this is particularly striking for the rodents. However, while there may be an element of real increase, much of the difference could be artifactual for two reasons. Firstly, the more reliable IFAT detection method was used exclusively for the 1995-98 survey, but only late in the 1992-94 work, and secondly, occurrence figures are not necessarily a good indication of prevalence because of the possibility of repeat sampling of the same animals. For example, rodents were not always marked successfully to identify recaptures in the 1992-94 survey. In addition, some rodent groups during the earlier survey were found to contain *Cryptosporidium muris*, the other species which infects mammals. The *C. muris* results have been omitted from Figure 14 because there are no records of this species causing cryptosporidiosis in livestock or humans.

**Figure 13: *Cryptosporidium* prevalence 1995-98**. Note that data are grouped by species or animal type, not geographical location.

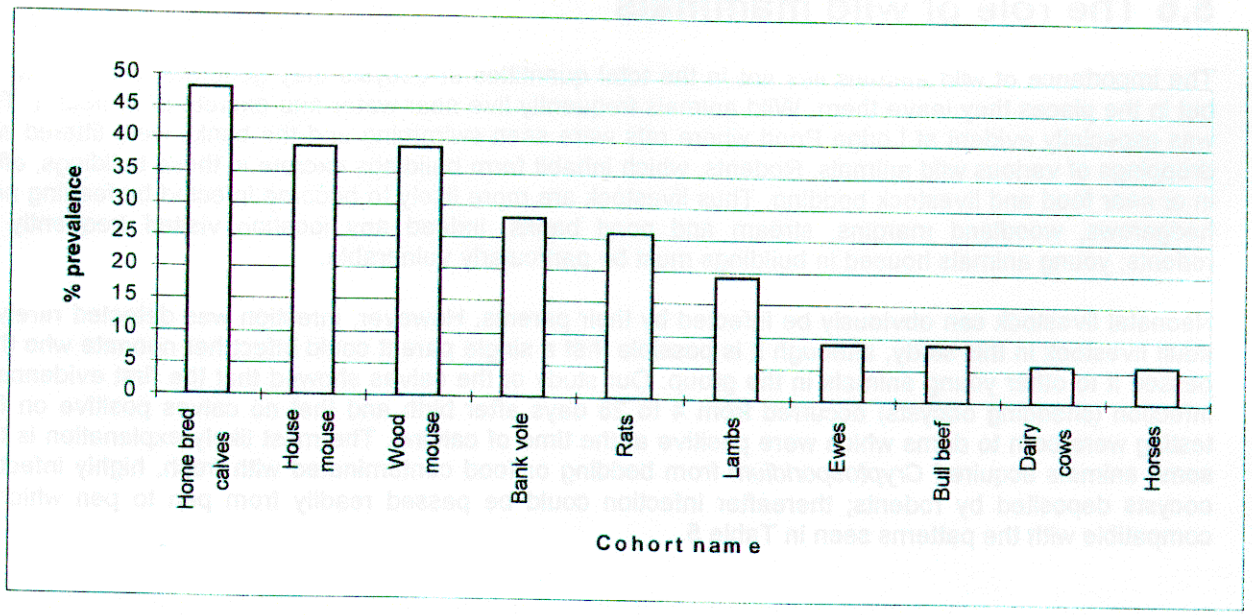
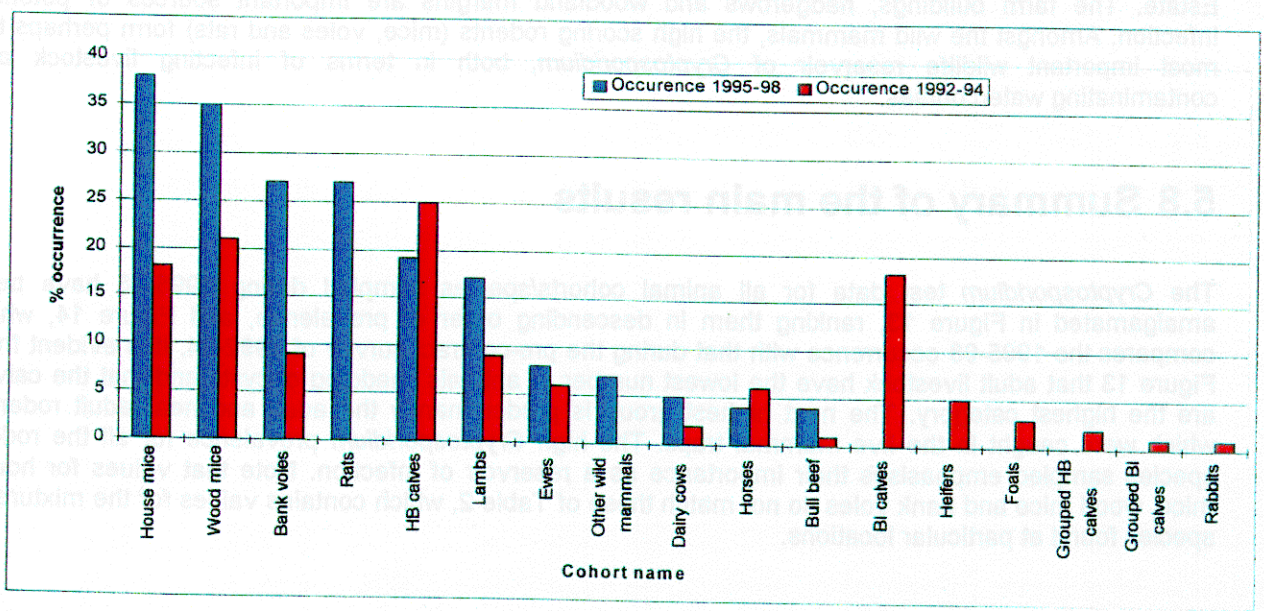



**Figure 14: Comparison of contract and pre-contract occurrence**



The main results from this study of animals and physical media can be summarised as follows:

1 All livestock groups contain animals shedding oocysts of *Cryptosporidium* in their faeces.

2 The proportion of adult livestock shedding oocysts is small, and the numbers of oocysts per gram of faeces is also small.

3 A high proportion of young livestock animals, particularly calves, become infected at an early age and shed large quantities of oocysts.

4 Several wild mammal species living on, or using the site, harbour *Cryptosporidium*. Mice and rats in particular have a high proportion of adult animals shedding oocysts, and mice exhibit the highest numbers of oocysts per gram of faeces.

5 Mice may be the main source of infection for very young calves, as suggested by the timing of first detected infection and the high numbers of mice positive for *Cryptosporidium* found in and around the calf pens.

6 A rat population, living near a pond on an arable farm without livestock or use of animal wastes, had the same proportion of infected animals as the Estate rats.

7 Surface waters draining the Estate contained *Cryptosporidium* oocysts on the majority of occasions tested.

8 Approximately $6 \times 10^{11}$ oocysts are produced by the animals of the Estate in a year, of which 98% are attributable to livestock and only 2% to wild animals.

9 The slurry lagoon was calculated to contain about $3 \times 10^{9}$ oocysts when full just before distribution, representing about $1/200^{th}$ of all those produced on the Estate annually.

10 Approximately $10^{9}$ oocysts leave the Estate in the stream each year, representing about $1/600^{th}$ of all those produced on the Estate annually. If only a third are assumed to be viable, it represents 333 million annually, just under a million viable oocysts per day. Up to a fifth of these could come from wildlife.

11 Approximately $10^{8}$ oocysts per year may enter the stream from the farm drainage ditch, $1/10^{th}$ of the number calculated to be leaving the Estate in the stream each year.

12 Autumn (September, October, November) and winter (December, January, February) are the periods when levels of oocysts on this Estate are highest amongst its animals and in the stream.

## 5.9 Further investigations

The prediction of $10^{9}$ oocysts in total and a third of these viable leaving the Estate per year via the stream can be confirmed only by further study. It would involve concentrating oocysts from large (hundreds of litres) water samples followed by enumeration and testing of oocysts for viability. It would also require better assessment of flow rates in the stream. It would then be possible to refine the calculations to define more accurately the impact of oocysts from the Estate on the stream.

Discovering the ratio of wildlife to livestock oocysts in the stream may be possible by application of DNA typing techniques to oocysts found in surface waters. DNA typing of oocysts could also be used to explore the sources of infection for young calves; newly infected calves may shed oocysts carrying rodent-specific signatures.

# 6 CONCLUSIONS

The aims and objectives of this project have been met by a detailed 3 year survey of the Estate which has established the quantities of oocysts generated by the animals using the Estate and shown that its surface waters routinely carry oocysts throughout the year, probably peaking in the autumn and winter. An important route of contamination of the stream with fresh viable oocysts (the farm drainage ditch) has been identified.

This study, and reports from the literature (Chalmers *et al.*, 1995; Chalmers *et al.*, 1997; Webster and MacDonald, 1995), strongly suggest that *Cryptosporidium* is ubiquitous amongst wild mammals in this country. This was emphasised by our investigation of Lodge Pond, a site without livestock influence where the rat population was infected. The parasite has been found in all the livestock groups examined on the Estate. Amongst the wildlife, rodents had the highest prevalence for the parasite. The period of the year when levels of Cryptosporidium were highest on the Estate was autumn and winter; this coincided with calving and was the time when rodent numbers were probably at their peak. This was the situation for a well managed, clean farm which emphasises current good practice. Nevertheless, to minimise entry of oocysts into surface waters and to reduce opportunities for infection of livestock it would seem sensible to:

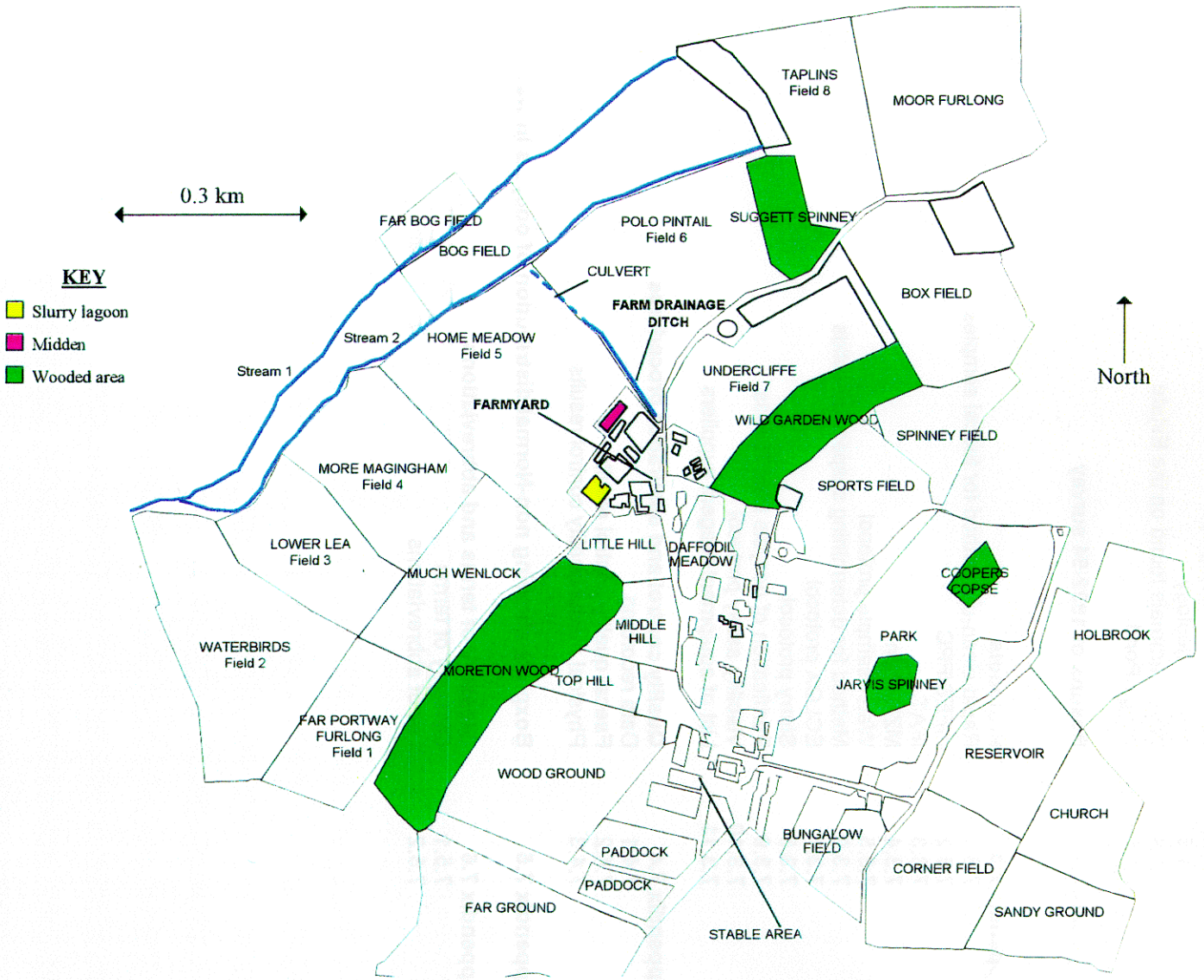
1 Restrict livestock access to streams, hedgerows and woodland margins to try to reduce the overall prevalence in grazing adult animals and to protect watercourses from input of livestock oocysts.
2 Minimise rodent presence, especially mice, in calf pens and other farm buildings to reduce the chances of infection of young animals.
3 Ensure that farm yard runoff carrying oocysts does not leak into nearby drainage systems but is directed into a holding facility such as a slurry lagoon.

*Cryptosporidium* is a natural part of the environment. It cannot be eliminated, but its impact might be further reduced by strict adherence to current guidelines and perhaps restricting access to, and improving control of, animal reservoir 'hot spots'.

# 7. APPENDICES

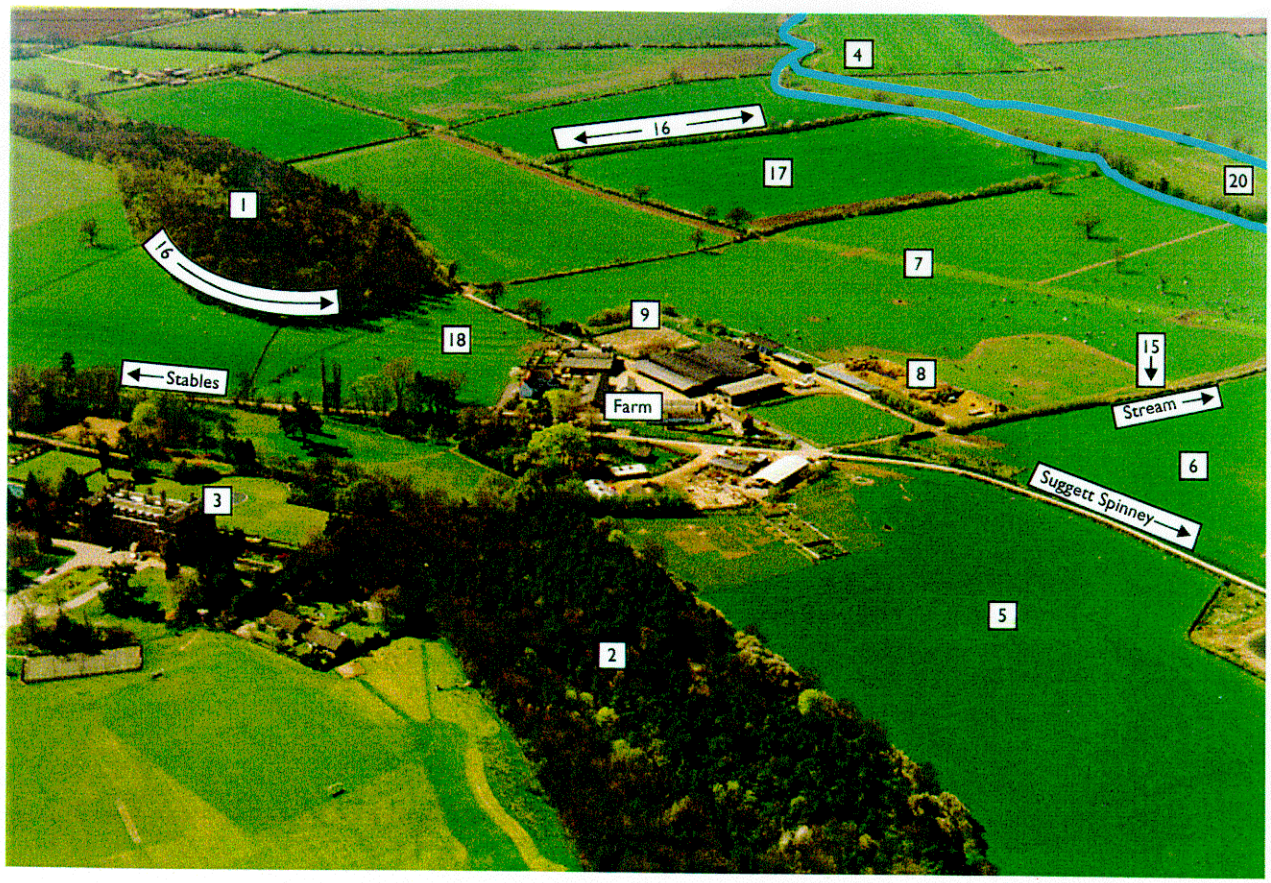
## 7.1.1 Site map

**Appendix 7.1**



# Appendix 7.1.2

**Figure 7.1.2: Aerial view of the Estate showing the main features.** Key: 1, Moreton Wood; 2, Wild Garden Wood; 3, Main House; 4, Streams outlined in blue; 5, Undercliffe Field; 6, Polo Pintail Field; 7, Home Meadow; 8, Farmyard manure pad; 9, Slurry Lagoon; 15, Farm Drainage Ditch; 16, Examples of small mammal trapping sites; 17, More Magingham Field; 18, Little Hill Field; 20, Edge of Bog Field.

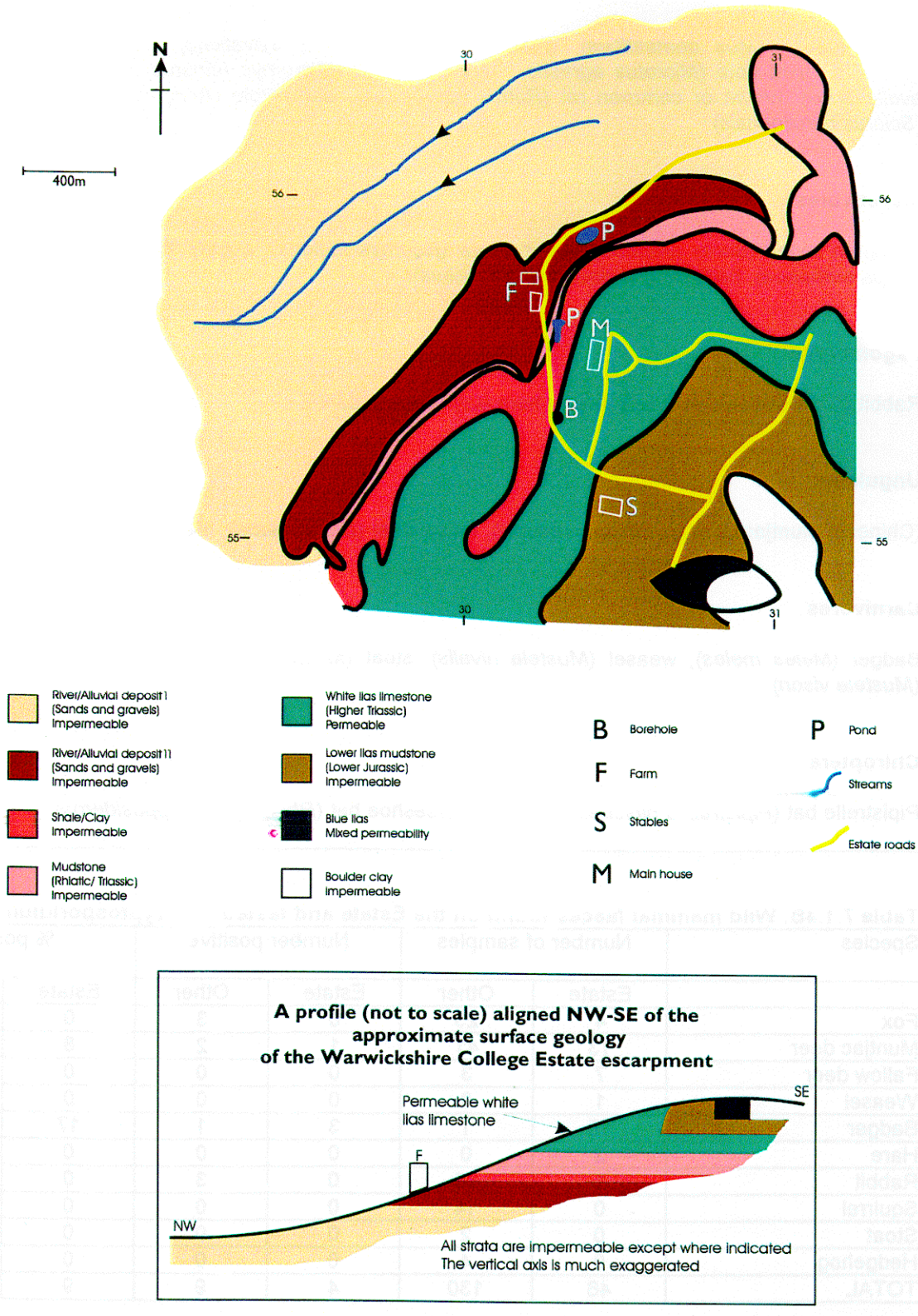


# Appendix 7.1.2A

**Figure 7.1.2A: Aerial view of the farm showing buildings and waste storage areas.** Key: 1, Moreton Wood; 7, Home Meadow; 8, Farmyard manure pad; 9, Slurry Lagoon; 10, Milking Parlour; 11, Dairy Cow Cubicles; 12, Heifer Barn; 13, Bull Beef Unit; 14, Calf Unit; 15, Farm Drainage Ditch; 17, More Magingham Field; 18, Little Hill Field; 19, Silage Barn; 21, Game Unit; 22, Lambing Shed.

# Appendix 7.1.3

**Figure 7.1.3: An approximate surface geology for the Estate**



# Wildlife found on the Estate

# Appendix 7.1.4

**Table 7.1.4A: Types of wild mammal found on the Estate**

**Rodents**

House mouse (*Mus domesticus*), wood mouse (*Apodemus sylvaticus*), bank vole (*Clethrionomys glareolus*), field vole (*Microtus agrestis*), harvest mouse (*Micromys minutus*), dormouse (*Muscardinus avellanarius*), brown or common rat (*Rattus norvegicus*), water vole (*Arvicola terrestris*), grey squirrel (*Sciurus carolinensis*)

**Insectivores**

Common shrew (*Sorex araneus*), water shrew (*Neomys fodiens*), pygmy shrew (*Sorex minutus*), mole (*Talpa europaea*), hedgehog (*Erinaceus europaeus*).

**Lagomorphs**

Rabbit (*Oryctolagus cuniculus*), brown hare (*Lepus europaeus*).

**Ungulates**

(Chinese) Muntjac deer (*Muntiacus reevesi*), fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*).

**Carnivores**

Badger (*Meles meles*), weasel (*Mustela nivalis*), stoat (*Mustela erminae*), fox (*Vulpes vulpes*), mink (*Mustela vison*).

**Chiroptera**

Pipistrelle bat (*Pipistrellus pipistrellus*), lesser horseshoe bat (*Rhinolophus hipposideros*).

**Table 7.1.4B: Wild mammal faeces found on the Estate and tested for Cryptosporidium, 1995-97.**

| Species | Number of samples | | Number positive | | % positive | |
|---|---|---|---|---|---|---|
| | Estate | Other | Estate | Other | Estate | Other |
| Fox | 4 | 25 | 0 | 3 | 0 | 12 |
| Muntjac deer | 13 | 41 | 1 | 2 | 8 | 5 |
| Fallow deer | 7 | 3 | 0 | 0 | 0 | 0 |
| Weasel | 1 | 0 | 0 | 0 | 0 | 0 |
| Badger | 18 | 1 | 3 | 1 | 17 | 100 |
| Hare | 2 | 0 | 0 | 0 | 0 | 0 |
| Rabbit | 1 | 40 | 0 | 3 | 0 | 7.5 |
| Squirrel | 0 | 14 | 0 | 0 | 0 | 0 |
| Stoat | 0 | 2 | 0 | 0 | 0 | 0 |
| Hedgehog | 0 | 4 | 0 | 0 | 0 | 0 |
| TOTAL | 46 | 130 | 4 | 9 | 9 | 7 |

# Lodge Pond

## Appendix 7.1.5

**Figure 7.1.5: Photograph showing Lodge Pond, sample sites L2 and L6, and the position of the rat colony (R).**

# Results of the 1992-94 survey Appendix 7.2

**Table 7.2.1: Summary of *Cryptosporidium* occurrence for the 1992-94 pre-contract survey of the Estate.**

| Animal type | Total specimens tested | % positive for *C. parvum* | % positive for *C. muris* |
|---|---|---|---|
| Home bred calves before weaning | 378 | 25% | 0 |
| Wild wood mice | 188 | 21% | 5% |
| Wild house mice | 218 | 18% | 11% |
| Bought in calves before weaning | 349 | 18% | 0 |
| Wild bank voles | 96 | 9% | 2% |
| Lambs | 376 | 9% | 0 |
| Horses | 837 | 6% | 0 |
| Ewes | 411 | 6% | 0 |
| Heifers | 132 | 5% | 0 |
| Foals | 39 | 3% | 0 |
| Dairy cows | 768 | 2% | 0 |
| Home bred calves after weaning | 267 | 2% | 0 |
| Bull beef | 712 | 1% | 0 |
| Bought in calves after weaning | 107 | 1% | 0 |
| Wild rabbits | 109 | 1% | 0 |
| TOTAL SPECIMENS TESTED | 4987 | | |

1) Some rodents groups contained individuals shedding *C. muris*, but few shed both species.
2) The data in the table represent **occurrence** not prevalence.

**Table 7.2.2 Number of oocysts detected in positive faecal specimens during the survey of 1992-94.** Low = ≤20 oocysts per 20 µl concentrate inspected, Medium = 20 - 50 oocysts and High = >50 oocysts. The percentage represented by each category is in brackets.

| Animal type | Low count | Moderate count | High count | Not counted |
|---|---|---|---|---|
| Home-bred calves before weaning | 38(40%) | 5(5%) | 51(55%) | 0 |
| Wild wood mice | 27(68%) | 6(15%) | 7(17%) | 0 |
| Wild house mice | 23(58%) | 4(10%) | 13(32%) | 0 |
| Bought-in calves before weaning | 25(40%) | 7(12%) | 30(48%) | 0 |
| Wild bank voles | 8(89%) | 0 | 0 | 1(11%) |
| Lambs | 17(49%) | 0 | 17(49%) | 1(2%) |
| Horses | 51(100%) | 0 | 0 | 0 |
| Ewes | 23(92%) | 0 | 0 | 2(8%) |
| Heifers | 5(83%) | 0 | 0 | 1(17%) |
| Foals | 1(100%) | 0 | 0 | 0 |
| Dairy cows | 7(58%) | 0 | 0 | 5(42%) |
| Home-bred calves post-weaning | 4(100%) | 0 | 0 | 0 |
| Bull beef | 7(70%) | 0 | 0 | 3(30%) |
| Bought-in calves post-weaning | 1(100%) | 0 | 0 | 0 |
| Wild rabbits | 1(100%) | 0 | 0 | 0 |

# Protocols Appendix 7.3

## 7.3.1 Rapid clean-up method for faecal samples containing vegetable matter e.g. horse specimens (D. Casemore, pers. comm.).

**Materials:** 60 ml pots, applicator sticks, 0.5 mm sieves, conical 15 ml tubes, 0.1% $Tween_{80}$/10% formalin.

1. Weigh 3 g well mixed faeces into a 60 ml pot. Add 20 ml 0.1% $Tween_{80}$/10% formalin and macerate with an applicator stick. Cap the pot and shake well. Leave to stand.
2. Shake well and strain through a 0.5 mm sieve into a glass beaker. Rinse the pot and material in the sieve with 0.1% $Tween_{80}$/10% formalin.
3. Discard the sieve and solid material. Divide the liquid between two conical 15 ml centrifuge tubes. Rinse the glass beaker into one of the tubes and level with 0.1% $Tween_{80}$/10% formalin. Cap and centrifuge at 1000 x g (MSE Centaur 2, 2400 rpm) for 10 min.
4. Aspirate the supernatant as far as the "cone" of the tube to waste. Resuspend and pool the two pellets in one tube, rinse the other tube into it with 0.1% $Tween_{80}$/10% formalin, up to 3 ml. Process by the FPC method or store at +4°C.

## 7.3.2 Modified formol-ether sedimentation (mFES) - adapted for use with faecal parasite concentrator (FPC) kit (adapted from Casemore *et al.*, 1985).

**Materials:** FPC kit (Evergreen Scientific, Los Angeles, USA), Pasteur pipettes, 0.1% $Tween_{80}$, 0.1% $Tween_{80}$/10% formalin, diethyl ether, 10% formalin, 10% hydrogen peroxide, (10% potassium hydroxide).

1. As soon as the specimen is received, assess whether it is suitable for dilution prior to pipetting a volume for testing or whether it should be weighed.
2. If it is to be pipetted, dilute the sample in an equal volume of 0.1% $Tween_{80}$ and emulsify. Store at +4°C.
   Just prior to use in the FPC protocol add an equal volume of 0.1% $Tween_{80}$ to the already diluted specimen (thus original faeces has been diluted ¼). By using 2 ml of diluted faeces in the test method, about 0.5 g of original faeces are tested).
3. Label two flat-bottomed tubes and one conical tube per sample. Work in batches of 12 i.e. 11 test specimens and a positive control.
4. In the first flat-bottomed tube, either:
   - mix 2 ml diluted faeces (see dilution instructions) with 1 ml 0.1% $Tween_{80}$/10% formalin, or
   - weigh 0.5 g solid faeces, emulsify with a little 0.1% $Tween_{80}$/10% formalin and make the volume up to 3 ml.
   - if the specimen is mucoidal and difficult to emulsify, add 0.5 ml 10% KOH, mix and then make the volume up to 3 ml.
5. In a fume cabinet, add 3 ml diethyl ether, cap and shake vigorously for 10 s, vortex with pulsing for 20 s, and shake vigorously for a further for 10 s.
6. Bring the volume up to 15 ml with 0.1% $Tween_{80}$/10% formalin, assemble the green sieve section with the second flat-bottomed tube and screw it on to the first tube. Shake vigorously for 10 s and invert the first tube over the second.
7. Unscrew the green sieve with the first tube, dismantle and place in 10% hydrogen peroxide solution. Top the remaining tube up to 15 ml with 0.1% $Tween_{80}$/10% formalin and cap the tube.
8. Centrifuge at 450 x g (MSE Centaur 2, 1600 rpm) briefly.
9. With a pastette carefully remove the column of liquid between the ether layer and the sediment to the conical tube.
10. Pour the ether into a waste jar for correct disposal and retain the pastette in the flat bottomed tube. Top the conical tube up to 15 ml with 0.1% $Tween_{80}$/10% formalin and cap.

11. Centrifuge at 1000 x g (MSE Centaur 2, 2400 rpm) for 10 min.
12. Aspirate the supernatant carefully into the discard tub using the pastette to just above the pellet. Vortex the pellet to resuspend it and add up to 2-3 ml 10% formalin.
13. Cap the tubes and store at +4°C.

## 7.3.3 Immunofluorescent antibody test (IFAT) for *Cryptosporidium.*

**Materials:** anti-*Cryptosporidium* monoclonal antibody, 4-well slides, Gilson pipette (P20), hot plate, humidified chamber, 37°C incubator, slide carrier, staining trough, cold acetone, (PBS 0.1M, pH 7.4), antifadent mountant, coverslips, epifluorescence microscope.

1. Label PTFE-coated 4-well slides (Shield Diagnostics, Dundee, UK) with the reference numbers of the specimens to be tested.
2. Vortex the specimen (usually prepared by FPC) and pipette 20 µl into the appropriate well on the slide. Spread over the whole well. If the specimen is very turbid, pipette 10 µl on to each of two wells.
3. Allow to dry on a hot-plate, then fix by placing 1 drop of cold acetone on each well. Allow to air dry.
4. Store the slide(s) at -20°C until ready to stain.
5. Bring the prepared slide(s) and the FITC-conjugated anti-*Cryptosporidium* monoclonal antibody (mAb) (TCS Ltd., Loughborough, UK) to room temperature prior to use.
6. Carry out staining procedure according to manufacturers instructions. Briefly:
7. Invert the antibody container gently a few times to mix.
8. Add 15 µl of mAb to each sample, spreading it out to cover the entire well.
9. Place on the wire tray over wet tissue in the incubation box and cover with aluminium foil, sealing it all round. Incubate at 37°C for 30 min.
10. Place the slide(s) in a slide carrier and lower into a glass staining trough filled with PBS (0.1M, pH 7.4), or use a Coplin jar. Agitate gently and repeat two or three times. Leave the slides to air dry.
11. Place 1 drop of antifadent mountant (Citifluor, Canterbury, UK) on each well of the slide(s) and place a coverslip over the top. Store the slides in the dark.
12. Inspect using epifluorescence microscopy (Nikon Optiphot 2) with a blue filter block at x200 and x400 magnification. Use x600 magnification for confirmation of suspect bodies (Appendix 7.3.10A/B).

## 7.3.4 Modified Ziehl-Neelsen (MZN) stain to confirm/measure *Cryptosporidium* oocysts.

**Materials:** microscope slides, PAP pen, Coplin jar/staining rack, strong carbol fuchsin, acid methanol, 0.4% malachite green.

### 7.3.4.1 If confirming/measuring a positive after FPC/IFAT:

1. Using the hydrophobic "PAP pen" (The Binding Site, Birmingham, UK), draw two 1 cm squares ("wells") on a microscope slide. Allow to dry.
2. Centrifuge the suspended FPC product at 1000 x g (MSE Centaur 2, 2400 rpm) for 10 min and use a pastette to aspirate the supernatant to just above the pellet.
3. Use the pastette to add 3 or 4 drops of the pelleted material to each of the wells created on the microscope slide. Allow to air dry.

### 7.3.4.2 If doing a quick screening for *Cryptosporidium* from a "raw" specimen:

Mix the specimen well and make a smear on a microscope slide, avoiding the edges and having some thick and some thin areas. Allow to air dry.

### 7.3.4.3 MZN stain procedure (Casemore, 1991).

(Proceed with the remaining stages for all specimens:)

4. Place the slide in a Coplin jar or staining rack and fix in methanol for 3 min.

5. Place the slide on a staining rack over the sink and flood with strong carbol fuchsin (BDH, Poole, UK) for 20 min. Rinse well in tap water.
6. Decolourise by washing with acid methanol (1% HCl : 99% methanol), agitating, and alternating with tap water until colour ceases to come out of the "smear".
7. Counterstain in 0.4% Malachite Green (BDH, Poole, UK) for 30-60 s. Rinse well in tap water and air dry.
8. Examine by bright field microscopy at x400 and x1000, using a calibrated eyepiece graticule to measure oocysts.

## 7.3.5 Water sample protocol.

**Materials:** 10 litre polyethylene barrels, thermometer, pH meter, metre rule, sieves, pipettes, vacuum pump, 1 litre centrifuge bottles, 50 ml centrifuge tubes, 1 and 3 ml disposable Pasteur pipettes, 4-well slides, coverslips, 1M $CaCl_2$, 1M $NaHCO_3$, 1M Na OH, 10% sulphamic acid, 0.1% $Tween_{80}$, saturated saline solution, mAb, 2 µg/ml DAPI in PBS solution, antifadent mountant, PBS (0.1M, pH 7.2).

### 7.3.5.1 Sample collection.

1. Take sample from furthest point downstream first, then work upwards to prevent contamination of sample points downstream. Collect sample carefully so as not to disturb sediment. Ensure barrel is full to capacity (>10 l).
2. Measure and record additional parameters such as temperature, pH, depth, flow rate (as appropriate) and comments.

### 7.3.5.2 Water Sample Preparation.

3. Remove sufficient water from the barrel for suspended solids test. Leave to stand for 30 min.
4. Pour through a sieve and funnel assembly into a clean barrel, leaving any large sediment behind to be discarded. Discard any water in excess of 10 l. Note the volume to be flocculated.

### 7.3.5.3 Oocyst concentration: Calcium carbonate flocculation (Vesey *et al.*, 1993).

5. Add 100 ml freshly made up 1M $CaCl_2$.
6. Add 100 ml 1M $NaHCO_3$. Mix.
7. Using 1M NaOH adjust the pH to 10 (±0.1). This takes about 130 ml 1M NaOH. Mix.
8. Leave to stand for at least 4 h, or overnight.
9. Aspirate supernatant leaving calcium carbonate floc residue. Pipettes should be labelled and retained for Step 13.
10. Add sufficient 10% sulphamic acid to dissolve the floc (about 350 ml). It will stop effervescing when fully dissolved.
11. Decant into a 1 l centrifuge bottle and rinse the barrel with 0.1% $Tween_{80}$. Add to the centrifuge bottle. Rinse the barrel again if there is room in the centrifuge bottle. Label each centrifuge bottle.
12. Balance the centrifuge bottles and spin at 2500 rpm (MSE Mistral 6L, swing out head) for 20 min.
13. Aspirate supernatant to just above the pellet. Resuspend pellet in remaining supernatant and pour into a 50 ml centrifuge tube. Rinse the first centrifuge tube with 0.1% $Tween_{80}$ into the second.
14. Centrifuge at 1000 g (2400 rpm, MSE Centaur 2) for 10 min.
15. Aspirate supernatant, resuspend the pellet in the small volume remaining, measure and note the final volume.
16. If final pellet <300 µl, remove 20% and proceed to Step 25.
17. If the pellet is >300 µl, divide it between 15 ml tubes so that no tube contains >1 ml, ready for saturated saline flotation.

### 7.3.5.4 Further purification - Saturated saline flotation (adapted from Weber *et al.*, 1992).

18. Add 11 ml saturated saline solution, mix well with the sample and carefully layer about 3 ml RO water on top of the salt.
19. Centrifuge at 1000 g (MSE Centaur 2, 2400 rpm) for 10 min.
20. Swirl the water on top of the salt with a disposable Pasteur pipette to set up a vortex and suspend any oocysts in this phase. Transfer to a 15 ml tube and resuspend in RO water.
21. Centrifuge at 1000 g (MSE Centaur 2, 2400 rpm) for 10 min.
22. Aspirate supernatant, pool replicate pellets from the same specimen in a 15 ml tube, resuspend in RO water and centrifuge at 1000 g (MSE Centaur 2, 2400 rpm) for 10 min.
23. Aspirate supernatant, transfer to an Eppendorf tube and rinse the 15 ml tube with sufficient RO water to fill the Eppendorf. Spin at 13 000 rpm for 1 min in a microfuge (MSE Microcentaur).
24. Aspirate supernatant and resuspend the pellet in the small volume remaining. Note this volume.

### 7.3.5.5 Staining and Microscopy.

25. Spot entire volume over about 4 wells of a PTFE slide, depending on turbidity.
26. Record the volume spotted out.
27. Stain with anti-*Cryptosporidium* mAb (TCS Ltd., Loughborough, UK) according to manufacturer's instructions.
28. Rinse mAb off using PBS (0.1M, pH 7.2), then post-stain with DAPI (Sigma Ltd., Poole, UK) by spotting 30 µl of 2 µg/ml DAPI in PBS (0.1M, pH 7.4) solution on to each well (Grimason *et al.*, 1994). Incubate at 37°C for 15 min. Rinse in PBS, air dry, mount in antifadent mountant and apply a cover slip.
29. Inspect using epifluorescence microscopy, using the blue filter to locate the oocysts and ultra-violet excitation to visualise the sporozoite nuclei (Appendix 7.3.10).
30. Count the total number of oocysts and the number of DAPI positive (i.e. 3 or 4 sporozoite nuclei visible) oocysts over all the wells for each specimen. Record the results.

## 7.3.6 Method for determining suspended solids (Anon., 1980).

**Materials:** Glass fibre filter pads (Whatman GF/C, 47 mm diameter, 1.2µm nominal pore size), 105°C oven, membrane filtration kit (Whatman), 250 ml side-arm flask, vacuum pump, desiccator, balance (accurate to 0.001 g).

1. Before leaving to collect water samples, label the very edge of the appropriate number of glass fibre filter pads using a pencil and place them in an oven at 105°C for 1-2 h to dry. Cool in a desiccator.
2. Once cool, weigh the pad accurately (to 0.001g) & record the weight (**A**).
3. Assemble the membrane filtration kit, ensuring that the filter pad is in the correct position.
4. Attach the assembly to a side-arm flask which itself is connected to a vacuum pump.
5. Measure the turbidity of the sample to be tested. Ideally, 250 ml of sample should be filtered. However, if the sample is very turbid, filter less but record the volume. Ensure that the sample to be tested is thoroughly mixed.
6. Switch vacuum pump on, then carefully pour test sample into funnel of membrane filtration kit.
7. When all of the sample has passed into the side-arm flask, wash funnel & filter pad a few times with RO water.
8. Remove the pad & place it in an oven at 105°C for 2 h, then place in a dessiccator to cool.
9. Weigh filter pad and again record the weight (**B**).
10. Return filter pad to the dessiccator for a further 30 min.
11. Weigh filter pad again to ensure it has dried to constant weight, +/-0.005g (**C**). If it has not dried to constant weight return to dessiccator for a further 30 min then re-weigh.

$$\text{Suspended solids} = C\text{-}A \times 4 \times 1000$$

$$= \text{S.S. in mg } l^{-1}$$

## 7.3.7 ECLOX™ protocol.

(ECLOX™ testing was kindly carried out by Karen Sawcer, Wolfson Applied Technology Laboratory, Queen Elizabeth Medical Centre, University of Birmingham).
**Materials:** Universal bottles, ECL kits (Aztec Environmental Control Ltd., Didcot, UK), reaction cuvette (Sarstedt 3.5 ml, 55 x 12 mm), Gilson pipettes.

### 7.3.7.1 Sample collection

1. Samples were collected by direct immersion of the Universal bottle, or by holding it beneath an outfall, as appropriate, at the time of collecting the 10 l grab samples.
2. Samples were refrigerated (usually for no more than 24 h) then mailed in protective wrapping to the laboratory for analysis. They were refrigerated upon arrival and were normally analysed with ECLOX™ within four days of collection.

### 7.3.7.2 ECLOX™ test procedure (Billings *et al.*, 1994).

3. Sequentially, 1000 µl sample* or (1000 µl deionised water for control), 100 µl Aztec reagent one and 100 µl reagent two were pipetted into the reaction cuvette (Sarstedt 3.5 ml, 55 x 12 mm) and initiated with 50 µl Aztec reagent three. (*Sample volumes may need to be adjusted - the sum of volumes must = A µl of sample + B µl deionised water = 1000µl. The sample volume (A µl) is important for the calculation of EU).
4. The cuvette was shaken by hand for two seconds and introduced into the luminometer (Bio-Orbit 1250, Bio-Orbit, Finland). The intensity and kinetics of light emission were recorded from the moment the glowing sample was moved into the measuring position of the luminometer and continued for 4 min.
5. Quality control and correct operation of instrumentation was maintained by running daily controls

### 7.3.7.3 ECLOX™ data analysis.

6. Subtraction of the computer generated integral values of sample light output between 0 and 4 minutes from that of the control allows calculation of percentage light inhibition:

$$\text{Inhibition} = \frac{\text{pure water integration value - sample integration value}}{\text{pure water integration value}} \quad (1)$$

7. ECLOX Units (EU) were calculated to take into account any variations in sample volume or dilution used during a study. Determined from the following formulae (multiplication of the value by ten rounds figures to whole numbers):

$$EU = \text{inhibition} \times \text{total working volume} / \text{sample volume (A)} \times \text{dilution} \times 10 \quad (2)$$

The total working volume = 1250 µl.

## 7.3.8 Slurry protocol.

**Materials:** large disposable cat litter trays, 60 ml pots, muslin, funnels, masking tape, swab sticks, 15 ml conical tubes, 4-well slides, coverslips, 0.1% $Tween_{80}$, saturated saline solution, saturated saline solution, mAb, 2 µg/ml DAPI in PBS solution, antifadent mountant, PBS (0.1M, pH 7.2).

### 7.3.8.1 Sample collection.

1. Place disposable cat litter trays on the ground in the path of the slurry spreading rain gun, and wait for them to partially fill.
2. Retrieve the trays and carefully transfer the slurry into 60 ml pots and label them.

### 7.3.8.2 Slurry filtering.

3. Stretch a piece of muslin across a large funnel and secure with masking tape. Place the funnel in a tripod above a clean 60 ml pot.
4. Pour slurry onto the muslin and agitate carefully with a swab stick. When most of the liquid has passed through the muslin wash any remaining solids with a small amount of 0.1% $Tween_{80}$.

### 7.3.8.3 Slurry processing.

N.B. Various methods to clean up and concentrate these samples were assessed and the following was chosen.

5. Put 10 ml of filtered slurry into each of two 15 ml conical tubes
6. Centrifuge at 1000 g (MSE Centaur 2, 2400 rpm) for 10 min.
7. Discard supernatant* and apply pellet for saturated saline flotation (see earlier)
8. Spot out final pellet (or a portion of it) onto a 4-well slide and stain using the standard IFAT procedure followed by DAPI post-staining, then view using epifluorescence microscopy.

* It was established during method development that the pellet retained the oocysts.

## 7.3.9 Purification of oocysts from faeces (adapted from Campbell *et al.*, 1992).

**Materials:** highly positive faeces, 50 ml tubes, 15 ml tubes, RO water, PBS, diethyl ether.

1. Make 5 g of highly positive faeces up to 50 ml with RO water.
2. Emulsify by vortexing vigorously.
3. Centrifuge at 900 g (MSE Centaur 2, 2200 rpm) for 5 min.
4. Discard supernatant.
5. Repeat washing procedure 3-5 times until supernatant is clear.
6. Resuspend pellet in 10 ml RO water.
7. Add equal volume diethyl ether & shake vigorously to mix.
8. Centrifuge at 900 g (MSE Centaur 2, 2200 rpm) for 5 min.
9. Discard supernatant layers
10. Resuspend sediment in a small volume of RO water and transfer to a clean 15 ml tube, rinse 50 ml tube with 2 ml RO water and add this to the 15 ml tube, make volume up to 15 ml with RO water.
11. Wash resuspended pellet twice in RO water to remove traces of diethyl ether.
12. Store oocysts in RO water..
13. (Occasionally further clean-up was required in which case sample was put through saturated saline flotation procedure - see Water methodology, Step 18).

## 7.3.10 Viability assay protocol (Campbell *et al.*, 1992).

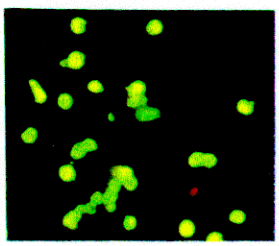
**Materials:** Oocyst suspension (at least $1 \times 10^6$ oocysts/ml), 1.5 ml Eppendorf tubes, 37°C water bath, microscope slides, coverslips, nail varnish, Hanks balanced salt solution (HBSS) pH 7.2, acidified HBSS (pH 2.75), DAPI working solution (2 mg/ml in methanol), PI working solution (1 mg/ml in 0.1M PBS, pH 7.2), anti-*Cryptosporidium* mAb, fluorescence microscope with blue, green & UV filter blocks.

1. Place 100 µl oocyst suspension in a 1.5 ml Eppendorf, top up with acidified HBSS (pH 2.75) and vortex vigorously to mix.
2. Incubate in a 37°C water bath for 60 min.
3. Spin in a microfuge (MSE Microcentaur) at 13 000 rpm for 20 s, aspirate supernatant to just above the pellet, then top up to line with HBSS (pH 7.2) & resuspend pellet by briefly vortexing. Repeat wash twice more, leaving the pellet resuspended in approx. 100 µl HBSS following the final wash.
4. Add 10 µl DAPI (Sigma Ltd., Poole, UK) working solution (2 mg/ml in absolute methanol) and 10 µl PI (Sigma Ltd., Poole, UK) working solution (1 mg/ml in 0.1M PBS, pH 7.2). Gently tap the bottom of the Eppendorf a few times to mix.

5. Incubate in a 37°C water bath (Grant Instruments (Cambridge) Ltd., UK) for 90 min.
6. Add 100 µl anti-*Cryptosporidium* mAb & tap bottom of Eppendorf a few times to mix.
7. Incubate in a 37°C water bath for 30 min.
8. Wash twice with HBSS pH 7.2 (as in Step 3), leaving pellet resuspended in approximately 100 µl after final wash.
9. Spot 10 µl out onto a microscope slide, carefully place a cover slip over the sample and seal the edges with nail varnish.
10. View under epifluorescence, using a microscope equipped with blue, UV and green filter blocks to visualise mAb, DAPI, and PI respectively (Figures 7.3.10A, B and C).
11. Quantify the proportions of oocysts which have included/excluded DAPI or PI by scoring them in a table as shown below. Ideally, at least 100 oocysts should be counted.

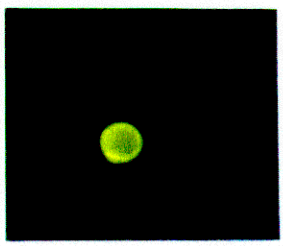
| DAPI+ PI- | DAPI+ PI+ | DAPI- PI- | DAPI- PI+ |
|---|---|---|---|
| Viable at assay | Non-viable | Potentially viable | Excysted |

## *Cryptosporidium* oocyst micrographs



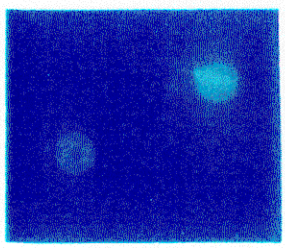
20 µm

**Figure 7.3.10A**



10 µm

**Figure 7.3.10B**



10 µm

**Figure 7.3.10C**

**Figure 7.3.10A:** *Cryptosporidium* oocysts stained using the IFAT technique, viewed at x 200

**Figure 7.3.10B:** *Cryptosporidium* oocyst stained using the IFAT technique, viewed at x 600

**Figure 7.3.10C:** *Cryptosporidium* oocysts stained with DAPI, viewed at x 600. Four sporozoite nuclei are clearly visible in the oocyst to the left, whilst the one on the right can be seen undergoing excystation

## 7.3.11 List of equipment/reagent suppliers.

**Antifadent mountant** - Citifluor Ltd., Canterbury, UK.

**Carbol fuchsin (strong)** - BDH Ltd., Poole, UK.

**Cryptocel IF antibody** - TCS Ltd., Loughborough, UK.

**DAPI (4',6-diamidino-2-phenylindole)** - Sigma Ltd., Poole, UK.

**Four-well PTFE slides** - Shield Diagnostics Ltd., Dundee, UK.

**FPC (faecal parasite concentrator) kit** - Evergreen Scientific, Los Angeles, USA.

**Hauptner tags (miniature)** - Brookwick Ward Ltd., Fife, UK.

**HBSS (Hanks balanced salt solution)** - Life Technologies Ltd., Paisley, UK.

**Longworth traps** - Penlon Ltd., Abingdon, UK.

**Malachite Green** - BDH Ltd., Poole, UK.

**MapMaker Pro** - Mapmaker Ltd., Mull of Kintyre, UK.

**Minitab** - Minitab Inc., Pennsylvania, USA.

**PAP pen** - The Binding Site, Birmingham Research Park, Birmingham, UK.

**PI (propidium iodide)** - Sigma Ltd., Poole, UK.

# Quality control and assurance procedures

# Appendix 7.4

## 7.4.1 Data recording

Information concerning field samples, whether animal or physical media, were noted initially on standard record sheets. All samples received a unique label. Progress of samples through the treatment methods, identification and enumeration tests was recorded on laboratory procedure sheets. The results of tests and full details about the sample and source (animal or physical media site) were later transferred to a Microsoft Access database. Examples of the database input forms are shown in Figure 7.4.1.

An important part of the quality assurance for this project involved quantifying losses of oocysts during processing of faeces. This was achieved via an elaborate calibration experiment referred to in section 4.5.3. The design of the exercise is described in the flow diagram of Figure 7.4.2.

## 7.4.2 Faecal quality control results

### 7.4.2.1 Internal faecal quality control results

Every batch of faecal samples passing through the test procedure included a standard positive control faeces. More elaborate positive controls were also employed, consisting of a made-up faecal base, similar to that used for the calibration experiment, seeded with known concentrations of oocysts, to produce either a high or low oocyst count. Each batch then included either a high or low positive control, but persons analysing the final slides did not know which. Table 7.4.2.1A shows the results of the high/low controls, and Table 7.4.2.1B shows a summary of all faecal positive controls. A total of 10% of all faeces tested were QC/QA samples.

### 7.4.2.2 External faecal quality control results

Material from 15 faecal samples (twelve IFAT slides and three vials of processed faeces) were sent for external analysis. The internal and external assessments are shown in Table 7.4.2.2. The numbers of oocysts observed on slides were very similar on 11/15 occasions. Mismatches were discussed and agreed to be due to fading of the fluorescence signal or the inherent problems of finding of oocysts on low scoring samples. The faecal concentrate results were in agreement.

## 7.4.3 Physical media quality control results

### 7.4.3.1 Internal physical media quality control results

A total of 55 negative controls were employed during the seventeen months of testing for physical media. These controls involved putting 10 litre samples of tap water through the same procedures using the same barrels and other equipment that was employed for the field samples of physical media. In the first month, one such control gave a positive result (2 oocysts seen). Following this the washing procedures for barrels and other equipment were made considerably more stringent; all negative controls since then have been negative on testing for oocysts. The summary of these tests is in Table 7.4.3.1.

### 7.4.3.2 External physical media quality control results

IFAT slides prepared and tested by ourselves were periodically submitted for external analysis. With rare exceptions all samples of physical media result in low scoring slides (<10 oocysts). A mixture of positive and negative slides were sent. The results from fourteen such slides are shown in Table 7.4.3.2. The two mismatches were for slides with three and one oocyst respectively scored negatively by the external assessor. In both cases it was agreed that slight fading of fluorescence during the interval of several days between the internal and external counts involving so few oocysts would be very likely to result in negative counts.

**Table 7.4.2.1A: Oocyst counts for positive control "faeces" processed and counted during routine testing of faecal samples for *Cryptosporidium* oocysts for internal QC.**

| qa code >> | qc a (high) | qc b (low) | qc c (high) | qc d (low) | qc e (high) | qc f (low) |
|---|---|---|---|---|---|---|
| | 206 | 20 | 168 | 7 | 126 | 11 |
| | 126 | 7 | 164 | 9 | 77 | 8 |
| | 162 | 21 | 158 | 32 | 123 | 21 |
| | 198 | 17 | 147 | 7 | 54 | 4 |
| | 225 | 21 | 83 | 17 | 163 | 10 |
| | | 9 | 162 | 12 | 143 | 5 |
| | | | 150 | 20 | 105 | 35 |
| | | | 76 | 5 | 105 | 2 |
| | | | 209 | 8 | 135 | 3 |
| | | | 151 | 14 | 85 | 15 |
| | | | 245 | 15 | 174 | 20 |
| | | | 140 | 3 | 178 | 20 |
| | | | 73 | 17 | 264 | 11 |
| | | | 81 | 4 | 76 | 7 |
| | | | 35 | 7 | 171 | 5 |
| | | | 104 | 6 | 180 | 3 |
| | | | 138 | 5 | 52 | 7 |
| | | | 110 | 6 | 175 | 4 |
| | | | 218 | 15 | 135 | 15 |
| | | | 81 | | 66 | 12 |
| | | | 84 | | 148 | 8 |
| | | | | | 30 | 8 |
| | | | | | 314 | 7 |
| | | | | | 279 | 14 |
| | | | | | 134 | 11 |
| | | | | | 116 | 7 |
| | | | | | 54 | 3 |
| | | | | | 73 | 10 |
| | | | | | 168 | 13 |
| | | | | | 76 | 11 |
| | | | | | 76 | 7 |
| | | | | | 95 | 11 |
| | | | | | 108 | 12 |
| | | | | | 112 | 11 |
| | | | | | | 7 |
| | | | | | | 9 |
| | | | | | | 6 |
| **n** | **5** | **6** | **21** | **19** | **34** | **37** |
| **mean** | **183** | **16** | **132** | **11** | **129** | **10** |
| **range** | **126-225** | **7-21** | **35-245** | **3-32** | **30-314** | **2-35** |
| **sd** | **39.39** | **6.27** | **54** | **7.2** | **65** | **6.3** |
| **95% confidence** | **183 ± 34** | **16 ± 5** | **132 ± 23** | **11 ± 3** | **129 ± 22** | **10 ± 2** |

**Table 7.4.2.1B: Summary of all internal faecal QC/QA results**

| | |
|---|---|
| **Total no. basic faecal positive control samples** | **212** |
| **Total no. high/low faecal positive control samples** | **122** |
| **Total no. external faecal control samples** | **15** |
| **Total no. faecal samples tested** | **3374** |
| **Total no. faecal control samples (%)** | **349 (10)** |

**Table 7.4.2.2: External faecal QC\QA results**

| Sample ref. | Sample type | CRG result | External result |
|---|---|---|---|
| qc e | IFAT slide | positive | positive |
| 9307 | IFAT slide | positive | negative |
| 9308 | IFAT slide | negative | positive |
| 9309 | IFAT slide | negative | negative |
| 9310 | IFAT slide | positive | positive |
| 9311 | IFAT slide | positive | positive |
| 9312 | IFAT slide | positive | positive |
| 9313 | IFAT slide | negative | negative |
| 9314 | IFAT slide | negative | negative |
| 9315 | IFAT slide | positive | negative |
| 9316 | IFAT slide | positive | negative |
| 9317 | IFAT slide | positive | positive |
| qc 1 | faecal concentrate | low positive | low positive |
| qc 2 | faecal concentrate | high positive | high positive |
| qc 3 | faecal concentrate | high positive | high positive |

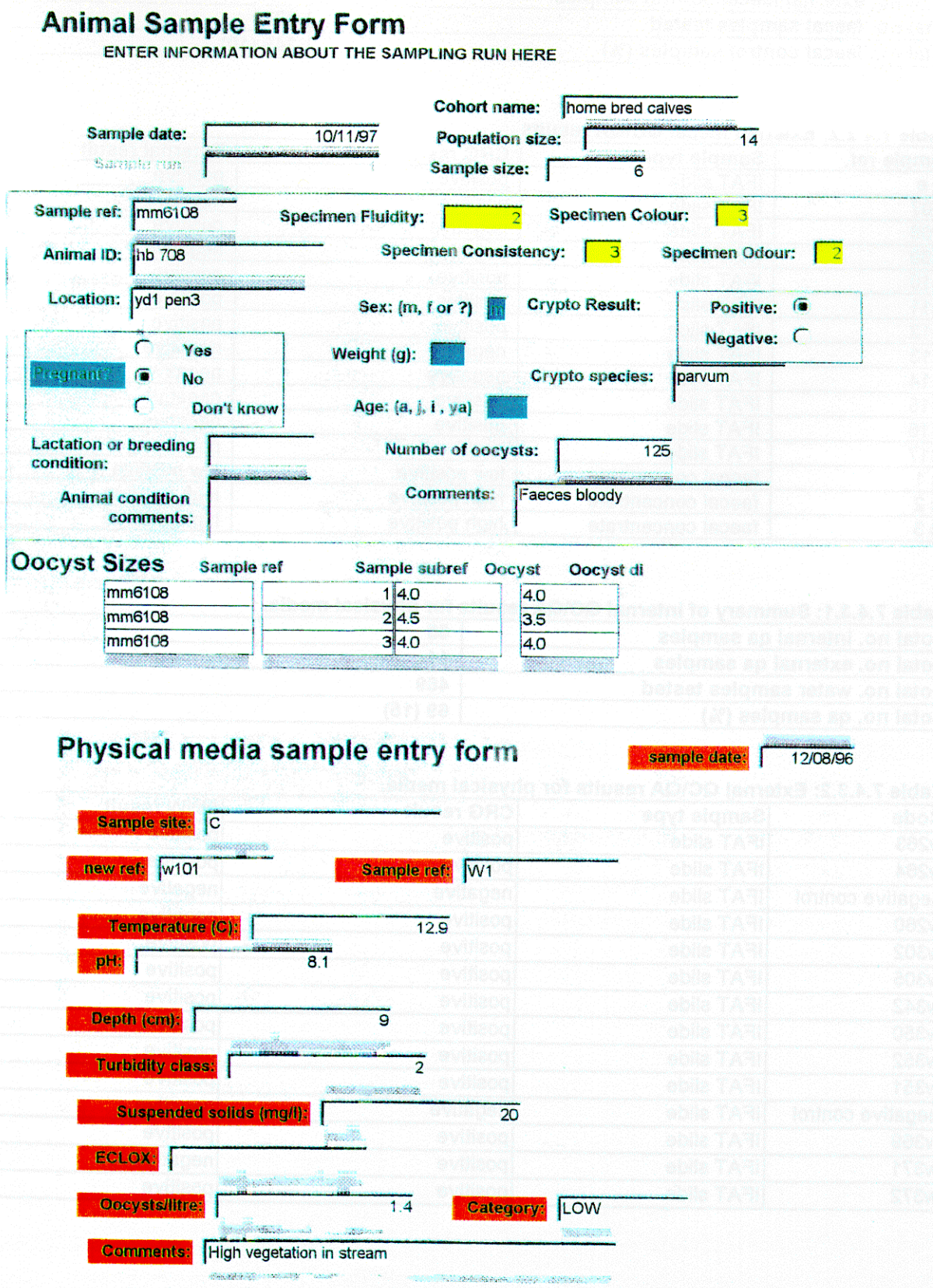
**Table 7.4.3.1: Summary of internal QC\QA results for physical media.**

| | |
|---|---|
| **Total no. internal qa samples** | **55** |
| **Total no. external qa samples** | **14** |
| **Total no. water samples tested** | **469** |
| **Total no. qa samples (%)** | **69 (15)** |

**Table 7.4.3.2: External QC\QA results for physical media.**

| Code | Sample type | CRG result | SWW result |
|---|---|---|---|
| w263 | IFAT slide | positive | positive |
| w264 | IFAT slide | positive | positive |
| negative control | IFAT slide | negative | negative |
| w260 | IFAT slide | positive | negative |
| w302 | IFAT slide | positive | positive |
| w305 | IFAT slide | positive | positive |
| w342 | IFAT slide | positive | positive |
| w350 | IFAT slide | positive | positive |
| w352 | IFAT slide | positive | positive |
| w351 | IFAT slide | positive | positive |
| negative control | IFAT slide | negative | negative |
| w369 | IFAT slide | positive | positive |
| w371 | IFAT slide | positive | negative |
| w372 | IFAT slide | positive | positive |

**Figure 7.4.1: Examples of database input forms for faecal and physical media samples.**



## Animal Sample Entry Form

ENTER INFORMATION ABOUT THE SAMPLING RUN HERE

Sample date: 10/11/97
Cohort name: home bred calves
Population size: 14
Sample size: 6

Sample ref: mm6108
Specimen Fluidity: 2
Specimen Colour: 3
Animal ID: hb 708
Specimen Consistency: 3
Specimen Odour: 2
Location: yd1 pen3
Sex: (m, f or ?)
Crypto Result: Positive / Negative
Pregnant? Yes / No / Don't know
Weight (g):
Crypto species: parvum
Age: (a, j, i , ya)
Lactation or breeding condition:
Number of oocysts: 125
Animal condition comments:
Comments: Faeces bloody

**Oocyst Sizes**

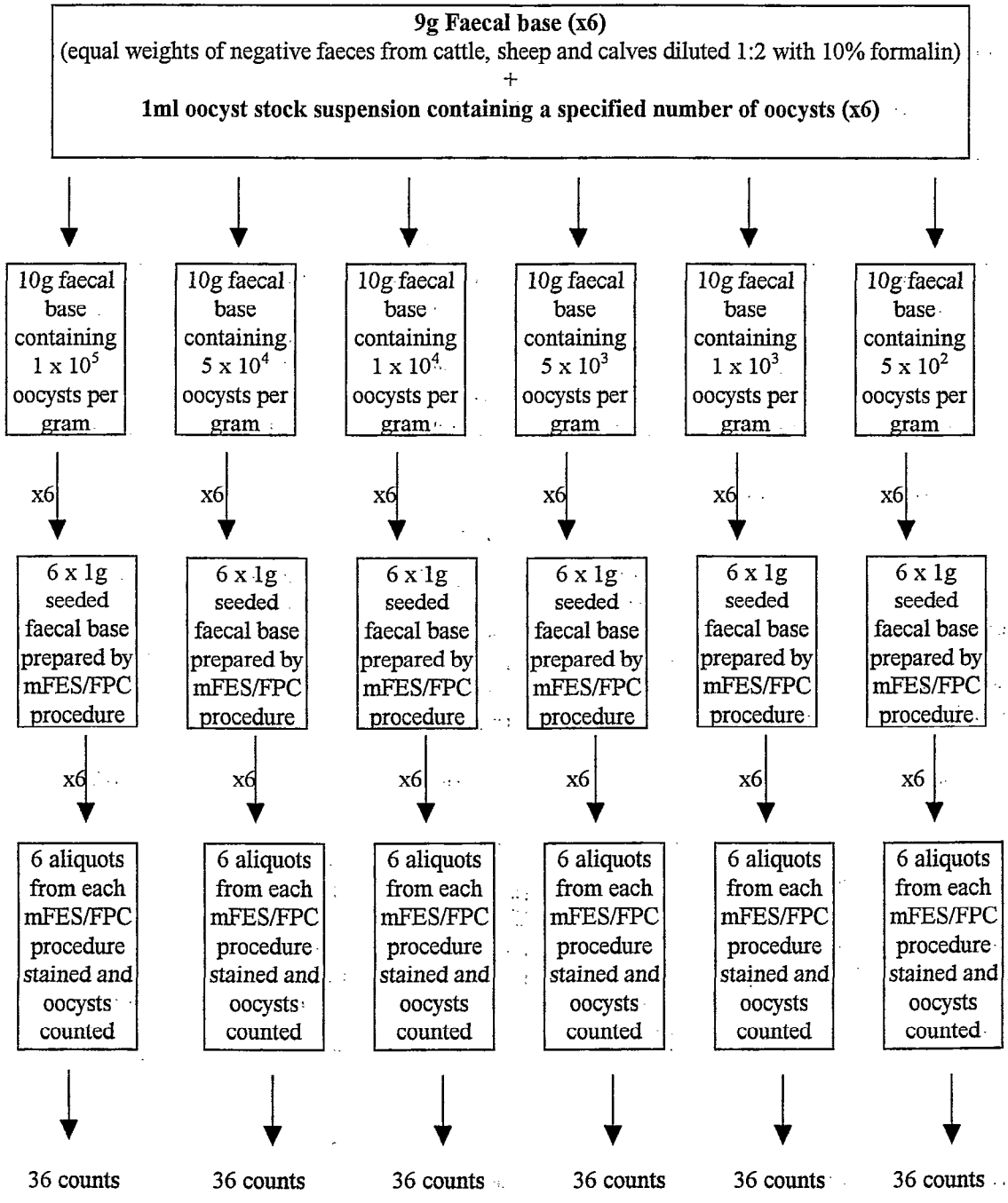
| Sample ref | Sample subref | Oocyst | Oocyst di |
|---|---|---|---|
| mm6108 | 1 | 4.0 | 4.0 |
| mm6108 | 2 | 4.5 | 3.5 |
| mm6108 | 3 | 4.0 | 4.0 |

## Physical media sample entry form

sample date: 12/08/96

Sample site: C
new ref: w101
Sample ref: W1
Temperature (C): 12.9
pH: 8.1
Depth (cm): 9
Turbidity class: 2
Suspended solids (mg/l): 20
ECLOX:
Oocysts/litre: 1.4
Category: LOW
Comments: High vegetation in stream

**Figure 7.4.2: Flow diagram showing the design of the calibration experiment used to assess oocyst recovery, detection limits and reliability of the mFES-FPC procedure.**



# Boxplots showing non-Normal distribution of oocysts in water

# Appendix 7.5

Boxplots are useful for illustrating the skewness of data, such as the non-Normal distribution of *Cryptrosporidium* oocysts in surface water samples. An annotated boxplot, of the type which will be produced by a Normal distribution, is shown in Figure 7.5A. Boxplots of the results obtained in the present survey are shown in Figures 7.5B and C. For the majority of sites the results are clustered below one oocyst per litre, but with numerous outlying high values (represented by asterisks).

**Figure 7.5A: An annotated boxplot.**

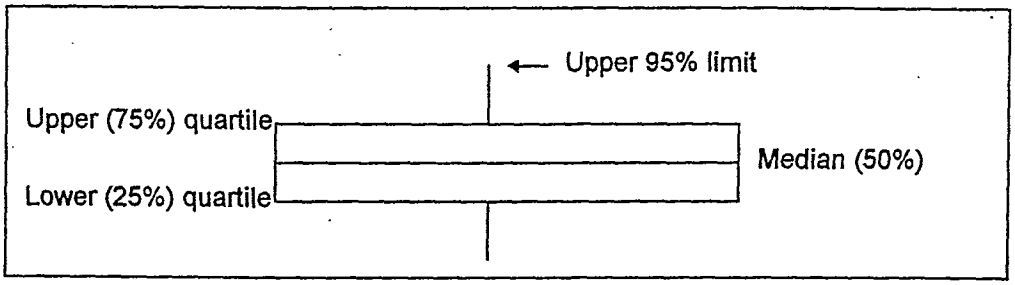



**Figure 7.5B: Boxplots for sites C, F, G and H - outlying values of >10 oocysts/l have been excluded.**

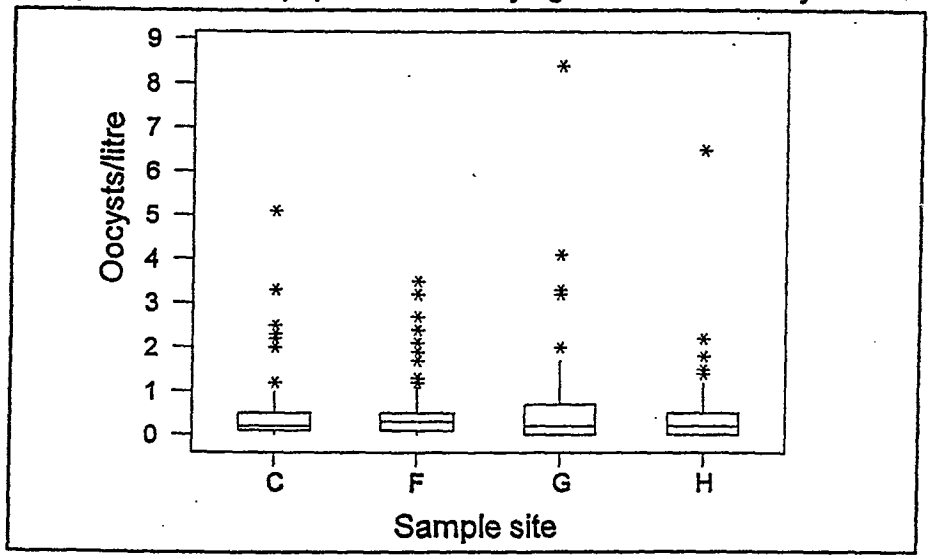
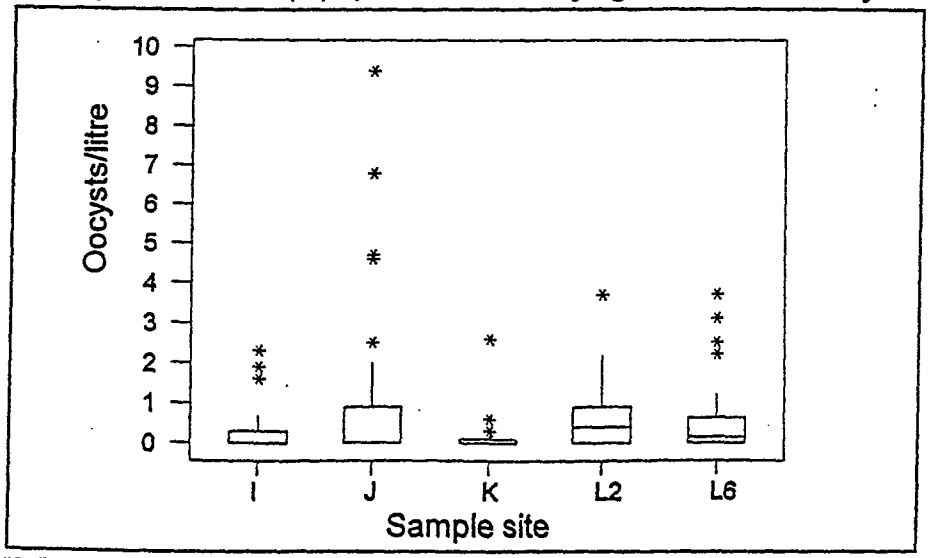



**Figure 7.5B: Boxplots for sites I, J, K, L2 and L6 - outlying values of >10 oocysts/l have been excluded.**



# Glossary of terms and abbreviations used in this report: Appendix 7.6

## 7.6.1 Glossary of terms used

**Antibody:** A protein produced by the body's immune system in response to an infection

**Antigen:** A molecule which may provoke the production of an antibody or other immune response

**Cryptosporidium:** The general descriptive term for the parasite (Greek for hidden spores)

***Cryptosporidium parvum (C. parvum):*** The species of *Cryptosporidium* known to cause disease in man

**Cryptosporidiosis:** The illness produced by infection with *Cryptosporidium*

**Estate:** Warwickshire College Estate

**Excystation:** The process by which sporozoites are released from *Cryptosporidium* oocysts

**Faecal coliforms:** A group of indicator organisms, commonly used to indicate faecal contamination

**Flocculation:** A technique for removing particles from water by the formation of a precipitate which then undergoes sedimentation

**Headwater:** Any watercourse which is within 2 km of its source

**Host:** An animal (including man) in which micro-organisms such as *Cryptosporidium* can grow or multiply

**Immunofluorescent antibody test:** A laboratory technique employing fluorescent dyes linked to specific antibodies

***In vitro*:** Referring to investigations using tissues or cells removed from the host

**Micro-organism:** An organism of microscopic size, such as a bacterium, protozoan or virus.

**Monoclonal antibody:** An antibody produced by *in vitro* techniques, often with high specificity for target antigen

**Occurrence:** The number of positive samples divided by the number of samples tested(expressed as a percentage)

**Oocyst:** The environmentally resistant transmissible form of *Cryptosporidium* excreted in the faeces of an infected host

**Parasite:** An organism that lives on or in another organism (the host) sometimes to the detriment of the host from which it obtains its nutrition

**Pathogen:** A micro-organism (protozoan, bacterium, or virus) capable of causing disease

**Prevalence:** The number of individuals of a host species infected with a particular parasite species, divided by the number of hosts examined (expressed as a percentage) (Margolis *et al.*, 1982; Thrusfield, 1995)

**Protozoan:** A single-celled micro-organism, which may be free-living or parasitic. *Cryptosporidium* is a parasitic protozoan

**Reverse osmosis (RO) water:** Made by a process which uses high pressure to force water through a membrane leaving behind solutes

**Slurry:** A semi-liquid composed mainly of cattle faeces and urine

**Sporozoite:** The motile stage of *Cryptosporidium* which is released from an oocyst following excystation

**Suspended solids:** Material suspended in water with a particle size larger than dissolved molecules or ions

**Turbidity:** This is an expression of the optical property of a liquid that causes light to be scattered and absorbed rather than transmitted undeflected through the sample

**Zoonosis:** A disease which can be transmitted naturally between animals and man

## 7.6.2 List of abbreviations used

| | |
|---|---|
| **BI** | bought-in (calf) |
| **BOD** | biochemical oxygen demand (mg/l) |
| **COD** | chemical oxygen demand (mg/l) |
| **cfu** | colony forming unit |
| **CRG** | Cryptosporidium Research Group (Coventry University) |
| **CS** | cattle slurry |
| **CSL** | Central Science Laboratory |
| **d** | days |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **DOB** | date of birth |
| **EA** | Environment Agency (formerly NRA - National Rivers Authority) |
| **ECL** | enhanced chemiluminescence |
| **ECLOX™** | enhanced chemiluminescent oxidation reaction |
| **FITC** | fluoroscein isothiocyanate |
| **FPC** | faecal parasite concentrator |
| **FYM** | farmyard manure |
| **h** | hour |
| **HB** | home-bred (calf) |
| **HBSS** | Hanks balanced salt solution |
| **HRI** | Horticulture Research International |
| **ID no.** | unique identification number |
| **IF** | immunofluorescent |
| **IFAT** | immunofluorescent antibody test |
| **mAb** | monoclonal antibody |
| **MAFF** | Ministry of Agriculture, Fisheries and Food |
| **mFES** | modified formol-ether sedimentation |
| **min** | minute |
| **MNA** | minimum number alive |
| **MZN** | modified Ziehl-Neelsen |
| **n** | negative |
| **na** | not applicable |
| **nd** | not done |
| **ns** | not sampled |
| **nt** | not tested |
| **PBS** | phosphate buffered saline |
| **PI** | propidium iodide |
| **PTFE** | polytetrafluoroethene |
| **s** | second |
| **SCA** | Standing Committee of Analysts |

# 8. REFERENCES


Anon. (1989). "Isolation and identification of *Giardia* cysts, *Cryptosporidium* oocysts and free living pathogenic amoebae in water etc.," 1/Ed. HMSO, London.

Anon. (1980). "Suspended, settleable and total dissolved solids in waters and effluents," HMSO.

Billings, C., Lane, M., Watson, A., Whitehead, T. P., and Thorpe, G. (1994). A rapid and simple chemiluminescent assay for water quality monitoring. *Analysis* **22**, 27-30.

Bukhari, Z., Smith, H. V., Humphreys, S. W., Kemp, J., and Wright, S. E. (1995). Comparison of excretion and viability patterns in experimentally infected animals: Potential for release of viable *C. parvum* oocysts into the environment. *In* "Protozoan parasites and water" (W. B. Betts, D. Casemore, C. Fricker, H. Smith and J. Watkins, eds.), pp. 188-191. Royal Society of Chemistry Special Publication No. 168, Cambridge.

Bukhari, Z., and Smith, H. V. (1997). *Cryptosporidium parvum*: oocyst excretion and viability patterns in experimentally infected lambs. *Epidemiology and Infection* **119**, 105-108.

Campbell, A. T., Robertson, L. J., and Smith, H. V. (1992). Viability of *Cryptosporidium parvum* oocysts: Correlation of *in vitro* excystation with inclusion or exclusion of fluorogenic vital dyes. *Applied and Environmental Microbiology* **58**, 3488 - 3493.

Campbell, A. T., Robertson, L. J., Smith, H. V., and Girdwood, R. W. A. (1994). Viability of *Cryptosporidium parvum* oocysts concentrated by calcium carbonate flocculation. *Journal of Applied Bacteriology* **76**, 638 - 639.

Cannon, R. M., and Rowe, R. T. (1982). Livestock surveys: A field manual for veterinarians. *Australian Bureau of Animal Health.*

Casemore, D. P., Armstrong, M., and Sands, R. L. (1985). Laboratory diagnosis of cryptosporidosis. *Journal of Clinical Pathology* **38**, 1337-1341.

Casemore, D. P. (1991). Laboratory methods for diagnosing cryptosporidiosis. *Journal of Clinical Pathology* **44**, 445 - 451.

Chalmers, R. M., Sturdee, A. P., Bull, S. A., and Miller, A. M. (1995). Rodent reservoirs of *Cryptosporidium*. *In* "Protozoan parasites and water" (W. B. Betts, D. Casemore, C. Fricker, H. Smith and J. Watkins, eds.), pp. 63 - 66. The Royal Society of Chemistry Special Publication No. 168, Cambridge.

Chalmers, R. M. (1996). The distribution of *Cryptosporidium* in livestock and wild animal populations on a Warwickshire farm. PhD thesis, Coventry University.

Chalmers, R. M., Sturdee, A. P., Bull, S. A., Miller, A., and Wright, S. E. (1997). The prevalence of *Cryptosporidium parvum* and *C. muris* in *Mus domesticus, Apodemus sylvaticus* and *Clethrionomys glareolus* in an agricultural system. *Parasitology Research* **83**, 478-482.

Fayer, R., Speer, C.A., and Dubey, J.P. (1990). General biology of *Cryptosporidium*. *In* "Cryptosporidiosis of Man and Animals" (J. P. Dubey, C. A. Speer and R. Fayer, eds.), pp. 2 - 29. CRC Press, Boston.

Grimason, A. M., Smith, H. V., Parker, J. F. W., Bukhari, Z., Campbell, A. T., and Robertson, L. J. (1994). Application of DAPI and immunofluorescence for enhanced identification of *Cryptosporidium* spp oocysts in water samples. *Water Research* **28**, 733 - 736.

Gurnell, J., and Flowerdew, J. R. (1994). "Live Trapping Small Mammals - A Practical Guide," 3/Ed. The Mammal Society, London.

Johnson, E., Atwill, E. R., Filkins, M. E., and Kalush, J. (1997). The prevalence of shedding of *Cryptosporidium* and *Giardia spp.* based on a single faecal sample collection from each of 91 horses used for backcountry recreation. *Journal of Veterinary Diagnostic Investigation* **9**, 56 - 60.

Kemp, J. S., Wright, S. E., and Bukhari, Z. (1995). On farm detection of *Cryptosporidium parvum* in cattle, calves and environmental samples. *In* "Protozoan parasites and water" (W. B. Betts, D. Casemore, C. Fricker, H. Smith and J. Watkins, eds.), pp. 154-157. Royal Society of Chemistry Special Publication No. 168, Cambridge.

Krebs, C. J. (1966). Demographic changes in fluctuating populations of *Microtus californicus. Ecological Monographs* **36**, 239-273.

Margolis, L., Esch, G. W., Holmes, J. C., Kuris, A. M., and Schad, G. A. (1982). The use of ecological terms in parasitology (report of an *ad hoc* committe of the American Society of Parasitologistis. *Journal of Parasitology* **68**, 131 - 133.

Meehan, A. P. (1984). "Rats and mice: Their biology and control," Rentokil Ltd., East Grinstead.

Olson, M. E., Thorlakson, C. L., Deselliers, L., Morck, D. W., and McAllister, T. A. (1997). *Giardia* and *Cryptosporidium* in Canadian farm animals. *Veterinary Parisitology* **68**, 375-381.

Ongerth, J. E., and Stibbs, H. H. (1987). Identification of *Cryptosporidium* oocysts in river water. *Applied and Environmental Microbiology* **53**, 672-676.

Poulton, M., Colbourne, J., and Dennis, P. J. (1991). Thames water's experiences with *Cryptosporidium*. *Water Science and Technology* **24**, 21 - 26.

Quilez, J., Sanchez-Acedo, C., Cacho, E. d., Clavel, A., and Causape, A. C. (1996). Prevalence of *Cryptosporidium* and *Giardia* infections in cattle in Aragon (northeastern Spain). *Veterinary Parisitology* **66**, 139 - 146.

Robertson, L. J., Cambell, A. T., and Smith, H. V. (1992). Survival of *Cryptosporidium parvum* oocysts under various experimental pressures. *Applied and Environmental Microbiology* **58**, 3494 - 3500.

Ruxton, G. D. (1995). Mathematical modelling of ammonia volatilization from slurry stores and its effect on *Cryptosporidium* oocyst viability. *Journal of Agricultural Science* **124**, 55-60.

Sawcer, K. E., Bodley-Tickell, A. T., Kitchen, S. E., Sturdee, A. P., and Thorpe, G. H. G. (Submitted). The application of enhanced chemiluminescence to water quality monitoring in an agricultural environment.

Smith, H. V., and Fricker, C. R. (1997). How effective is the current method for the analysis for waterborne *Cryptosporidium*. *In* "2nd UK Symposium on Health-Related Water Microbiology" (R. Morris and A. Gammie, eds.), pp. 170-184, University of Warwick.

Smith, H. V., and Hayes, C. R. (1997). The status of UK methods for the detection of *Cryptosporidium* spp oocysts and *Giardia* spp cysts in water concentrates. *Water Science and Technology* **35**, 369-376.

Smith, H. V., and Rose, J. B. (1998). Waterborne cryptosporidiosis: Current status. *Parasitology Today* **14**, 14-22.

Thrusfield, M. (1995). "Veterinary Epidemiology," 2nd/Ed. Blackwell Scientific Ltd., London.

Vesey, G., Slade, J. S., Byrne, M., Shepherd, K., and Fricker, C. R. (1993). A new method for the concentration of *Cryptosporidium* oocysts from water. *Letters in Applied Microbiology* **17**, 276 - 279.

Weber, R., Bryan, R. T., and Juranek, D. D. (1992). Improved stool concentration procedure for detection of *Cryptosporidium* oocysts in fecal specimens. *Journal of Clinical Microbiology* **30**, 2869 - 2873.

Webster, J. P., and MacDonald, D. W. (1995). Cryptosporidiosis reservoir in wild brown rats (*Rattus norvegicus*) in the UK. *Epidemiology and Infection* **115**, 207 - 209.

Xiao, L., Herd, R. P., and Rings, D. M. (1993). Diagnosis of *Cryptosporidium* on a sheep farm with neonatal diarrhea by immunofluorescence assays. *Veterinary Parasitology* **47**, 17 - 23.

Xiao, L., and Herd, R. P. (1994). Epidemiology of equine *Cryptosporidium* and *Giardia* infections. *Equine Veterinary Journal* **26**, 14 - 17.