CRYPTOSPORIDIUM SP. OOCYSTS IN THE AQUATIC ENVIRONMENT: OCCURRENCE, REMOVAL AND DESTRUCTION

By

JEREMY FRANÇOIS WILLIAM PARKER

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Department of Zoology,

University of Glasgow,

Glasgow.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DECLARATION:

I declare that this thesis describes work carried out by myself unless otherwise cited or acknowledged. It is of my own composition and has not been presented, in whole or part, for any other degree.

Jeremy F. W. Parker

June 1993.

ABSTRACT

In the past 10 years the protozoan parasites *Cryptosporidium* and *Giardia* have been recognised as important gastrointestinal pathogens of man. Both parasites have been responsible for numerous outbreaks of waterborne diarrhoeal disease in humans. Methods developed for the recovery and detection of these organisms in water and water-related samples involve the filtration of large volumes of water (100 - 1000 L), the elution and concentration of the transmissive stage and their detection using immunofluorescent antibody techniques. During the course of this study *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts were detected in surface water at ranges of 0.006 - 15.6 oocysts/L and 0.009 - 2.1 cysts/L respectively, in treated water at ranges of 0.008 - 1.36 oocyst/L and 0.007 - 0.34 cyst/L respectively. In raw sewage, the concentrations ranged from 2.5 - 75 oocysts/L and 242.5 - 792.5 cysts/L and, in treated sewage effluent, from 0.024 - 26.5 oocysts/L and 0.095 - 361.7 cysts/L. Removal of these organisms by water treatment processes such as slow sand filtration, rapid sand filtration and microstraining ranged from 62 % to 92 % and removal by sewage treatment processes ranged from 79.6 % to 99.3 %.

Cryptosporidium sp. oocysts were detected in water at the abstraction point of an upland water supply and in potable water in the distribution network. Information available on human cryptosporidiosis in the communities served by this water supply indicated that the small numbers of oocysts detected in both raw and final water could not be correlated with any identifiable outbreak of waterborne disease. The majority of oocysts detected at the abstraction point were non-viable. Oocysts were detected in human sewage effluent and bovine faecal specimens, implicating both as a source of contamination of tributaries to the loch from which water was abstracted.

Immunomagnetic separation techniques were used to improve the recovery of oocysts during sample processing and to recover oocysts in order to assess their viability. Recoveries of 33 % to 66% were achieved from environmental samples.

Ozone was used successfully to inactivate *Cryptosporidium parvum* oocysts, with 100 % inactivation achieved at ozone concentrations of 3 and 5 mg/L for between 2 and 10 min at pH 5, 7 and 9 at temperatures of 5°C and 20°C. Shaking oocysts with sand for time periods between 5 and 90 min inactivated 50 % to 99.7 % of the oocysts. Exposing oocysts to 1 mg/L chlorine for 5 min after 5 min treatment with sand gave a further 15 % inactivation.

Oocysts are many times more resistant to disinfectants, including ozone, than other pathogens encountered in water treatment. Ozone produces oxidants such as superoxide ions during its decomposition. The antioxidant enzyme superoxide dismutase was detected in freeze-thawed fractions of oocysts at 5.44 to 9.27 U/mg protein. This may provide the parasite with a mechanism which can neutralise immune and non-immune oxidant mediated attack both during its development within the host and its exposure to disinfectants in water treatment.

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ABBREVIATIONS

AP	-	auramine phenol
BSA	-	bovine serum albumin
CDC	-	Centres for Disease Control
DAPI	-	4',6-diamidino-2-phenylindole
DIC	-	Differential Interference Microscopy
DPD	-	diethyl-*p*-pheylene diamine
EDTA	-	ethylene-diamine-tetra-acetic acid
ELISA	-	enzyme linked immunosorbent assay
FITC	-	fluorescein isothiocyanate
HBSS	-	Hanks' Balanced Salt Solution
HMEM	-	Hanks' Minimal Essential Medium
IFA	-	immunofluorescent assay
MAb	-	monoclonal antibody
MPC	-	magnetic particle concentrator
PBS	-	phosphate buffered saline
PHLS	-	Public Health Laboratory Service
PI	-	propidium iodide
RO	-	reverse osmosis
SDS	-	sodium dodecyl sulphate
SOD	-	superoxide dismutase
SPDL	-	Scottish Parasite Diagnostic Laboratory
TEM	-	trasmission electron microscopy
TRITC	-	tetramethylrhodamine B isothiocyanate
ZN	-	Ziehl Nielsen

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INTRODUCTION

The parasitologist Ernest Edward Tyzzer, in 1907, described a protozoan parasite which he frequently found in the gastric glands of laboratory mice (Tyzzer, 1907). He recognised stages of asexual and sexual reproduction, followed by oocyst production. Tyzzer also described faecal transmission of the 'spores', which contaminated food. The name he suggested was *Cryptosporidium muris*. Tyzzer credited J. Jackson Clarke with the first published description resembling *Cryptosporidium*. However, neither the dimensions of the organisms described, or the figures presented by Clarke support this acreditation by Tyzzer.

Tyzzer's original description of the life cycle of *C. muris* was very accurate. More recent studies by investigators using electron microscopy have confirmed this. Tyzzer did, however, interepret the location of developmental stages as extracellular.

In 1912, Tyzzer identified a second new species, which he called *Cryptosporidium parvum* (Tyzzer, 1912). He distinguished this as a new separate from *C. muris* by experimentally infecting mice and showing that *C. parvum* was smaller and developed only in the small intestine. He concluded that *C. parvum* was not strictly extracellular, but could not be considered intracellular, because it appeared to protrude from the host cell. The term "intracellular-extracytoplasmic" coined by Goebel and Brandler (1982) is often used now to describe the location of this parasite in the cell.

Tyzzer was also the first to report on an avian cryptosporidiosis (Tyzzer, 1929), finding all the developmental stages in the caecal epithelium of a chicken.

For nearly 50 years after the work of Tyzzer, *Cryptosporidium* was not regarded as economically or medically important. During that time, however, a total of 21 species, found in fish, reptiles, birds and mammals were described. These were named mainly on the assumption that each host species supported a separate species of the parasite (Fayer *et al*, 1990). In 1955, Slavin reported the first link between *Cryptosporidium* and morbidity and mortality, naming a new species in the process - *C. meleagridis*. Diarrhoea and a low death rate were found in 10 to 14 day old turkey poults. It was only in 1971 when *Cryptosporidium* was found to be associated with bovine diarrhoea (Panciera *et al*, 1971) that interest in *Cryptosporidium* was stimulated in the veterinary profession.

In 1976, Nime *et al*, and Meisels *et al*, independently reported the first cases of human cryptosporidiosis. Thereafter, relatively few cases were reported until 1982, when physicians in various cities in the USA reported severe protracted watery diarrhoea in males, caused by

infection with *Cryptosporidium*, in association with the acquired immunodeficiency syndrome (AIDS). Medical and veterinary interest in the epidemiology, diagnosis, treatment and prevention increased considerably on a worldwide basis after these events.

In the early 1980's the development of numerous techniques and methods to recover and identify the transmissive oocyst stage in faecal samples provided health care workers and scientists with reliable methods of non-invasive diagnosis. These techniques have, among other things, enabled large scale epidemiological surveys to be undertaken, allowed the testing of chemotherapeutic agents and disinfectants for their efficacy against *Cryptosporidium* and permitted experimental studies on the biology of the organism.

In the last decade the protozoan parasites *Cryptosporidium parvum* and *Giardia intestinalis* have been shown to be major causes of diarrhoeal illness in humans. Transmission between humans is by the faecal-oral route. Studies have indicated that the prevalence of infection with these parasites is greater in developing countries than in developed countries (Davidson and Cerda, 1986; Current and Garcia, 1991; Adam, 1991).

In the last 30 years more than 108 outbreaks of waterborne giardiasis and cryptosporidiosis have occurred (Craun, 1991). These outbreaks indicated the potential for transmission via the waterborne route, which has now become a cause for particular concern. Surveys in the U.S.A. and U.K. have indicated the widespread occurrence and distribution of these parasites in the aquatic environment (Rose *et al*, 1987; Ongerth *et al*, 1987a; Smith *et al*, 1990; Gilmour *et al*, 1990; LeChevallier *et al*, 1991a & b).

Outbreaks of waterborne cryptosporidiosis have been attributed to both pre- and post-treatment contamination of the water supply (Hayes *et al*, 1989; Smith *et al*, 1989; Anon, 1990a; Richardson *et al*, 1991), demonstrating that oocysts, the transmissive stage of *Cryptosporidium*, can pass through water treatment processes, including disinfection, into the distribution network. The source of oocysts in the aquatic environment has been attributed to the discharge of oocyst contaminated sewage effluent and animal waste into water courses (Madore *et al*, 1987; Anon, 1990a).

Methods for the recovery, isolation and detection of *Giardia* cysts in water were developed in the 1970's (Jakubowski, 1976; Jakubowski and Ericksen, 1979) and were later used for the recovery, isolation and identification of *Cryptosporidium* (Musial *et al*, 1987, Smith *et al*, 1989, Gilmour *et al*, 1989; Anon, 1990b). These methods are not only time consuming, but are also inefficient in their recovery of organisms from water. Nonetheless, they have provided a means of assessing the extent of contamination in the aquatic environment. However, there is

no indication of the threat posed to human health by the presence of low numbers of oocysts in potable water. The viability of oocysts detected in the environment is an issue which has only recently been possible to address.

As a consequence of the ability of these organisms to pass through water treatment processes, either alternative means for the removal, inactivation and destruction of oocysts must be sought, or methods which enhance the existing treatments must be devised. Ozone has recently been shown to demostrate some efficacy as a disinfectant for inactivating oocysts, at the laboratory scale (Peeters *et al*, 1989; Korich *et al*, 1990; Perrine *et al*, 1990; Labatiuk *et al*, 1991). Despite the promise shown by ozone for inactivating oocysts, the latter do show a high degree of resistance, many times that of other organisms detected in water, suggesting the possible existence of antioxidant defence mechanisms in the parasite.

This project aimed to:

- assess the occurrence, distribution and concentration of *Cryptosporidium* spp. oocysts in the aquatic environment.

- assess the removal of oocysts from water by water treatment.

- investigate the source of oocysts entering the aquatic environment, their fate, viability and possible impact on human health.

- investigate alternative methods for the recovery of oocysts from environmental water samples, with a view to assessing the viability of the oocysts detected.

- investigate methods for the destruction and inactivation of oocysts.

- investigate aspects of oocyst resistence to inactivation by disinfectants and oxidants.

When investigating the occurrence and distribution of *Cryptosporidium* spp. oocysts in the aquatic environment, and their removal by water (and sewage) treatment, techniques also permitted the assessment of the occurrence of *Giardia* spp. cysts in water, and their removal by water treatment. A short review of *Giardia* and giardiasis is included at the end of chapter one in order to provide a background for the work.

CHAPTER 1. LITERATURE REVIEW.

1. *Cryptosporidium* and cryptosporidiosis

1.1. Taxonomy and Classification

Species of the genus *Cryptosporidium*, meaning hidden spore in Greek, are protozoan parasites assigned to the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidiorida, suborder Eimeriorina, family Cryptosporidiidae (Levine, 1984). Species of *Plasmodium*, the parasites which cause malaria, are in the same order (Eucoccidiorida), but different suborder (Haemosporirina) than species of *Cryptosporidium*. More closely related are other coccidia (suborder Eimeriorina), including *Isospora belli*, *Sarcosystis* spp. and *Toxoplasma gondii* which infect humans and *Eimeria* which infect mammals and birds.

Most species of *Cryptosporidium* named in the literature, after Tyzzer created the genus, were named assuming that these coccidia were as host specific as species of *Eimeria*, to which they are taxonomically closely related. Cross transmission studies in the early 1980's demonstrated little or no host specificity for the apparent 'species' of *Cryptosporidium* isolated from mammals. Tzipori *et al* (1980) suggested *Cryptosporidium* as a single species, because of the lack of host specificity. Levine (1984) grouped them into four species, *Cryptosporidium nasorum*, which infects fish, *Cryptosporidium crotali*, reptiles, *Cryptosporidium meleagridis*, birds and *Cryptosporidium parvum* which infects mammals. *C. crotali* is now considered to be a species of *Sarcocystis*, a genus of coccidian parasites commonly found in snakes and mammals. It is now agreed that there are in fact two valid species which infect birds, *C. baileyi* and *C. meleagridis* (Current *et al*, 1986) and two which infect mammals, *C. parvum* and *C. muris*. On the basis of oocyst morphology it is *C. parvum*, not *C. muris*, which is associated with the well documented cases of cryptosporidiosis in mammals (Upton and Current, 1985). Current and Reese (1986) and Uni *et al* (1987) suggest from ultrastructural studies that *C. parvum* and *C. muris* are distinct species. Two further species have been documented, *C. serpentis* in reptiles (Levine, 1980) and *C. nasorum* in fish (Hoover *et al*, 1981).

Table 1.1.1. Characteristics of currently recognised *Cryptosporidium* spp. oocysts.

Species	size	site of infection	host
C. parvum	4 x 6 μm	small intestine	mammals (including man)
C. muris	7.4 x 5.6 μm	gastric glands	mice, cattle
C. baileyi	6.2 x 4.6 μm	bursa of Fabricus	poultry (chickens)
C. meleagridis	5.2 x 4.6 μm	small intestine	poultry (turkeys)
C. serpentis	6.2 x 5.3 μm	gastric mucosa	reptiles
C. nasorum	3.6 x 3.6 μm	gastric mucosa	fish

1.2. Host Specificity

Transmission studies have been performed with oocysts taken from a particular species of animal and inoculated into animals of other species. Generally, isolates of *Cryptosporidium* taken from mammals have been infective for other mammals. It is most likely that *Cryptosporidium parvum* was the species transmitted in all such studies. In transmission studies performed with avians, mixed results were obtained. Chickens inoculated intratracheally with *C. parvum* oocysts had light infections in the trachea, larynx and primary bouchi. Also, small numbers of oocysts were found in the faeces of a few birds (Lindsay *et al*, 1987a). Chickens inoculated orally with *C. parvum* had no developmental stages in mucosal smears, tissue sections or faeces. Transmission from mammals to other vertebrate classes has not been reported.

Cryptosporidium oocysts have been successfully transmitted from one avian species to another. Oocysts from quail and pheasant were infective for chickens (O'Donoghue *et al*, 1987), although there was no mention of which species of *Cryptosporidium* this might have been. *C. baileyi* were mildly infective for turkeys when inoculated orally (Current *et al*, 1986; Lindsay *et al*, 1987b) and they developed extensively throughout the respiratory tract of turkeys when inoculated intratracheally. Oocysts from chickens (Lindsay *et al*, 1986; Current *et al*, 1986), quail and pheasant (O'Donoghue, 1985) were not infective to mammals. Transmission between other vertebrate classes has not been reported. There are several reported failures to transmit from one host to another (Current *et al*, 1986; Lindsay *et al*, 1987a; O'Donoghue *et al*, 1987). It is not known whether these differences in cross-transmission were a result of differences in host specificity of different parasite species or whether they were due to strain differences between the same parasite species. However, transmission, whether successful or not, can be affected not only by species specificity, but also the route of inoculation, age and condition of the oocysts used and the age and immune status of the recipient. As such negative transmission is not always indicative of host specificity.

1.3. Epidemiology

Cryptosporidium has been described in immunocompetent people in 26 countries, with a prevalence from large scale surveys of selected populations from 0.6 to 20 % in developed countries and 4 to 20 % in developing countries (Soave and Armstrong, 1986). In a review by Fayer and Ungar (1986) of 36 large scale surveys of selected populations, e.g. children and adults seeking medical attention for diarrhoea and gastrointestinal symptoms, it was considered that *Cryptosporidium* is associated with diarrhoeal illness worldwide, and that the prevalence is highest in poorly developed regions. The prevalence figures ranged, in Europe, from 1 to 2 %, and in North America, from 0.6 to 4.3%, which are lower than those reported in Asia, Australia, Africa and Central and South America, where the prevalence ranges from 3 to 20 %. In most of the surveys reviewed, *Cryptosporidium* was the most common parasite found and in several it was considered to be the most significant of all known enteropathogens causing diarrhoea. Many of the surveys reported higher prevalence in children than in adults, especially in the under 2 year olds. In addition infection was seasonal, being higher during the warmer, wetter months. It was also noted that a small number of oocysts may be present in faeces for up to 2 weeks following resolution of diarrhoea. Crawford and Vermund (1988) compared the worldwide occurrence of *Cryptosporidium* compiled by Navin (1985) from pre-1985 studies with studies from 1985 to 1988. Data were similar from the pre-1985 and post 1985 studies. The studies prior to 1985 suggested an overall prevalence of cryptosporidiosis in people with diarrhoea was 2.5 % in industrialised countries and 8.5 % in developing countries.

Current and Garcia (1991) presented a summary of more than 100 geographically based surveys from at least 40 countries. They showed, as did the others, that mean prevalence rates in the more industrialized countries of N. America and Europe are between 1 and 3 %, while in underdeveloped countries it ranged from 5 % in Asia to 10 % in Africa. The higher prevalence may be a result of a lack of clean water and sanitary facilities, crowded housing facilities and a large number of potential reservoir hosts (domestic mammals) near the homes. Walsh and Warren (1979) suggested that an estimated 5 billion episodes of diarrhoea and 5 to 10 million diarrhoea associated deaths occur annually in Asia, Africa and Latin America alone. If this is accurate one can predict 250 to 500 million *Cryptosporidium* infections annually in people living in these countries.

Limited serologic surveys support the concept that cryptosporidiosis is more common in developing countries. Seroprevalence rates in Europe and N. America are between 25 and 35 %. In Lima (Peru) and Maracaibo and Caracas (Venezuela), 64 % of children and adults had serological evidence of previous infection, that is, the sera contained antibodies specific for *Cryptosporidium* antigens (Ungar *et al*, 1988). Seroconversion data from studies (Ungar,

1989) suggest that *Cryptosporidium* infection is more common in most regions than faecal oocyst surveys indicate. They also show an increased risk of infection for unexposed people who travel to or go to work in areas of high prevalence.

Table 1.3.1. Summary of prevalence, (Ungar, 1990)

Country	Range %	Mean %
Africa	2.6 - 21.3	10.4
Asia	1.3 - 13.1	4.9
Carribbean	1.4 - 16.7	7.6
C/S. America	3.2 - 31.5	9.7
Pacific	0.1 - 22.2	6.0
Europe	0.1 - 14.1	3.5*
N. America	0.3 - 4.3	1.6

*(if 2 surveys with prevalences of 10.1 and 14.1 % are excluded, then the mean = 2.6 %)

Large scale surveys generally examined faecal specimens from selected and not necessarily comparable populations. In more developed countries (other than in outbreaks) specimens are most often from adults and children submitting samples to a specific diagnostic laboratory, or who sought medical attention for gastrointestinal complaints. Any survey detecting oocysts gives information on active infection. The accuracy of these depends on the number of stools submitted per person and the diagnostic techniques used. Serologic prevalence, detecting anti *Cryptosporidium* antibodies using IFA or ELISA techniques, correlates with infection at sometime in life, not necessarily active infection.

Data reported to the Centres for Disease Control (CDC) in the U.S.A. estimated a 2 to 5 % prevalence rate of cryptosporidiosis in HIV infected patients (Current and Garcia, 1991). In patients with AIDS and diarrhoea, 15 to 16 % were infected with *Cryptosporidium* (Laughon *et al*, 1988; Smith *et al*, 1988). In one U.K. hospital 11 % of AIDS patients had cryptosporidiosis (Connolly *et al*, 1988). Of the AIDS patients in the U.K. infected with *Cryptosporidium*, 19 % were thought to have died from cryptosporidiosis. In France 21.2 % of 132 AIDS patients had cryptosporidiosis (Rene *et al*, 1989).

The increased prevalence of cryptosporidiosis in immunocompetent people in developing countries compared to industrialized countries is probably also the case for AIDS patients. Soave and Armstrong (1986) reported 3 to 4 % of AIDS patients in the USA to have cryptosporidiosis whereas in Haiti and Africa this figure was 50 %.

In the UK, early reports of cryptosporidiosis were often infections in adults (Casemore, 1990a), reflecting the high proportion of immunocompromised subjects (Casemore *et al*, 1985) . There is a peak of cases in children between 1 and 5 years old, with a secondary peak in adults between 20 and 40 years (which may be a result of contact with infected children, or possibly occupational exposure). Sixty percent of positives came from children, and 30 % from adults under 45 years old. Asymptomatic infections have been found in adults who are close family contacts of cases (Casemore 1990a). There is no evidence of increased incidence in the elderly.

In a large PHLS UK survey (Palmer and Biffin,1987), infection was most common in the 1 to 5 years age group, and less common in the under 1 year olds, being infrequent in children under 6 months old. This is similar to findings in other studies, although some studies did find that infection occurred more commonly in children under 1 year old. The distribution of cases by age and sex was generally unremarkable.

In England and Wales laboratory reports began in 1983. From 1985 to 1989 the number of cases has risen, which may be a result of improved detection methods and an increase in awareness. In Scotland, where cryptosporidiosis became a reportable disease in 1989 (Forbes, 1988), there has also been an increase. In 1989, among cases of gastrointestinal infection, where diagnosis was confirmed by examination of stools, *Cryptosporidium* was the fourth most commonly identified cause (to *Campylobacter*, *Salmonella*, *Rotavirus*), accounting for 8 % of all cases in England and Wales and 13 % in Scotland (Anon, 1990a).

There are several epidemiological features of cryptosporidiosis. These include transmission by environmentally robust oocysts, the numerous potential reservoir hosts for zoonotic transmission which exist and person to person transmission (e.g. in day care centres). In addition, the occurrence of asymptomatic infections and the widespread distribution of the parasite in the environment, resulting in waterborne transmission are important features.

1.4. Transmission

Initial cases of cryptosporidiosis were thought to have come from animals. Current *et al*, (1983) noted an association between human infection and exposure to infected calves. Cattle can excrete up to 10^{10} oocysts per day (Blewett, 1989) and this not only provides large numbers of oocysts for infection by contact, but also causes large scale environmental contamination and so the risk of environmental transmission. *C. parvum* oocysts have been isolated from a large number (40) of host species (Fayer and Ungar, 1986; Current and Garcia, 1991) including companion animals such as rodents, pups and kittens (Current *et al*, 1983),

which can act as reservoir hosts. *C. parvum* readily crosses host species barriers as has been demonstrated by cross transmission studies previously discussed. All this evidence points to transmission from animals to man - zooanthroponosis.

It is reasonable to accept that animal to man transmission may be the common form of transmission in rural areas where contact with animals is common. However, the large number of infections in urban areas cannot really be explained by zoonosis. Present evidence shows that person to person transmission is common (Fayer and Ungar, 1986; Casemore, 1990a; Current and Garcia, 1991). The accidental infection of a laboratory worker (Blagburn and Current, 1983) demonstrated the transmission of one human isolate to another human. More common and perhaps clearer in demonstrating person to person transmission is the occurrence of cryptosporidiosis in children in daycare centres and their caretakers (Ungar, 1990) and also transmission between family members. Hospital acquired infections have also been recorded (Baxby *et al*, 1983; Koch *et al*, 1985; Marshall *et al*, 1987) in both patients and medical staff. Spread between sexual partners, both homosexual and heterosexual also occurs. The higher prevalence of the disease in young children (who often put their hands and other objects in their mouths) may well be a result of poorer hygiene than that of older children and adults, amongst whom there is a lower prevalence of disease. This lower prevalence may also be due, in part, to acquired immunity from previous exposure and infection.

Transmission has also been associated with travel, for example to Pakistan (Flegg, 1987). It is probably a result of increased exposure due to a greater prevalence of the disease in the community and perhaps a greater degree of environmental contamination.

Foodborne transmission has been implicated as a route of transmission, with food such as sausages, raw milk and tripe being associated with the transmission of cryptosporidiosis. The only proven case was from tripe (Nichols and Thom, 1985).

Airborne transmission of cryptosporidiosis may explain the location of the parasite in the respiratory tract in animals and in the respiratory epithelium and sputum of AIDS patients (Ma *et al*, 1983; Miller *et al*, 1984; Højlyng and Jensen, 1988). In one recorded incident where airborne transmission was postulated, a veterinary scientist who was treating a calf with cryptosporidiosis smelled the air from the open end of a tube inserted into the stomach of the calf and later developed clinical intestinal cryptosporidiosis. It was probably a result of inhaling and subsequently ingesting droplets containing oocysts (Højlyng *et al*, 1987).

Waterborne transmission of cryptosporidiosis has, in the last few years, been the cause of a number of cases and outbreaks of cryptosporidiosis. Surveys conducted of various types of water indicate the extent of environmental contamination. The high number of oocysts excreted by infected livestock provides a great potential for contamination of the environment and runoff from agricultural land may well account for the level of contamination in the aquatic environment. Waterborne transmission of cryptosporidiosis and the occurrence and prevalence of oocysts in water will be reviewed and discussed in later sections in this chapter and in chapter 3.

1.5. Life Cycle

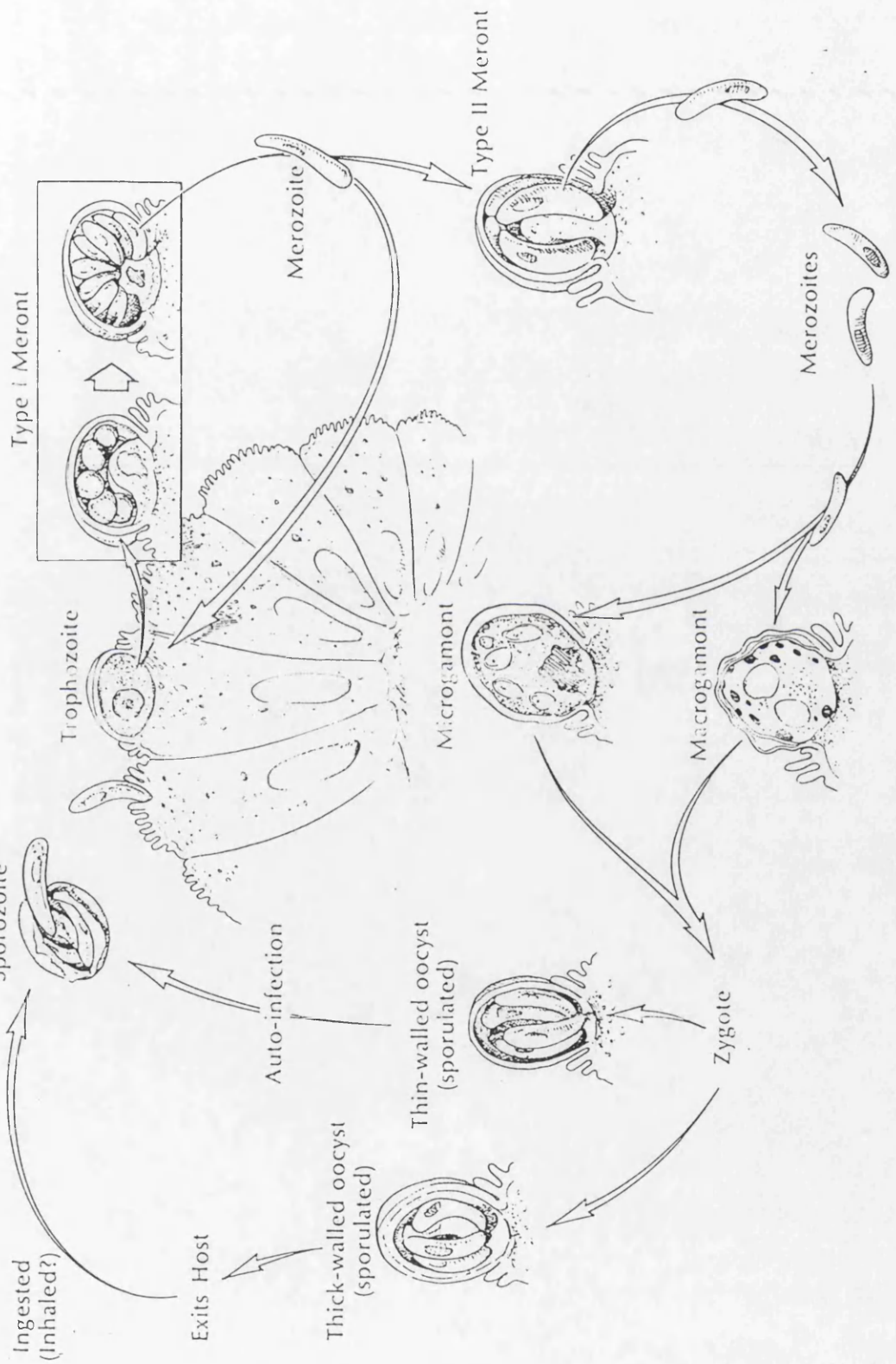
The life cycle has been outlined by various authors. The most detailed studies and descriptions are those by Tyzzer in 1910 and 1912. Basically it follows the pattern characteristic to other coccidia such as *Eimeria* and *Isospora* spp. which infect mammals and birds.

The life cycle can be divided into six major developmental events,

- excystation - the release of infective sporozoites
- merogony - asexual multiplication within the host cells
- gametogony - formation of microgametes and macrogametes
- oocyst wall formation - produces environmentally resistant stage that transmits infection from host to host
- sporogony - formation of infective sporozoites within oocyst wall.

The infectious stage is the sporulated oocyst, which excysts following ingestion, releasing up to 4 sporozoites into the small intestine. Each sporozoite can infect a columnar epithelial cell, becoming embedded at the base of the microvilli. This is the beginning of the asexual cycle. The intracellular but extracytoplasmic sporozoite differentiates into the trophozoite and then transforms into the schizont, which produces eight merozoites. The merozoites leave the host cell and can infect other adjacent cells, thus repeating the asexual life cycle. Alternatively, a merozoite differentiates into either a microgametocyte or a macrogametocyte: this is the sexual cycle. Microgametocytes produce microgametes which burst out of the host cell and fertilize a macrogametocyte, still within the host cell, to form the zygote. Most (80%) of the zygotes develop into environmentally resistant thick walled oocysts, which undergo sporogony to become sporulated oocysts, which are excreted in the faeces. Approximately 20% of the zygotes develop into thin walled oocysts, which are autoinfective.

Figure 1.5.1. Life cycle of *Cryptosporidium parvum*.



Each intracellular stage resides in a parasitophorous vacuole, confined to the microvillous region of the host cell, and although it is intracellular, it is extracytoplasmic. The sporozoites approach and enter the microvillous epithelial border in the small intestine by flexing and twisting movements to initiate infection. They indent the microvillous membrane, invaginating it. The double unit membranes of the host cell extend along the length of the parasite, finally covering and enclosing it in the parasitophorous vacuole (Tzipori, 1988). The parasite undergoes sporogony within the host cell and are infective when excreted in the faeces.

Studies in experimentally infected mice (Current and Reese, 1986) have shown that approximately 20 % of oocysts of *C. parvum* do not form a thick, two layered, environmentally resistant oocyst wall, but that the four sporozoites are surrounded by a single unit membrane. After release, the membrane ruptures and the sporozoites penetrate the microvillous region of other enterocytes, reinitiating the life cycle and perpetuating the infection. The other 80 % of oocysts develop a thick, environmentally resistant walls and are passed in the faeces. This is the form which transmits infection from one host to another.

The autoinfective, thin walled oocysts and the type I meronts that can recycle are believed to be the forms in the life cycle which are responsible for the development of severe infections in hosts exposed to low numbers of thick walled oocysts and for persistent, life threatening disease in immunodeficient people who are not repeatedly exposed to these environmentally resistant forms (Current and Garcia, 1991).

The time scale for the development of specific stages of the parasite in the host is not known with any degree of certainty, but mature type I schizonts, mature type II schizonts, gamonts and oocysts have been found *in vitro* at 12, 24, 48 and 72 hours respectively after cell cultures were inoculated with sporozoites (Current and Haynes, 1984).

1.6. Structure and morphology

Ultrastructurally, oocysts are similar to other coccidia. The oocyst wall consists of distinct inner and outer layers. *Cryptosporidium* oocysts also have a suture on the oocyst wall (not found in other coccidia) which dissolves when exposed to excystation stimuli (temperature, bile salts), . Sporozoites are released through the suture. Other coccidian oocysts (eg. *Eimeria* spp.) have a thin area at one pole, called the micropyle, which is resistant to trypsin and bile. However, similar trypsin and bile sensitive sutures to those found in *Cryptosporidium* oocysts have also been found in the sporocyst walls of other coccidians, for example *Sarcocystis*, *Toxoplasma* and *Isospora*. It should be noted that the suture observed in *Cryptosporidium*

oocysts is in the oocyst wall, whereas in other coccidians the suture is in the sporocyst wall; sporocysts not being present in *Cryptosporidium* oocysts.

The sporozoites and merozoites are ultrastructurally similar to other coccidia and possess most of the organelles typical of Apicomplexan organisms, eg. pellicle (plasmalemma plus double inner membrane complex), micronemes, electron dense bodies, nucleus, ribosomes, apical rings and subpellicular microtubules. However, they lack some organelles which are characteristically found in most coccidians, for example conoids, rhoptries, mitochondria, micropores and typical polar rings. Sporozoites and merozoites do have a cylindrically shaped, electron dense collar, which may be analogous to a polar ring, located at the anterior tip, close to the inner membrane complex.

Sporozoites and merozoites attach to epithelial cells via their anterior end, become enveloped by host microvilli and are eventually retained in a parasitophorous vacuole. After attachment to epithelial cells, changes in the ultrastructure are seen in both the apex of the host cell and in the parasite, which results in the formation of a feeder or attachment organelle.

Trophozoites contain undifferentiated cytoplasm, a well developed feeder organelle and nucleus with prominent nucleolus. The trophozoites give rise to schizonts or gamonts, which undergo two to four nuclear divisions, before the fomation of merozoites or gametes. Macrogamonts do not undergo nuclear division.

During schizogony the nucleus divides two or three times, depending on whether four or eight merozoites are formed. Host cell membranes surrounding mature schizonts lyse, releasing merozoites, which are able to infect other host cells.

Microgamonts are relatively rare compared to other stages in the life cycle of *Cryptosporidium* spp. Immature microgamonts are similar to schizonts, but can usually be distinguished from schizonts by their more compact and smaller nuclei. Early microgamonts are surrounded by a single surface membrane. In later stages a double membrane appears at various sites around the margin of the gamont, where microgametes have started to form. The body of each microgamete is formed by the protrusion of a nucleus from the gamont surface. An apical cap forms over the anterior end of microgametes. As they mature, microgametes pinch off, leaving the gamont surface. The residual body is surrounded by a single membrane and contains numerous ribosomes, endoplasmic reticulum and a few micronemes.

Microgametes are bullet shaped and small, approximately 1.4 μm x 0.5 μm for *C. parvum*. They lack flagella and mitochondria which are usually found in the microgametes of other coccidia. The condensed nucleus fills most of the microgamete.

Macrogametes have a relatively large, central nucleus, with a prominent nucleolus, lipid bodies, amylopectin granules and wall forming bodies.

Little is known about the fertilization of macrogamonts by microgametes. Penetration of the microgamete into the macrogamont has not been observed, although portions of microgametes have been observed within macrogamonts. Fusion of the gamete nuclei occurs rapidly.

After fertilization, the zygote develops into either a thick or a thin walled oocyst. Approximately 20% of the oocysts of *C. parvum* form thin walled oocysts, in which the sporozoites are surrounded by a single unit membrane. They are autoinfective and soon after leaving the host cell they penetrate the microvillous region of other enterocytes and reinitiate the life cycle (Current and Reese, 1986). The remaining 80% are thick walled oocysts and are shed in the faeces. The formation of the oocyst wall begins with the separation of the sporont from the feeder or attachment organelle, with the simultaneous appearance of two unit membranes, external to the zygote plasmalemma. A thin moderately coarse outer layer and a finely granular inner layer are formed by material transported across the plasmalemma and the inner membrane (pellicle). The two layers are separated by a zone that consists of the two oocyst membranes sandwiched between the outer and inner layers of the oocyst wall. The outer layer of wall is continuous and of uniform thickness, while the inner layer has a suture at one pole.

1.7. Clinical Signs and Symptoms

People become infected after oral ingestion of oocysts, either from other humans, from animals, or from the environment, especially water. Depending on the species and isolate of the parasite and the age and immune status of the host, the severity of the disease ranges from subclinical to severe. Generally young humans and animals are more susceptible and have the most severe clinical signs. Most reports of disease in domesticated animals, companion animals and poultry are from those less than one month old. Most of the reports concerning immunologically competent children are for those less than two years old. Out of all the animals which acquire infection, the only immunologically normal adults which become clinically ill (or are routinely susceptible) are humans, pigs and guinea pigs. In immunocompetent animals and humans the disease is self limiting and recovery from clinical signs which can last from a few days to a few weeks, is spontaneous. Immunodeficient people can become chronically and terminally ill.

The best determination of the length of the incubation period comes from cases when the initial time of exposure is known. For example, four Finnish veterinary students who were exposed to the same infected calf, on the same day, all developed symptoms six to seven days later (Pohjola *et al*, 1986). In other similar examples (either laboratory or veterinary animal exposure) the time from ingestion of oocysts to onset of symptoms ranges from 5 to 28 days, most often 7 days. In cases where the exact time of exposure is not certain, e.g. travel or patient contact, the average incubation period is still in the region of 7 to 10 days (Ungar, 1990).

In various mammals including humans the most prominent sign is watery, cholera like diarrhoea, associated with anorexia, weight loss, dehydration and abdominal discomfort. The diarrhoea is voluminous. Passage of up to 71 stools and 12 to 17 litres of fluid per day have been reported (CDC, 1982). Mucous is sometimes present in the stool, but rarely blood or leucocytes. Loss of appetite, nausea and vomiting may occur and weight loss of as much as 25 kg has been noted. There may be low grade fever, perhaps less than 39^{o}C, and general malaise, weakness or fatigue. Respiratory problems have also been described/noted, but these are mostly in immunocompromised/immunodeficient patients.

Fayer and Ungar (1986) reported diarrhoea as the most commonly noted symptom (92%), with nausea and vomiting (51%), abdominal pain (45%) and low grade fever (36%) being the other most often noted symptoms.

On biopsy of immunocompromised patients with diarrhoea, organisms have been seen throughout the gastrointestinal tract, from the oesophagus and stomach to the rectum and even the appendix. In immunocompetent patients, the infection is usually confined to the small and large intestine.

Symptoms wax and wane, and are usually more persistent in immunologically impaired patients. Jokipii and Jokipii, (1986 and 1987) reported that in 50 normal patients, duration of symptoms lasted for 2 to 26 days, with a mean of 12 days. Current and Garcia, (1991) suggested that oocyst shedding often parallels the waxing and waning of symptoms, although Ungar, (1990) pointed out that there is uncertainty as to whether the number of oocysts shed correlates with clinical symptoms.

In immunocompetent patients, malnutrition is a possible factor prolonging cryptosporidiosis. In a study in Jerusalem (Sallon *et al*, 1988), children with diarrhoea and *Cryptosporidium* positive stools were significantly more malnourished than children with diarrhoea and no *Cryptosporidium*. Also, the malnourished children with diarrhoea and oocysts in their stools

had a longer duration of diarrhoea than similarly malnourished children without *Cryptosporidium* oocysts in their stools.

Diarrhoeal illness is a major cause of morbidity and mortality in developing countries and *Cryptosporidium* (based on stool and serologic surveys, and increased reports of *Cryptosporidium* and malnourishment) may play an important role in the overall health status of children. *Cryptosporidium* may also have a role in respiratory disease which often accompanies diarrhoea in malnourished children (Egger *et al*, 1990).

In immunodeficient people the duration and outcome of intestinal cryptosporidiosis depends on the immune status of the individual. In AIDS patients cryptosporidiosis becomes progressively worse and is a major factor leading to death in such patients. First the ileum and jejunum become infected, then a large portion of the gastrointestinal mucosa, leading to a life threatening condition. Fluid loss is excessive, often ranging from 3 to 6 litres, although 17 litres has been reported (CDC, 1982).

In other immunodeficient people, such as those undergoing immunosuppressive therapy e.g. for cancer or transplants, or whose immune status may be affected for other reasons, such as malnourishment or patients with a concurrent viral infection (measles, chickenpox, CMV), the length and severity of illness depends on the ability to reverse the immunodeficiency (Ungar, 1990).

In immunodeficient patients, cryptosporidiosis is not always confined to the gastrointestinal tract. Symptoms in respiratory cryptosporidiosis include a cough, shortness of breath, wheezing, croup and hoarseness. Oocyst can be seen in the sputum, tracheal aspirates, bronchoalveolar lavage fluid, brush biopsy specimens and alveolar exudate from lung biopsies. Most patients with severe immunodeficiency and cryptosporidiosis in the respiratory tract do not recover (Ungar, 1990; Current and Garcia, 1991).

Gallbladder and biliary tree disease has been reported in HIV infected patients (Gross *et al*, 1986; Current and Garcia, 1991). Diagnosis is by histologic examination of gallbladder epithelium or by demonstration of oocysts in the bile. Pancreatic duct cryptosporidiosis has also been reported, again in AIDS patients (Gross *et al*, 1986).

1.8. Diagnosis

Prior to 1980, human cryptosporidiosis was diagnosed histologically by finding small spherical life cycle stages of *Cryptosporidium parvum* in the microvillous region of the intestinal mucosa

obtained by biopsy or in tissue obtained at necropsy. Haematoxylin and eosin were used to stain sections. Developmental stages appear as small, spherical, basophilic bodies (2 - 5 μm, depending on the stage of the life cycle) within the microvillous region of the intestinal mucosa. Transmisson electron microscopy (TEM) can be used for confirmation and reveals distinct life cycle forms, each within a parasitophorous vacuole, confined to the microvillous border of the host cell. These are intracellular but extracytoplasmic. Special staining procedures have proved to be of no advantage over haematoxylin and eosin staining for histological diagnosis. There are various problems associated with biopsy specimens, which include the need for invasive procedures, rapid fixation and careful processing of samples to avoid losing the organisms. The size of the organism and the fact that not all regions of the intestine are infected give rise to sampling errors. These invasive, expensive and time consuming techniques are no longer required for diagnosis because a variety of alternative techniques are now available to identify oocysts in faeces, sputum and bile. The use of biopsy and light or electron microscopy is still useful to investigate histopathology and cytoarchitectural changes associated with infection.

The laboratory diagnosis currently relies on the demonstration of oocysts in faecal specimens, using a variety of stains. In 1910, Tyzzer descibed the Romanowski stain, which showed endogenous stages of the parasite in gut mucosal smears. Pohlenz *et al* (1978) used Giemsa's stain to detect oocysts in smears of cattle faeces, and Tzipori *et al* (1980) used it with human faeces. A rapid version of this stain is often used, called Giemsa "diff quick", which reduces the staining time required. The acid fast stain Ziehl-Nielsen (ZN), described by Henriksen and Pohlenz (1981) who used it for staining calf faecal material, and which was futher modified by Casemore *et al* (1984), is one of the more commonly used stains nowadays. Faecal smears are stained using strong carbol fuchsin, decolourised in an acid/alcohol solution and counterstained in a weak solution of malachite green. Oocysts stain red, with internal structures and sporozoites sometimes visible (depending on the degree of staining), on a green background.

Auramine phenol (AP), used as a fluorescent stain, has been described, and modified to include negative staining with strong carbol fuchsin (Casemore *et al*, 1984 and 1985b). Oocysts appear as green fluorescent discs, with what is described as a "characteristic doughnut appearance". Auramine phenol appears to be more sensitive than ZN (Casemore *et al*, 1985b) as it stains outer walls as well as internal structures (Casemore *et al*, 1985a)

More recently fluorescent antibody detection has been shown to be a specific and sensitive way to detect *Cryptosporidium* oocysts in faecal smears (Sterling and Arrowood, 1986; Stibbs and Ongerth, 1986; Garcia *et al*, 1987; M^{c}Lauchlin *et al*, 1987) and also in environmental samples (Ongerth and Stibbs, 1987). The antibodies are raised against surface exposed epitopes on the oocyst wall. Indirect fluorescent antibody (IFA) detection has been shown to be more sensitive

than carbol fuchsin and auramine staining (Stibbs and Ongerth, 1986). Direct and indirect monoclonal antibody stains were shown to be highly sensitive in detecting oocysts when they were compared to other conventional staining methods (Arrowood and Sterling, 1989). Fluorescent monoclonal antibodies are now used routinely for the detection of oocysts in environmental samples (Smith and Rose, 1990) and various commercial kits are now available for this purpose (e.g. Northumbria Ltd., UK; Meridian Diagnostics, USA; Cellabs, Australia).

Various concentration techniques have been reported, which are used to increase the sensitivity of the current diagnostic methods (Casemore *et al*, 1985a). Concentration techniques are not so necessary when infection is heavy, but are useful for follow up samples. The most common methods include Sheather's (1953) concentrated sucrose flotation method and formol ether concentration (Ritchie *et al*, 1952), modified by Allen and Ridley (1970). Some reports suggest that Sheather's is the more sensitive method (Ma and Soave, 1983; Garcia *et al*, 1983), but when formol ether is suitably modified, it is more sensitive than Sheather's. In addition sucrose may interfere with staining and adherence of material to the microscope slide (Casemore 1985 a and b).

1.9. Pathogenesis

The pathophysiological mechanisms of *Cryptosporidium* induced diarrhoea are poorly defined at present.

Studies in germ free calves, monoinfected with *C. parvum* suggest that malabsorption and impaired digestion in the small bowel, coupled with malabsorption in the large intestine are major factors responsible for diarrhoea in calves with cryptosporidiosis (Heine *et al*, 1984). Similarly, malabsorption has been reported in a neonatal pig model, as a result of villous damage induced by parasites (Argenzio *et al*, 1990). The malabsorption and impaired digestion may result in overgrowth of intestinal microflora, a change in osmotic pressure across the gut wall, and an influx of fluid into the lumen of the intestine. Malabsorption and impaired digestion has also been reported in humans infected with *Cryptosporidium parvum*. The secretory (cholera like) diarrhoea common in immunodeficient patients with *Cryptosporidium* infection suggests a toxin mediated hypersecretion into the gut, but there are, as yet, no reports of such a toxin.

Definitive systematic studies are needed to determine mechanisms by which *Cryptosporidium parvum* and its metabolites or toxins alter the normal intestinal function of susceptible animal models.

In the small intestine, sporozoites released from oocysts infect the epithelium, causing moderate diffuse villous atrophy and crypt hyperplasia, mainly in the jejunum and ileum (Moon *et al*, 1985). All stages of the life cycle of *Cryptosporidium* are found in the apex of absorptive cells, beneath the host cell membrane, but outside the cytoplasm. Parasites are also seen in the ileum of infected guinea pigs, within the cytoplasm of M cells, overlying Peyer's patches (Marcial and Madara, 1986). Mild to moderate inflammatory infiltrates of lymphoid cells, macrophages and polymorphonuclear leucocytes have been seen in affected villi and in the underlying lamina propria (Kim *et al*, 1988; Argenzio *et al*, 1990). Depletion of lymphoid cells has been described in Peyer's patches in experimental infections in a foetal lamb (Kim *et al*, 1988). The degree of histopathologic changes in the gut does not correlate well with the severity of diarrhoea in humans, but enteric lesions do relate to extent and severity of diarrhoea in other infected animals.

The underlying mechanisms of the malabsorptive or secretory diarrhoea caused by *Cryptosporidium* infection are not known. Significant impairment of glucose stimulated sodium and water absorption in both jejunum and ileum has been observed in experimentally infected pigs, but absorption of electrolytes and water from Ringer's solution without glucose is not significantly affected (Argenzio *et al*, 1990). Enterotoxin-like activity found in cell free medium from *Cryptosporidium* infected tissue (Garza *et al*, 1986) has not been confirmed by others.

Zu *et al* (1992) observed diarrhoea in *Cryptosporidium* infected neonatal piglets 3 - 5 days after infection, with oocyst shedding and some decrease in ileal lactase, sucrase and alkaline phosphatase, but without increases in cyclic AMP or cyclic GMP, or alterations in cholera toxin. Lactase deficiency and xylose malabsorption have been found in infected calves (Moon *et al*, 1985).

Although the parasite is located within the brush border, its superficial microvillous attachment and the electron dense "feeder organelle" may contribute to the reduction in brush border enzymes, or the delivery of an unidentified toxic product to the epithelial cells. The specific roles of the mediators and mechanisms by which *Cryptosporidium* induces fluid and electrolyte loss remain to be clarified.

1.10. Immunology

Antibodies against *Cryptosporidium* have been reported in a range of animal species (Tzipori and Campbell, 1981) and in immunocompetent and immunocompromised people (Ungar *et al*, 1986; Casemore, 1987). Ungar and Nash (1986) reported an antigen of approximately 23 kDa was recognized by antibody from most infected individuals.

Age related susceptibility and resistance has been reported (Fayer *et al*, 1990). In general, young immunologically immature mammals have a greater prevalence of infection and experience more severe illness than adults. Although there is an age range of 3 days to 95 years, the greatest prevalence is seen in the under 5 year olds. If sanitation and nutrition are poor, there is a high prevalence in children. Where sanitation and nutrition are good, outbreaks may occur in such places as day care centres. In these situations children become infected first and adults may then become infected from subsequent contact. Contaminated water also leads to infection. These episodic infections indicate susceptibility may be influenced more by previous exposure than by age.

Most infected bovines are neonates, although the role of exposure resulting in immunity has not been ruled out, suggesting that susceptibility to infection may be a combination of age, immune status and resistance after previous exposure to the organisms (Fayer *et al*, 1990). Exposure of virtually all laboratory raised suckling rodents to *Cryptosporidium* results in cryptosporidiosis. Unexposed mice over 3 weeks old were not susceptible to infection (Fayer *et al*,1990). Exposure of neonatal nude mice with greatly reduced T-cell function results in persistent infection (Heine *et al*, 1984), although suckling nude mice still develop heavier infection than adult nudes. Immunosuppressed adult rats (using e.g. hydrocortisone), become susceptible to intestinal cryptosporidiosis after oral inoculation with *Cryptosporidium parvum*, suggesting that acquired immunity may play a role in resistance to infection in rats. The effect of malnutrition suppressing immunity may affect the susceptibility of individuals to infection, or the severity of disease.

Bovine immunoglobulins (Ig) Ig A, Ig G, Ig M antibodies to *Cryptosporidium* sporozoites and oocysts have been identified by enzyme linked immunosorbent assay (ELISA) (Ungar *et al*, 1986; Fayer *et al*, 1989). Human Ig A, Ig G, Ig E and Ig M have been identified in sera by ELISA (Ungar *et al*, 1986; Casemore, 1987; Fayer *et al*, 1990).

Sporozoite infectivity can be neutralised by exposure to immune serum (Riggs and Perryman, 1987; Riggs *et al*, 1989). However, measurable immune resistance does not necessarily provide protection. This is shown by the fact that some HIV patients produce IgM and all produce IgG antibodies during the course of chronic cryptosporidiosis. Studies in other coccidia suggest that immunity to coccidiosis is T-cell dependent (Rose *et al*, 1989). Humans (as well as mice) with T-cell dysfunction are unable to develop a protective immunity against *Cryptosporidium*. A protective immune response might require a T-cell dependent induction that results in specific humoral and cellular mechanisms (Current and Bick, 1989). The nature and identity of antigens which stimulate a protective response is not known. Monoclonal antibodies (MAb) recognise

the same epitope on oocyst proteins from different species of *Cryptosporidium*. One MAb against *C. parvum* reacts with *C. muris* and *C. meleagridis*, but not with *C. baileyi* (Meridian). *C. muris* and *C. baileyi* share four proteins of different molecular weight. *C. parvum* and *C. baileyi* share one , and *C. parvum* and *C. muris* share one also (Fayer *et al*, 1990).

Attempts to provide protection or therapy through passive immunity have yielded mixed results. In an initial, uncontrolled study, bovine transfer factor from lymph node lymphocytes of calves immunized with *Cryptosporidium* provided a spectrum of response for 5 out of 8 AIDS patients with cryptosporidiosis (Louie *et al*, 1987). In a controlled study 6 out of 7 patients given immune transfer factor responded with decreased frequency of bowel movements and weight gain. In five of the patients, oocysts were not detected in stools (McMeeking *et al*, 1990). Epidemiological studies on diarrhoea and *Cryptosporidium* in Costa Rica (Mata *et al*, 1984), Ecuador, Guatemala, Haiti and Liberia (Fayer and Ungar, 1986), indicated that breast fed children rarely or never had cryptosporidiosis. It was not possible, however, to tell from the data, whether antibody, or another biologically active factor in milk was responsible, or whether it was reduced exposure to contaminated food, water or utensils which was responsible for the lower prevalence in breast fed children.

Scant information is available on the immune response to *Cryptosporidium* in humans. Cellular and humoral aspect of the immune response are important from evidence of chronic cryptosporidiosis in HIV infections and in patients with gammaglobulin deficiencies, which contrasts with the transient nature of infection in immunologically normal individuals. The role of antibodies, T helper cells (CD4+), T suppressor cells (CD8+) and T cell products (e.g. γIFN or interleukin-2) remain to be investigated. The importance of immunologic mediators in the clearance of organisms during infection or subsequent protection, either from infection or symptomatic expression of disease is unknown (Ungar, 1990).

The antibody response has been assessed using immunofluorescent assays (IFA) (Casemore, 1987) and enzyme linked immunosorbent assays (ELISA) (Ungar *et al*, 1986, 1988 and 1990), with IgM, IgG and IgA having been detected. Using IFA, and oocysts as antigen, specific anti *Cryptosporidium* IgG, IgA, IgM and IgE were detected in serum samples from patients at different stages of *Cryptosporidium* infection (Casemore, 1987)

Immunologically competent patients show an early rise and fall of IgM and a later elevation (within 6 weeks) of IgG. The IgG response may disappear within a few months after infection, or may persist, perhaps signifying continued exposure, or undetected infection (Campbell and

Current, 1983). Some AIDS patients produced IgM, and all produced IgG, which remained high throughout the course of infection (Ungar *et al*, 1986).

In humans, age and immune status at the time of primary exposure to *C. parvum* do not appear to be the primary factors influencing susceptibility to infection. However, host immune status has a marked impact on length and severity of disease. Immunocompetent people develop short term (< 2 weeks) self limiting diarrhoeal illness following oral exposure to oocysts. This is initially the case in immunocompromised patients, but it becomes progressively worse, resulting in prolonged, life threatening, diarrhoeal illness. The difference in outcome between these two groups suggests the development of an acquired immune response of sufficient magnitude to clear the parasite from the intestinal mucosa. This concept is also supported by reports of people who rapidly cleared *Cryptosporidium parvum* infections when their immune function was restored following discontinuation of immunosuppressive therapy (Miller *et al*, 1983). In a recent study, ELISA was used to measure IgA, IgG and IgM antibody levels in serum, stool and duodenal fluid samples from 15 children. The authors concluded that the immune response in their subjects was probably an antibody dependent, cell mediated, cytotoxic effect of unknown mechanism (Laxer *et al*, 1990).

1.11. Treatment

A total of 94 therapeutic modalities have been tested for efficacy against *Cryptosporidium* in man and animals (Fayer and Ungar, 1986; Fayer *et al*, 1990). Supportive care with oral or intravenous hydration, possibly with parenteral nutrition, is a therapeutic intervention that offers a clear benefit for most immunocompetent patients. For such patients the disease is self limiting, so hydration therapy may be sufficient, if any treatment is provided. For immunocompromised patients the disease can be life threatening, unless the cause of immunosuppression can be removed. Few chemotherapeutics have shown any efficacy.

Spiramycin has been tested, but the results are inconclusive, with some reduction in diarrhoea and in a few cases oocysts shedding was stopped. Spiramycin may help control diarrhoea to some extent in patients treated for cryptosporidiosis during the early stage of AIDS, but it does not seem to have an effect on the course of disease in later stages of those with AIDS (Soave and Armstrong, 1986). Other studies have been performed using αDFMO (difluoromethylornithine), but side effects such as bone marrow suppression and gastrointestinal irritation have limited its use. Antidiarrhoeal compounds are of some use in controlling fluid loss. Several antidiarrhoeal compounds provide symptomatic improvement. A number of drugs (15 anticoccidial compounds) have been tested in animal models, but none prevented infection in a suckling mouse model, even at high doses (Angus *et al*, 1984).

Given that immune status has an effect on the severity and duration of disease, then immune intervention may be one method of control. One trial (McMeeking *et al*, 1990) suggested oral administration of an uncharacterized dialyzable extract prepared from lymph node cells obtained from calves immune to *C. parvum* (immune DLE) may produce sustained improvement in patients with AIDS. Antibody neutralization sensitive epitopes have been demonstrated, and several laboratories are investigating the potential immunotherapeutic utility of hyperimmune colostrum (Current and Garcia, 1991). Mata *et al* (1984) showed a lower prevalence of cryptosporidiosis in breast fed children in Costa Rica, but the data did not show if factors in milk helped or if it was just reduced exposure to contaminated food, water and utensils. In other studies, no protection was given to calves and humans from colostrum or milk from dairy cows exposed naturally to *C. parvum.*, while some reports indicate that colostrum from hyperimmunized cows does provide some protection. In addition, calves acquire cryptosporidiosis while suckling from cows, most of which have colostral antibody to *C. parvum.* This supports the idea that natural exposure does not give significant lactogenic immunity. In contrast, there are some reports that colostrum from cows hyperimmunized with oocyst and sporozoite antigen protects humans and mice (Tzipori, 1988; Fayer *et al*, 1989; Fayer *et al*, 1990).

1.12. Control and Prevention

The key to control and prevention is to remove, reduce or destroy the oocyst stage from the environment, or to avoid contact with known sources of contamination. Certain groups of people are at greater risk of contact and infection (Crawford and Vermund, 1988). These include people who may be exposed to, or more susceptible to, infection through

- occupation, for example veterinarians, farmers, medical and day care workers.
- a social group, for example by contact with an infected person or place such as other family members or a sexual partner.
- traveling to particular areas, e.g. underdeveloped countries.
- immune status, i.e. immunodeficient or immunosuppressed, old or young.

Favourable conditions of moisture and temperature enable oocysts to remain viable and infectious for 6 - 18 months (Tzipori, 1983; Current and Haynes, 1984; Blewett, 1989). Freezing kills oocysts (Current, 1989; Robertson *et al*, 1992a), moist heat > 45°C for 10 min. decreases oocyst infectivity (Anderson, 1985) and periods of drying (dessication) also result in loss of sporozoite viability (Anderson, 1986; Robertson *et al*, 1992a).

Various chemical agents have been tested for their disinfectant and oocysticidal properties, although few were found to be effective (Blewett, 1989). The four most effective compounds were

- hydrogen peroxide (10 vol.)
- Exspor - a chlorine dioxide based cold sterilant
- Oocide - a two phase product producing ammonia
- and ozone (Peeters *et al*, 1989; Korich *et al*, 1990).

Disinfection is discussed later in this chapter and in chapter 6.

1.13. Incidence of *Cryptosporidium* oocysts in water/the environment

Despite the problems involved in sampling and identification, information on the occurrence of *Cryptosporidium* spp. oocysts in a variety of waters has emerged in the literature in the past few years.

1.13.1 Surface and ground waters.

Madore *et al*, (1987) found oocysts in selected surface water, with likely contamination from sewage or faecal outfall, at a concentration of 0.8 - 5800 oocysts/L . In a study which looked at 11 rivers in Washington and California, Ongerth and Stibbs (1987) found that all the rivers contained oocysts in a range of 2 - 112 oocysts/L. Twenty-four out of 101 surface water samples contained oocysts at a range of 0.005 - 252.7/L. (Sterling, 1990).

The Badenoch committee reported that oocysts were found in all types of surface water, including pristine water (i.e. water with no source of human or animal contamination) at concentrations between 0.006 - 2.5 oocysts/L (Anon, 1990a). Oocysts were detected less often in ground water, 1 out 13 in the USA (0.005/L) and 2 out of 10 boreholes in the UK at a mean concentration of 0.012 oocyst/L. Out of 7 samples of spring water, 4 contained oocysts at a mean concentration of 0.04/L (Anon, 1990a).

In a survey of waters in the Western USA (Rose, 1988), 77% of rivers and 75% of lakes were found to contain oocysts and 28% of pristine waters were also positive. Average levels detected in surface waters ranged from 0.02 - 1.3 oocysts/L.

Table 1.13.1. Concentrations of oocysts detected in water in the U.S.A.

Type of Water	Average oocysts/L (geometric mean)
Pristine rivers	0.02, 0.08
Reservoirs/lakes	0.91, 0.58
Streams/rivers	0.94, 1.3, 1.09

(From Rose 1990, in Drinking Water Microbiology,McFetters).

In the UK Smith *et al,* (1990) found oocysts in 40.5% of samples of the untreated waters tested, most of which were surface waters, at a range of 0.006 - 2.3 oocysts/L. There were peaks in oocyst numbers, which occurred in July and February.

In a more recent study in the USA (LeChevallier, 1991a), 87% (74/85) samples taken from raw water locations contained between 0.007 - 484 oocysts/L, with a geometric mean concentration of 2.70 oocysts/L. In this study, the authors found that 32% of the 242 oocysts observed contained sporozoites, giving an indication of low viability, an important point when considering the risk to human health of oocysts in water. There was no significant difference in oocyst levels between protected watersheds and water receiving sewage treatment plant effluents. However, oocyst levels associated with sites receiving industrial (urban) sources of pollution were approximately 10 times higher than in protected sites. A correlation was shown between oocyst density and raw water quality parameters, such as turbidity, total coliform count, pH and temperature. The degree of watershed protection was deemed to be an important factor in oocyst levels present in water. In a study of the effects of time and watershed characteristics on oocyst concentration, Hansen and Ongerth (1991) found oocysts to be present in river water in inhabited and uninhabited areas and that oocyst occurrence was continuous, not intermittent. Seasonal factors, including run off from land drainage may affect the concentration of oocysts present by 10 fold, with concentrations in drier periods being significantly lower than those in wetter periods. The character and intensity of both human and domestic animal activities in a watershed may affect oocyst concentrations in surface water by as much 10 to 15 fold. Public water supply watershed management practices of limiting human activity may reduce oocyst concentrations by as much as 5 times (Hansen and Ongerth, 1991).

Contamination of water can occur via several routes, although agricultural sources seem to be the most likely. Slurry stores may leak, burst or overflow. Seepage from solid manure stores and run off from soiled yards may occur if they are not properly controlled or collected. This may be especially the case in areas of high rainfall. Landspreading with either solid manure or slurry can cause contamination by direct run off of liquid into water, especially during periods

of high rainfall or even following a heavy dew. Manure percolating into field drainage systems and animals grazing near water courses are also potential sources of contamination.

Animal sources may be the more likely source of contamination. In three studies in the USA samples of surface water receiving agricultural run off contained 2904, 1.53 and 1.09 oocysts/L compared to 1864, 1 and 0.58 oocysts/L which received treated human waste water (Madore *et al*, 1987; Ongerth *et al*, 1987; Rose *et al*, 1988).

The second main source of contamination, mainly of surface waters, is from the disposal of products from sewage treatment works when there is infection in the community. Sewage treatment uses a number of treatments to reduce pollution from the effluent, but these are not designed to remove pathogens or intestinal microorganisms, although there is some removal of these organisms. However, when oocysts are present in raw sewage, many of them will pass through treatment. There is also the likelihood that they will be present in sewage sludge which can be used in agricultural practice or deposited in land tip sites. The Sludge (Use in Agriculture) Regulations 1989 were introduced to control the use of sewage sludge in agriculture, but because oocysts remain viable in soil for months, adherence to these rules may not remove the possibility of contamination following the disposal of sludge onto land.

1.13.2. Treated water

LeChevallier *et al* (1991b) reported that out of 82 samples of finished drinking water 26.8% contained *Cryptosporidium* oocysts. Taking the geometric mean these workers reported *Cryptosporidium* to be present at 1.52 oocysts/100 litres. Rose *et al* (1988) reported *Cryptosporidium* oocysts in 2 out of ten filtered water samples at a mean concentration of 0.001 oocyst/L and in 2 out of 4 unfiltered potable water supplies at 0.006 oocyst/L. Smith and Rose (1990) quoted a range of 0.04 - 0.026 oocyst/L (36 samples) in drinking water in the USA and 0.006 - 0.26/L (65 samples) in the UK. These were not associated with any outbreaks. In samples associated with an outbreak of disease, 0.42 - 2.2 oocysts/L (20 samples) and 0.04 - 4.8 oocysts/L (7 samples) were detected in the USA and UK respectively (Smith and Rose, 1990). In an outbreak in Carrollton, Georgia, USA (Hayes *et al*, 1989) an average of 0.63 oocyst/L was detected within distribution, with the highest level of 2.2 oocysts/L.

A possible association with disease was reported following the finding of oocysts in water after microstraining, but this is as yet unconfirmed (Anon, 1990a). No conclusive association was made between the occurrence of disease in connection with finding oocysts in final water, although there was an increase in cases in the area at the time. The detection of oocysts in final

water, without disease in the population served suggests that they can be present in drinking water at low levels without risk to the community. In addition, the organisms detected may be inactivated by water treatment. LeChevallier *et al* (1991b) found that only 2 out of 23 oocysts detected in potable water had a viable type morphology.

Cryptosporidium oocysts have been reported at 0.07 - 484 oocysts/L (Rose, 1988; LeChevallier *et al* 1991) in surface waters in the USA. Grimason *et al* (in preparation) reported a range of 0.006 - 15.6 oocysts/L in surface waters in the UK. These figures suggest that the level of these parasites found in drinking water prior to treatment may be sufficiently high for low numbers to pass through treatment. Organisms have been found in potable water after filtration. In one case (Smith *et al*, 1989) post treatment contamination was held to be responsible, while in other cases the treatment works in question were considered to be operating at an approved standard. (Hayes *et al*, 1989; Richardson *et al*, 1991). In other outbreaks some operational irregularities were reported (D'Antonio *et al*, 1985). These incidents indicate that current forms of water treatment (with or without operational irregularities) are not successful at removing all *Cryptosporidium* oocysts from water intended for drinking. In addition, contamination of potable water after it has received final treatment is also possible. Rose (1988) reported *Cryptosporidium* in 50% of samples from unfiltered potable water. These are at risk, with no effective type of filtration for organisms the size of *Cryptosporidium* (5μm). Oocysts have been shown to be resistant to disinfection (Angus *et al*, 1982; Campbell *et al*, 1982; Smith *et al*, 1989; Korich *et al*, 1990). Oocysts have been reported in the effluent from a newly designed slow sand filter (LeChevallier *et al*, 1991b). These factors all indicate the potential for waterborne transmission of cryptosporidiosis.

In third world countries there is little water treatment. In Peru levels of 13.2 oocystst/L were found in water cisterns serving individual residencies (Sterling, 1987). Low numbers were detected in water delivery trucks, and high numbers (198.2/L) in oxidation ponds, which are often a source of water for those too poor to pay for water from a water truck. Water often sits in containers for one week or more and contamination from other sources is possible. Faecal coliforms were also found in the water, which indicates that contamination has occurred but does not indicate contamination with oocysts.

1.13.3. Waste water

Waste water of both human and animal origin is obviously an important source of contamination of water sources, with sewage effluent often discharged into water courses which are also used to abstract water for human use. A few studies have investigated the occurrence of *Cryptosporidium* oocysts in both raw and treated sewage.

Madore *et al* (1987) reported levels of oocysts ranging from 850 - 13,700 oocysts/L, with a mean of 5180 oocysts/L, in raw sewage samples. Three of these samples were taken from the intakes of domestic sewage treatment plants and the fourth sample, containing the largest number of oocysts, came from the effluent wastes of a cattle slaughter house. In other studies, again with limited sampling, DeLeon *et al* (1988) reported a mean of 521 oocysts/L and Rose *et al* (1988) reported 4 oocysts/L in untreated waste water. In a more extensive study throughout the USA, Rose (1988) reported that 91% of raw and treated samples contained oocysts, with average concentrations of 28.4 and 17 oocysts/L respectively. In the study by Madore *et al* (1987), nine samples of treated sewage effluent (treated by activated sludge) were collected and the concentration of oocysts detected ranged from 140 to 3,960 oocysts/L, with a mean of 1297 oocysts/L. Two other samples of treated sewage effluent following sand filtration contained 4 to 16 oocysts/L, considerably fewer than the other levels reported, indicating that sand filtration may be beneficial in removing oocysts. DeLeon *et al* (1988) and Rose (1988) reported 39.7 and 4 oocysts/L respectively in activated sludge treated sewage effluent.

1.14. Recovery and detection of oocysts in water related samples

The current methods used for the recovery and detection of oocysts from water and water-related samples are based on those used for other waterborne protozoa. The method used for the detection of *Giardia* cysts (Jakubowski *et al*, 1978; Jakubowski and Ericksen, 1979) has been used for the detection of *Cryptosporidium* oocysts (Rose *et al*, 1986; Musial *et al*, 1987; Ongerth and Stibbs 1987; Rose, 1988). This method has been further modified by Smith *et al* (1989c) and is now the method of use recommended by the Department of the Environment, Standing Committee of Analysts (SCA) for the detection of *Cryptosporidium* oocysts in water supplies (Anon, 1990a and b). The cartridge filter system is the method suggested by the United States Environmental Protection Agency (USEPA) for the detection of protozoa in water (Jakubowski and Ericksen, 1979).

The technique involves the concentration of oocysts on yarn wound polypropylene cartridge filters of 1µm nominal porosity by filtration of large volumes of water, from 100 to 1000 litres. The actual volume of water sampled may vary depending on the turbidity of the water; the more turbid the water, the smaller the volume sampled. Oocysts are eluted from the filter by cutting, homogenizing and washing the filter in oocyst free water (approximately 4 litres). Elution efficiency may be improved by back flushing the filter before it is cut (Musial *et al*, 1987). The samples of water are concentrated by large scale centrifugation and the parasites are then clarified from the debris and sediment by flotation, usually on a sucrose solution of specific gravity 1.18, although other gradients such as potassium citrate (Ongerth and Stibbs,

1987) and percoll-sucrose and zinc sulphate (LeChevallier *et al*, 1990) have been used. Detection and identification of organisms is achieved by immunofluorescence staining techniques and observation of internal contents. (Jakubowski and Ericksen, 1979, Ongerth and Stibbs, 1987; Ongerth, 1989; Musial *et al*, 1987; Rose *et al* 1986 and 1988; Smith *et al*, 1989; Gilmour *et al*, 1989).

Most concentration methods use wound or pleated cartridge filters - polypropylene or fibreglass. Ongerth and Stibbs (1987) used polycarbonate membrane filters and sampled up to 80 litres of water. This restricts the turbidity of the water which can be sampled and involves the transportation of water to the laboratory. Most other workers have used polypropylene cartridge filters (Madore *et al*, 1987; Musial *et al*, 1987; Rose *et al*, 1986,1988 and 1990; Smith *et al*, 1990).

Initial techniques for detection involved conventional staining and brightfield, phase and differential interference contrast (DIC) microscopy. These are time consuming, labour intensive and require highly trained staff. The development of monoclonal antibodies (MAb) to *Cryptosporidium* oocysts and *Giardia* cysts has greatly enhanced the ability to detect these parasites in environmental and clinical samples (Riggs *et al,* 1983; Sauch, 1985; McLauchlin *et al*, 1987; Sterling and Arrowood, 1986; Rose *et al*, 1988; Arrowood and Sterling, 1989). These commercially available monoclonals are unable to distinguish between isolates and species (Smith and Rose, 1990) so species not relevant to human health may be detected.

Other methods are being developed for the detection of protozoan parasites. These are 1) antibody based and 2) nucleic acid based, involving the use of molecular biological techniques such as polymerase chain reaction (PCR) (Abbaszadegan *et al*, 1991; Laxer *et al*, 1991; Bej *et al*, 1991; Mahbubani, 1991 and 1992).

1.15. Viability and Infectivity of oocysts

Determination of oocyst viability is a crucial matter for water authorities and other departments concerned with the supply of water and/or the health of the general public. It is also particularly important for researchers studying the oocysticidal effects of a wide range of disinfectant and therapeutic agents. There are currently three methods for assessing the viability of oocysts. These are 1) animal infectivity, 2) *in vitro* excystation and 3) the inclusion or exclusion of fluorogenic vital dyes.

1.15.1. Animal Infectivity

There is no really reliable *in vitro* culture system for *Cryptosporidium* where the completion of the life cycle and proliferation of the parasite can be achieved. In situations such as this the use of animal models is common, not only for assessing the viability of the organisms, but also for producing sufficient numbers of oocysts for experimental work.

Numerous animals, including mice, rats and lambs have been used to determine the infectivity and viability of oocysts, after a variety of treatments, e.g. with ozone (Peeters *et al*, 1989; Korich *et al*, 1990; Perrine *et al*, 1990; Blewett *et al*, 1992). Using animals allows oocyst proliferation and makes their detection easier.

There are problems associated with the use of animal models. Obtaining a suitable animal may be a problem and immunosuppression may be required to allow infection to be established. The minimum infectious dose may vary for different animals and may be higher than is thought normal in the true host(s) of the parasite. In some studies on disinfection, control animals required between 66 and 10^4 oocysts to establish infection (Peeters *et al*, 1989; Korich *et al*, 1990; Perrine *et al*, 1990). Animal infectivity does not provide information on the numbers, or ratio of viable to non-viable organisms present in a sample, or whether there may be attentuated organisms present. What it does assess is whether there are viable organisms present in sufficient numbers to cause infection, i.e. the sample contains infective organisms, not just viable, but those with the ability to establish infection in the host animal.

The application of animal infectivity as a means of assessing oocyst viability in environmental samples, and surveys for instance, is limited both by practical considerations and for economic and ethical reasons. The number of organisms detected in a sample may be lower than the infectious dose for the animal, and while the oocysts may be infective and able to cause infection in humans, it may not be possible to extrapolate from animals to humans. Ethically, the number of animals required might not be acceptable. Also, there might be other similar organisms present in the final pellet (e.g. *C. muris*) which may also be infective to the animals.

1.15.2. *In vitro* excystation

In vitro excystation provides a quantitative method of assessing the viability and survival of oocysts after exposure to environmental and laboratory induced stresses. It is cheaper and requires less time than infectivity studies. Results can be obtained in approximately 4 hours, compared to days or weeks required to obtain results from infectivity trials.

This method involves the enumeration of totally excysted, partially excysted and unexcysted oocysts after stimulation to excyst by exposure to bile salts and NaH_2CO_3 and a temperature of 37°C. In addition a ratio of sporozoites to empty or excysted oocysts can be counted (Reducker and Speer, 1985; Sundermann *et al*, 1987; Woodmansee, 1987; Blewett, 1989; Robertson *et al*, 1992b). Good correlation has been demonstrated between *in vitro* excystation and infectivity to mice (Blewett, 1989; Korich *et al*, 1990).

1.15.3. Fluorogenic dyes

In general the success of using fluorogenic dyes as means of assessing the viabilty of *Giardia* cysts has been limited (Bingham *et al*, 1979; Schupp and Erlandsen, 1987; Smith and Smith 1989). Some success was obtained using *G. muris* by Schupp and Erlandsen (1987) with fluorescein diacetate (FDA) and propidium iodide (PI) and this correlated well with infectivity as a means of assessing oocyst viability. These dyes were found to be of little value for use in assessing viability of *Cryptosporidium* oocysts (Smith *et al*, 1988; Korich *et al*, 1990). However Campbell *et al* (1992) using the dyes 4', 6-diamidino-2-phenylindole (DAPI) and PI found a method of assessing oocyst viability which correlated well with *in vitro* excystation. This assay involves the inclusion or exclusion of the dyes by oocysts. It has not as yet been compared with infectivity to animals. There are several advantages of this method compared to *in vitro* excystation and animal infectivity. It is easy and rapid to perform, taking approximately only 3 hours before a result can be obtained. It has the ability to assess the viability of individual organisms and can be performed on low numbers of oocysts, so that experiments can be performed using fewer oocysts, which is an advantage in some situations. The viability of low numbers of organisms found in environmental samples (often as few as one or two) can be assessed using this method. This is extremely important not only for water authorities or companies who may need to act on the information provided but also for information about oocyst survival in the environment and following exposure to water treatment processes.

1.16. Waterborne Outbreaks of Cryptosporidiosis

The first recorded waterborne outbreak of cryptosporidiosis occurred in July 1984, in Braun Station, a suburb of San Antonio with a population of 6,000 in Texas in the USA (D'Antonio *et al*, 1985). Two outbreaks of gastroenteritis were in fact experienced in Braun Station during the period May - July 1984. Both were caused by contamination of the common artesian well which supplied the community with water. However, the first outbreak was attributed to the Norwalk virus retrospectively, and on the basis of limited serological data. The incidence of diarrhoea in the population affected was twelve times that of the neighbouring communities in

both the outbreaks of gastrointestinal illness (D'Antonio *et al*, 1985; Barer and Wright, 1990; Sterling, 1990). Oocysts were detected in stool samples from 47 out of 79 people with diarrhoea. The age of those affected ranged from 1 to 72 years. A random telephone survey of 100 out of 1079 households (a total of 346 people) revealed an attack rate of 34%. In a control group, not consuming the same water, 12 out of 194 were excreting oocysts. The water supply was an artesian well which received no filtration, the only treatment being disinfection with chlorine. Oocysts were not detected in the source water, which may, in part, reflect the fact that recovery and identification techniques were not well developed at this stage. Faecal coliforms were detected in the untreated water. A dye added to the community's sewage system appeared in the well water, which suggested that sewage may have been the source of contamination. However, attempts to identify the exact site of contamination were unsuccessful.

In January and February 1987 a large outbreak of cryptosporidiosis occurred in Carrollton county residents, Georgia, USA (Hayes *et al*, 1989). Approximately 13,000 cases of gastrointestinal disease were reported. A random telephone survey indicated an attack rate of 54% in Carrollton City and 40% overall in Carrollton County. The rate of infection was significantly higher in people drinking water from the city supply (61%) compared with those drinking from other well supplies (20%). Oocysts were detected in 39% of 147 stool samples examined. No other pathogens were detected. The only significant risk factor was the water supply, which received conventional water treatment; coagulation, sedimentation, rapid sand filtration and chlorination. The water source was a river fed by a lake and subsidiary streams, with a sewage outflow upstream of the water catchment area. Treated water met the relevant Environmental Protection Agency (EPA) guidlines, with turbidity values < 1 nephelometric turbidity units (NTU), (0.07 to 0.18 NTU), < 1 coliform per 100ml of water. Chlorine residuals were 1.5 mg/L at the treatment works and 0.5 mg/L in the distribution system. A boil water advice was given on 30th January and kept in effect until March 2nd. Oocysts were detected in 7 out of 9 samples taken in early February from the distribution system, with a mean concentration of 0.63 oocyst/L, and in dead end mains. The highest concentration was 2.2 oocysts/L in a 24 h sample taken after treatment. Oocysts were also detected in the streams draining into the main source river in low numbers (0.08/L), but not in the river itself. Oocysts were detected in some samples taken from dairy cattle nearby, though the cattle were not pastured in areas where the source water was positive. Afterwards investigation into water treatment practices revealed a number of faults in operational procedures, including poor mechanical agitation of the coagulant and failure to backwash filters before restarting. It was postulated that restarting more of the filters than usual without proper backflushing possibly discharged a bolus of infective oocysts trapped on the filters into the drinking water. Also, efficiency may have been impaired by equipment used to regulate water flow through the filters.

The first confirmed waterborne outbreak of cryptosporidiosis in the UK occurred in two towns in Ayrshire, Scotland, in April 1988 (Smith *et al*, 1989a). The incidence of diarrhoea was between 2 and 5 times that normally expected. Oocysts were detected in the stools of 27 patients. Ten of the 27 confirmed cases were aged 10 or over, and 21 of the 27 required some form of fluid replacement therapy. Enquiries made by Environmental Health Officers indicated that many hundreds of people had suffered a diarrhoeal illness at that time. Water treated at Camphill water treatment works was treated by coagulation, flocculation, rapid sand filtration and chlorination. Treated water was pumped to storage tanks at another water treatment works via break pressure tanks. Oocysts were detected in filter backwash water in the treatment works (300 oocysts/L), in pressed sludge "cake" (3 oocysts/g), but not in the raw inlet or in the final outlet water from the treatment works. Two out of 7 samples taken from mains supplies were positive, with 0.04 and 0.08 oocyst/L. Post treatment contamination was confirmed when oocysts were detected in a break pressure tank (0.04 oocyst/L) where an old fire clay pipe, which records incorrectly described as being disconnected, was discharging run-off into the tank. Oocysts were detected in samples taken from the surrounding stream, soil and grass. Slurry and muck spreading had taken place in the vicinity of the break pressure tank shortly before the outbreak, and although there had been little or no rain in the 3 months before the outbreak, there were 3 very wet days towards the end of March. The contamination of the surrounding area with oocysts combined with rainfall may have caused the run-off into the break pressure tank, resulting in post-treatment contamination. The presence of oocysts in the filter backwash and sludge "cake" samples suggests that the raw inlet water must at some stage have been contaminated, but that water treatment was successful in the removal of oocysts.

A second outbreak in the UK occurred in Swindon/Oxfordshire in December 1988 and early 1989 (Richardson *et al*, 1991), and 516 laboratory confirmed cases were recorded, although up to 5000 people were thought to have been affected. Backwash water from the rapid gravity sand filters in use contained 10^4 oocysts/L, water leaving Farmoor water treatment works contained 0.1 oocyst/L, and at the end of distribution, in Swindon, 24 oocysts/L (Anon 1990a). During the peak of the outbreak 30% of the distribution samples were positive (0.002 - 77 oocysts/L), raw reservoir water 0.002 - 14 oocysts/L, and water leaving the water treatment works 0.002 - 5 oocysts/L (Anon, 1990a). Removal efficiency of oocysts by filters was assessed. The rapid sand filters reduced oocyst numbers by over 99%, while the settlement treatment process for the filter backwash water and sludge reduced the number of oocysts by only 83%. This resulted in the supernatant fluid from the settlement tanks which was returned to the head of the treatment works containing 10^3 oocysts/L, which, combined with the numbers entering the treatment works (up to 10 oocysts/L) resulted in a daily load on each filter of 10^9 oocysts. Increased washing, non-recycling of backwash water and increased

filtration time resulted in oocyst free treated water within a week. No oocysts were seen in sediment samples taken from the raw water reservoir, in the disinfection contact tank or in the distribution system in Oxfordshire. Sediment in an underground treated water reservoir receiving Farmoor water contained 100 oocysts/g. Monitoring of other contamination indicators such as coliform bacteria, turbidity, chlorine demand, coagulant residuals and bacterial plate counts showed no adverse changes in the quality of the treated water. Severe diarrhoea occurred in cattle near the river above the intake to the Worsham treatment works.

It was postulated that contamination of the source water with animal waste containing high numbers of viable oocysts may have been responsible for this outbreak.

Shortly after this episode 442 cases of cryptosporidiosis were reported in Scotland (January to June 1989) with 244 cases occurring in 3 health board regions (Anon, 1990a). Out of those who had oocysts in their faeces, 39% received water from Loch Lomond, and statistical analysis showed a higher incidence of the disease in people receiving water from Loch Lomond compared to people in the same areas supplied with water from another source. Other factors must be considered, such as person to person spread, foreign travel and contact with farm animals and pets.

The water receives basic treatment (microstraining, 23μm, and chlorination) and oocysts were detected in the raw water at the abstraction point, Ross Priory (0.01 - 2.3 oocysts/L), and in distribution (0.008 - 0.4 oocyst/L). The finding of oocysts in the loch water and the epidemiological evidence indicates an association between the water and the spread of disease, but is not conclusive. A further study is underway to monitor cryptosporidiosis in the area and the presence of oocysts in the water. This is reported in chapter 4.

In December 1989 an outbreak of cryptosporidiosis occurred in North Humberside and was over by the end of May (Anon, 1990a). Oocysts were detected in 477 stools of patients between the ages of 3 months and 95 years. The suggested route of infection was the mains water from Barmby treatment works. The Communicable Disease Surveillance Centre (CDSC) showed a significant association between illness and consumption of water from the Barmby treatment works. Oocysts were detected in the raw water on 4 occasions (0.1 - 1 oocyst/L). A possible explanation offered was that brief, intermittent contamination of the supply occurred, but it could not be detected by the level of sampling undertaken.

An outbreak of cryptosporidiosis occurred in the Isle of Thanet between December 1990 and January 1991, with 47 recorded cases aged between 2 months and 85 years (Joseph *et al*, 1991). A cases controlled study showed a statistical association between illness and the

consumption of unboiled tap water from a particular source. No oocysts were detected in untreated or treated water samples, although the cause is thought to be treated river water used to supplement the normal borehole water supply. Treatment of the river water was flocculation (aluminium sulphate coagulant), flotation and filtration, then filtration through an activated carbon filter, superchlorination and then dechlorination before distribution.

1.16.1 Other outbreaks

There have been a number of outbreaks of cryptosporidiosis where water was thought to be the route of transmission, but these have not been confirmed as waterborne outbreaks (Anon, 1990a).

In Cobham, Surrey, 2 possible outbreaks occurred in 1983 and 1985. Water was suggested as the route of infection, but there was no conclusive evidence. Run off following rain was considered to have contaminated the source, with oocysts penetrating treatment.

Between March and May 1984, 19 cases of cryptosporidiosis were reported in Holywell, North Wales. Oocysts were detected in upland feeder streams and although the waterborne route was suspected there was no evidence to confirm this. No common source was identified and person to person, zoonotic and foodborne transmission were all considered to be possible causes.

A possible but unconfirmed waterborne outbreak occurred in Sheffield, Yorkshire between April and October 1986 (Rush *et al*, 1987 and 1990). 81% of 104 people with cryptosporidiosis had consumed water from the same reservoir complex. Oocysts were detected in faecal samples from cattle on farms near the reservoir, in surface water from the reservoir, in feeder streams and in the gut contents of brown trout caught in the reservoir.

In July to October 1986 in Bernalillo, New Mexico, consumption of untreated water was the main risk factor associated with 78 laboratory confirmed cases of cryptosporidiosis (Gallaher *et al*, 1989). A case controlled study showed that 5 out of 24 patients with cryptosporidiosis drank untreated surface water. None of the matched controls had done so. Attendance at daycare centres was also identified as a risk factor in acquiring cryptosporidiosis. Surface waters tested in the area were negative, although sampling was undertaken some time after the reports of illness.

1.16.2. Water Associated Outbreaks of Cryptosporidiosis

Waters used for recreational purposes have also been shown to be responsible for transmission of *Cryptosporidium* oocysts resulting in disease.

In Doncaster, between August and October 1988, there was a rise in cases of cryptosporidiosis observed, especially amongst young children (Joce *et al*, 1991). In a case controlled study a statistically significant association was seen between head immersion while swimming in the learner pool at Armthorpe leisure complex and diarrhoeal illness. Investigation revealed that pipes serving the filtration plant to the pool had been disconnected in several places. A blockage in the sewage system had caused backflow, allowing sewage containing oocysts to enter the water treatment system. In water from the learner pool a concentration of 500 oocysts/L was detected, and in sand from the water filters, oocysts were detected at a concentration of 76 oocysts/g.

In Scotland oocysts have been detected in at least four swimming pools since July 1989. Concentrations detected have ranged from 0.005 to 2.8 oocysts/L. Water analysis in these situations has usually been performed because an epidemiological association has been made between cryptosporidiosis in the community and the swimming pool in question. On none of these occasions has the water source supplying the swimming pool contained oocysts.

1.17. Water treatment

1.17.1. Coagulation

Coagulation is the process where alum (the coagulant, aluminium sulphate) is added to water to remove colour and some suspended matter and colloids which may be present. Within a specific pH range (5.7 - 6.2) the alum reacts to form an insoluble precipitate known as the floc.

1.17.2. Flocculation

In order to achieve efficient coagulation the alum must be added at a point of rapid mixing, which ensures thorough dispersion of the alum. The floc is formed instantaneously, but the floc particles, which at this stage are very small, do not settle out. The water is stirred to help the floc particles adhere to each other and grow to a settleable size. This is known as flocculation. Apart from aiding floc growth, the mixing at this stage helps with the formation of the floc blanket during clarification.

1.17.3. Clarification

Most clarification stages rely on the formation of a floc blanket, which acts like a filter, retaining most of the floc. Not all of the floc is retained in the floc blanket, some of the smaller particles being carried over into the clarified water channel.

1.17.4. Rapid Gravity Sand Filtration

Typically rapid sand filters (RSF) are between 45 and 80 cm deep, with sand of effective size 0.35 to 1mm (usually 0.5 mm) supported by gravel and underdrains. In recent years many rapid sand filters have been replaced by dual or multimedia filters, in order to increase the filtration rate. During filtration, residual particles of floc not removed during sedimentation and clarification are trapped in the interstices of the bed, which may induce further flocculation of particles. A limited amount of biological activity may occur if it is not suppressed by prechlorination or high flow rates. The filters are cleaned by backwash and the backwash water produced is discharged to sewer, or recycled. The performance of the filter in removing microorganisms and turbidity varies throughout the duration of the run between backwashings. Immediately after the filter has been backwashed, performance is poor until the bed has compacted. Sometimes water is filtered to waste for the first 15 to 30 min at the start of each run, or alternatively there is a 30 min slowstart to prevent initial breakthrough. Performance may progressively reduce around the time when backflushing is required.

1.17.5. Slow Sand Filtration

Slow sand filters (SSF) reach a depth of 1 to 1.5m of silica sand with effective size 0.35 mm. The upper layer is supported on gravel and a system of underdrains. Slow sand filtration is more reliable and easier to operate than rapid filtration, and the frequent backwashing required for RSF's is not required for SSF's. When a filter is first brought into use a microbial slime community (schmutzdecke) develops on the surface of the bed, which consists of bacteria, free living ciliated protozoa and amoebae, crustacea and invertebrate larvae, acting in food chains. This results in the oxidation of organic substances in water and of ammonical nitrogen to nitrate. Pathogenic bacteria, viruses and parasites are removed principally by adsorption to the schmutzdecke and subsequent predation. Slow sand filtration brings about the greatest improvement in water quality of any conventional water treatment process. Ninety eight to 99.5 % of bacteria are removed, with *E. coli* being removed by a factor of 1000 and removal of viruses is even greater. Slow sand filtration is more efficient than other processes at removing helminths and protozoa. It operates more efficiently when the water temperature is high.

Effluent from a slow sand filter may still contain a few *E. coli* and viruses, especially during the early phase of a run.

1.17.6. Pressure Filtration

There is little theoretical difference between rapid sand filters and pressure filters. Pressure filters are built to make use of the head available at the reservoir. The filter is enclosed in a steel pressure cylinder. The flow through a pressure filter is not always constant throughout the day, unlike rapid sand filtration. It is designed to cope with changes in demand, with increased demand having an increased flow and decreased demand having a decreased flow through the treatment works. Chemical dose rates must vary with the flow rate to maintain the filtered water quality. This is achieved by dosing with pumps driven by a water turbine or flow controlled electric dosing pumps.

Pressure filters are erected in batteries of three or more. Cleaning is by backwash, the water for which is obtained from other filters in the battery. Mechanical rakes are used to break stir and loosen dirt from sand while the filter is being backwashed. Water in the backwash water outlet is viewed through a sightglass and backwashing normally continues until this water runs clear.

1.17.7. Microstraining

The micromesh of a microstrainer is made of a twill type weave of fine stainless steel or plastic wires, the openings of which have a twisted triangular shape. Microstrainers used in water treatment usually have openings of 35μm or 25μm, especially when the micromesh is clean. The microstrainer drum is cleaned by high pressure water jets.

The drum rotates, immersed in water, the head difference driving water through the micromesh, straining out particles, often algae. These entrapped particles form a straining layer on the micromesh, so that smaller particles (than 35μm or 25μm) are retained.

1.17.7. Disinfection

In water treatment, terminal disinfection is used as a final barrier to pathogens, and as a means of retaining a residual concentration of disinfectant in distribution. Although chlorine and hypochlorite are the most commonly used disinfectants used for treating drinking water, others such as chloramines, chlorine dioxide, ozone and ultraviolet irradiation have been investigated and used. Disinfection is discussed in a later section (1.19).

1.18. Removal and inactivation of oocysts by water treatment

The primary role of water treatment is to prevent transmission of infectious diseases. Despite regulations and advances in treatment technology outbreaks still occur. Several outbreaks have been attributed to *Cryptosporidium*, but there have been a number of outbreaks of diahrroeal disease for which no aetiological agent was determined and many of those could have been *Cryptosporidium*. The most effective treatment to prevent microorganisms from passing through treatment into treated water involves pretreatment to coagulate and sediment suspended particles, followed by filtration and disinfection (Logsdon and Hoff, 1986). Limited information on the zeta (or electrokinetic) potential (small voltages of like sign influenced by surface charge in relation to pH) of oocysts, which causes electrostatic repulsion, suggests they should respond, like *Giardia*, to coagulation and therefore should be removed by filtration in proportion to similar sized particles (4-6 μm) (Logsdon *et al*, 1988).

In waste water undergoing secondary treatment by activated sludge, the number of oocysts present in the treated effluent was reduced by 74% (Rose *et al*, 1986). and 79% (Madore *et al*, 1987) and by a further 87% and 99% in those with sand filtration. The overall removal efficiency based on the average number of oocysts in raw and treated sewage was 96.6% and 99.8% respectively. Similar information is available for the treatment of drinking water. A survey of a drinking water treatment plant employing filtration reported a large number of oocysts in backflush water from a rapid sand filter (2,906 oocysts/L) indicating effective removal of oocysts and their concentration on the filter (Rose, 1988). Comparison of the levels of oocysts in the water before and after treatment indicated a removal efficiency of 91%. In tests performed on an in-house slow sand filtration plant, 99.96% removal was achieved (Fox, 1988).

Ongerth (1989) took monthly samples over one year from a slow sand filtration plant (operational for only 2 years) and reported 47 % removal of oocysts. Schuler *et al*, (1991) reported greater than 99.9% oocyst removal from water containing 2,000 oocysts/L using diatomaceous earth filtration (Schuler and Ghosh, 1990). Oocyst removal was further improved using alum. Fine grade diatomaceous earth or cationic polymers did not improve removal. Mechanical removal did not appear to be the method of removal because fine grade diatomaceous earth did not improve removal.

Other workers found that filtration without the use of coagulation achieved estimated oocyst removals of 54.6% and 91.4% from effluent and river water respectively. An 85% reduction was seen in a plant with direct filtration. This plant was new and just initiating operation, and

the rapid mixers and the ozonator were not operating properly. Oocysts were detected in water undergoing conventional treatment (coagulation, sedimantation, rapid sand filtration and chlorination) and removal was estimated at 93.3%. Reduction in oocyst numbers of 89.9% was achieved in spring water undergoing chlorination (0.45 mg/L) as the sole form of treatment. (Rose *et al*, 1986; Rose, 1988; DeLeon *et al*, 1988).

Smith and Smith (1990) state that slow sand filtration is considered to be more effective than rapid gravity filtration, because of the lower hydraulic flow through the slow sand filter and the smaller particle size that can be removed in a well designed and operated slow sand filtration plant. However most modern large scale sand filtration plants opertate as rapid gravity filters.

1.19. Disinfection

From two outbreaks (Hayes *et al*, 1989; Richardson *et al*, 1991) of cryptosporidiosis which have occurred following contamination of water before treatment, and where water treatment has failed to remove or destroy the oocysts, it is evident that routine terminal disinfection is ineffective for oocysts of *C. parvum*.

Disinfectant efficiency as the percentage inactivation of organisms, is quoted for the concentration of disinfectant required and for the duration of exposure to the disinfectant. This quotient is commonly called the Ct' value, and is measured in mg/l.min.

1.19.1. Chlorination

Smith *et al* (1989), found that concentrations of free chlorine of between 8,000 and 16,000 mg/l for 24 h at pH 6,7 and 8 and temperatures 5 and 20°C were required to reduce oocyst excystation to 0 %. Viability was assessed by excystation.

In other laboratory scale work in which oocysts were exposed to 80 mg/l of chlorine for between 90 min and 120 min, excystation was reduced to 0 % after 120 min (Ct' = 9600) while 99 % inactivation was achieved after 90 min. (ct' = 7200) (Korich *et al*, 1990).

1.19.2. Chloramination

Korich *et al* (1990) found that exposure of oocysts to 80 mg/l monochloramine (NH_2Cl) for 180 min (Ct' = 9600) reduced excystation to 0 %, but that the effect was less when infectivity in mice was used to assess viability. The same concentration of monochloramine for 90 min. (Ct' = 7200) gave only 90 % reduction in viability compared to chlorine.

1.19.3. Chlorine dioxide

Peeters *et al* (1989) reported that 0.43 mg/l of chlorine dioxide (ClO_2) reduced infectivity within 15 min., although some oocysts remained viable. Korich *et al* (1990) reported approximately 90 % inactivation of oocysts exposed to 1.3 mg/l of chlorine dioxide for 60 min. (Ct' = 78) and this was paralleled by results obtained from mouse infectivity.

1.19.4. Ozone

Ozone has shown the most promise as an effective disinfectant against oocysts. An initial concentration of 1.11 mg/l ozone for 6 min. was shown by Peeters *et al* (1989) to eliminate totally the viability of oocysts in water at a concentration of 10^4 oocysts/ml, while a concentration of 2.27 mg/l was required to inactivate 5 x 10^5 oocysts/ml in water. Viability was assessed by infectivity of oocysts for neonatal mice. The minimum infectious dose required to establish infection in controls was 10^3 oocysts. Korich *et al* (1990) reported that between 90 and 99 % of oocysts (2.8 x 10^5/ml) in water at 25°C were inactivated, as measured by infectivity, when exposed to a constant dose of 1 mg/l ozone for 5 min. When the contact time was increased to 10 min., 99 to 99.9% inactivation was achieved. The mean infectious dose for mice was 60 oocysts. Perrine *et al*, (1990) reported 99 to 99.99 % inactivation of oocyst in water at 20°C after 4 to 8 min. exposure to initial ozone concentrations of 0.44 to 1.09 mg/l. They also found that lower temperatures reduced the level of inactivation achieved. Immunosuppressed rats were used to assess viability and the minimaum infectious dose in controls was 10^4 oocysts.

A minimal residual concentration of 0.4 mg/l ozone for 6 min. is commonly used in water treatment where ozone is a part of treatment. This is achieved by injecting 1.5 to 4 mg of ozone per litre of water. Ozone is about the only form of disinfection which achieves high levels of oocyst inactivation within the conditions practicable for the water treatment industry.

Table 1.19.4. Comparison of C.t^n values required for 99 % inactivation of other waterborne organisms by ozone and chlorine.

Organism	Disinfectant	pH	temp. °C	C.t^n value
G. intestinalis	ozone	7.0	25	0.17
		7.0	5	0.53
	chlorine	7.0	25	<15
		7.0	5	~125
G. muris	ozone	7.0	25	0.27
		7.0	5	1.94
	chlorine	7.0	25	25.5-44.8
		7.0	5	449-1012
Poliovirus	ozone	7.2	20	0.08
		7.2	5	0.22
	chlorine	6.0	5	2.0
E. coli	ozone	7.2	1	0.02
	chlorine	6.0	5	0.04
N. gruberi	ozone	7.0	25	1.29
		7.0	5	4.23
	chlorine	7.0	25	9600

Data from Wickramanayake *et al*, (1984a); Jarrol *et al*, (1981); Wickramanayake *et al*, (1984b); Roy *et al*, (1982); Scarpino *et al*, (1974); Katznelson *et al*, (1974).

1.19.5. Other disinfectants

Blewett (1989) tested a number of disinfectants at 22°C and 37°C. The prime criterion for efficacy was a reduction in excystation to below 10 %. If agents met this criterion, infectivity trials were carried out using new born mice.

Two agents, 10 volume hydrogen peroxide, available as a domestic bleach of low toxicity and Exspor, a cold sterilant, were the most effective at reducing the infectivity of oocysts for mice. Oocide, a two phase ammonia releasing agent marketed for agricultural use, was found to be oocysticidal in another trial by Blewett (1989). At the recommended working strength of 5 %, excystation was reduced to less than 3 % at 22°C.

Other disinfectants which have been tested for their efficacy in inactivating oocysts in water, but without much success under their normal conditions of use. These include ultra violet irradiation, aqueous sulphur dioxide, ammonia, iodine, bromine, iodine bromide and a combination of hydrogen peroxide and ozone, called peroxone (table 1.19.5).

Table 1.19.5. Inactivation of oocysts achieved with alternative disinfectants.

Disinfectant	Normal conc.	Required conc.	contact time	% inactivation
U.V.	16-30 mw.s/cm^2	80 mw.s/cm^2		99
Sodium sulphite	2 mg/l	980 mg/l	1 h	24.1
aqueous SO_2	2-5 mg/l	1000 mg/l	1 h	37.1
Iodine	1-2 mg/l	100 mg/l	1 h	> 90
Bromine	1-2 mg/l	> 1000 mg/l	1 h	87
Iodine bromide	1-2 mg/l	39.1 mg/l	1 h	7

Data from Ransome *et al*, (1991).

Combinations of chlorine and ozone and hydrogen peroxide and ozone have been tested but were not found to be as effective for oocysts inactivation as ozone itself (Ransome *et al*, 1991).

From the work documented to date on the effects of disinfectants on the inactivation of *Cryptosporidium* oocysts, ozone shows the greatest effect and is worthy of further investigation. So far, little work has been done on the effects of other parameters such as temperature, pH, water quality (e.g. turbidity, organic content) and different ozonation regimes. Exprimentation with ozone and ozonation in pilot-scale plants is necessary to test the results obtained from laboratory scale experiments.

1.3. *Giardia* and Giardiasis

1.3.1. Taxonomy and classification

Members of the genus *Giardia* belong to the family *Hexamitidae*, order *Diplomonadida*, class *Zoomastigophora*, phylum *Sarcomastigophora* (Levine, 1980). The precise number of species, with potential strain differences, has yet to be determined. It is generally accepted now, based on morphological differences in the trophozoites, that there are at least 3 valid species; *G. agilis*, *G. muris* and *G. intestinalis* (also called *G. lamblia* or *G. duodenalis*) (Filice, 1952; Kulda and Nohynkova, 1978; Meyer, 1983; Feely *et al*, 1984; Meyer, 1985).

G. intestinalis is considered to be the cause of clinical disease in man. It is not known, however, if the *G. intestinalis* species has one or more strains with distinct antigenic and biological properties (Meyer, 1976). This is important, because it might play a part in the variability seen in pathogenicity, infectivity, clinical manifestations of the disease, host response and efficacy of treatment, and may, in addition, be of relevance to the development of diagnostic immunoassays.

1.3.2. Life cycle.

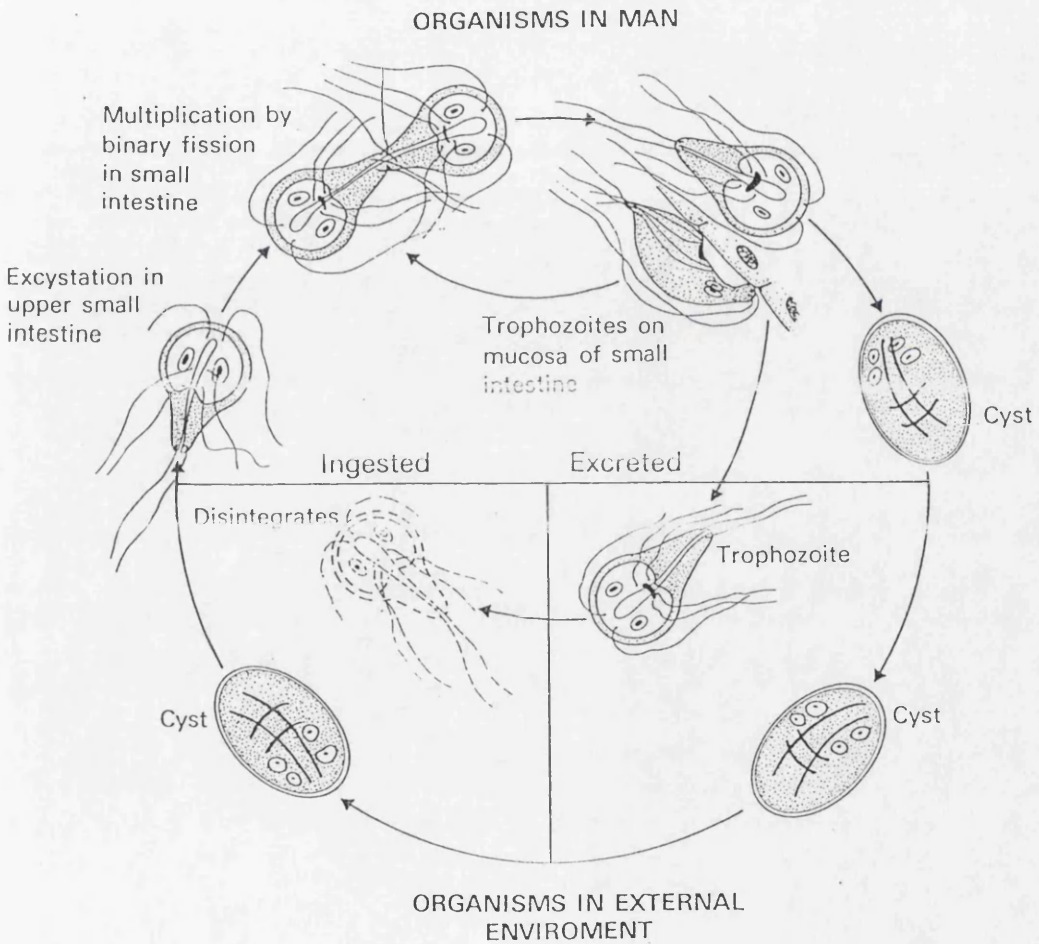
The life cycle of *Giardia* is characterised by two stages; an infective cyst stage and a vegetative trophozoite stage. The cysts are elliptical in shape, with slightly refractile cell walls and are smaller than the trophozoites.

Table 1.3.2. Characteristics of *Giardia* spp. trophozoites and cysts.

Species	Size of trophozoite	Size of cyst	Host
G. intestinalis	15 μm by 8 μm	8-14 by 6-10 μm	mammals, birds and reptiles
G. agilis	20 μm by 5 μm	range as above	amphibians
G. muris	10 μm by 7 μm	range as above	rodents, birds and reptiles

Identification of cysts in stools is based on the ovoid shape (of freshly encysted cysts), the presence of 2 - 4 nuclei found at one pole of the cyst and the appearance of intracellular structures; flagellar axonemes. Cysts excyst in the lumen of the small intestine following exposure to the acidic environment of the stomach, releasing two binucleate trophozoites which divide by binary fission.

Figure 1.3.2. Life cycle of *Giardia intestinalis*.



Morphologically, *Giardia* trophozoites resemble a pear cut in half longitudinally. The bodies are dorsoventrally flattened, pyriform and bilaterally symmetrical, with two anterior, vesicular nuclei and four pairs of flagella which are used for locomotion. The trophozoites usually live attached to the duodenal/jejunal mucosa, possibly by means of the adhesive disc, or ventral sucker. They feed by pinocytosis on the dorsal surface and ventrally in the centre of the disc region (Knight, 1978). The trophozoites detach from the epithelial cells, pass through the jejunum to the colon, some may transform into cysts (encystation) which are excreted in the faeces (Gillin *et al*, 1987, 1988; Sterling *et al*, 1988). Both cysts and trophozoites can occur in the faeces.

Cysts, when first formed, are reported to contain the same two nuclei which were present in the original trophozoite from which it was formed. A single subsequent division of each nucleus gives rise to a quadrinucleate cyst (Meyer, 1985).The cyst performs 3 main functions; (i) protects the trophozoites against unfavourable environmental conditions, (ii) functions as a site for reorganisation and nuclear division, which is followed by cytoplasmic multiplication upon excystation and (iii) it is the transmissive stage of the parasite (Cheng, 1986).

1.3.3. Epidemiology.

The first human case of giardiasis was described in 1681 by Antonie van Leeuwenhoeck who discovered the trophozoites in his own diarrhoeal stool (Dobell, 1920). However, most cases have been reported since 1960. Giardiasis has been reported to be the most commonly isolated intestinal parasite throughout the world and is especially prevalent in young children in developing countries (Feachem *et al*, 1983; Davidson and Cerda, 1987; Adam, 1991). The world wide prevalence is estimated at 7.2 % (Feachem *et al*, 1983)

In 1989, *Giardia* was identified as the aetiological agent responsible for 6.7 % of laboratory confirmed gastrointestinal infections in the U.K., representing over 7,000 cases (Anon, 1990a). Until 1986 giardiasis was the most commonly reported pathogenic protozoan infection. A review of cases reported to the Communicable Diseases (Scotland) Unit over a five year period (from 1984 to 1990) showed that the greatest number of cases reported occurred in young children, with approximately 40 % of the cases in children under 10 years old. The ratio of symptomatic to asymptomatic cases is unknown, although the number of asymptomatic cases is reported to vary between 17 and 100 % (Wolfe, 1984; Woo and Paterson, 1986).

Since January 1989, giardiasis has been an officially reportable disease in Scotland (Forbes, 1988) although this is not the case for the rest of the U.K. This almost certainly leads to an underestimate in the numbers recorded, although underestimation probably occurs also because

of asymptomatic infections and in cases where the infection is not severe enough for the patient to seek treatment.

1.3.4. Transmission.

Routes of transmission of *Giardia* are similar to those for *Cryptosporidium*, i.e. person to person, zoonotic, foodborne and waterborne. Initial cases of human giardiasis were thought to have been contracted from animals. However, animals can be infected experimentally with cysts of human origin (Hewlett *et al*, 1982), so it is likely that they can become naturally infected from humans excreta. Giardiasis has been reported in a variety of domesticated animals, livestock and wildlife, including companion animals which may act as reservoirs of human infection (Davidson and Cerda, 1987). Epidemiological evidence has implicated beavers as reservoirs for human infection associated with waterborne and water associated outbreaks of giardiasis (Dykes *et al*, 1980).

Infected individuals can excrete up to 10^9 infective cysts per day (Tsuchiya, 1931; Hewlett *et al*, 1982) which can lead to a high degree of environmental contamination, which increases the risk of infection for other susceptible animal and human hosts.

Person to person transmission is one of the most important routes of transmission and has been demonstrated in children attending day care centres, homosexuals, hospital personnel and household contact with infected people (Davidson and Cerda, 1987; Adam, 1991). This route of transmission may account for the high prevalence of infection in primary school children, possibly a result of poor hygiene. The lower prevalence in older children and adults may be a result of better hygiene and immunity resulting from previous exposure to the organism. Secondary cases and asymptomatic excretors may be a source of infection for other susceptible people. Increased sexual transmission has been reported, especially amongst homosexual men (Schmerin *et al*, 1978).

Foodborne transmission of giardiasis is less well documented and probably occurs as a result of contamination of food by infected food handlers (Osterholm *et al*, 1981; Adam, 1991). Foods which require handling and washing, such as fresh fruit and vegetables and salad are implicated in foodborne giardiasis.

In common with other organisms which contaminate and survive in the environment, and can be transmitted to humans via potable water, the waterborne route has also been documented for *Giardia*.

1.3.5. Clinical presentation

Humans acquire infection after ingesting cysts. Trophozoites released from the cysts adhere to epithelial cells lining the small intestine, the jejunum being the most infected site (Davidson and Cerda, 1987; Adam, 1991). Illness may vary depending on the immune status of the host, the infectious dose required to establish infection, the age of the host and the virulence of the organism (Adam, 1991). Rendtorff (1954) showed experimentally that as few as 10 cysts can cause infection in humans. The incubation period is approximately two weeks, with the majority of experimentally infected people asymptomatic and most infections cleared spontaneously within seven weeks (Rendtorff, 1954). Some people may proceed to chronic giardiasis which can last from six months to many years.

The symptoms experienced by individuals suffering from giardiasis are similar to those caused by other organisms which cause diarrhoeal illness. The usual presentation of giardiasis is with diarrhoea (93 %), malaise (70 - 80 %), abdominal pain (60 - 75 %), anorexia, nausea, bloating (approximately 67 %), flatulence, malabsorptive stools, vomiting and weight loss (up to 35 %) (Cerda, 1983). Stools are described as loose to watery, malodorous, light in colour and contain mucous. In chronic infections symptoms may be mild and recurrent. Symptoms and the severity of infection in immunocompromised hosts tend to be more chronic and last longer than in immunocompetent people (Adam, 1991).

1.3.6. Diagnosis

Diagnosis of infection with *Giardia* is usually by the demonstration of cysts (and trophozoites) in stool samples. Upper gastrointestinal sampling can be performed if stool samples are repeatedly negative and giardiasis is still suspected. Trophozoites can be detected in jejunal aspirates. The sensitivity of single stool examination is between 50 and 70 %, so it is recommended that two or three specimens over a period of a week should be examined (Heyman *et al*, 1987). Iodine can be used to stain cysts as an aid to identification (Wolfe, 1984). Recently direct and indirect immunofluorescence tests using monoclonal antibodies raised against the surface-exposed epitopes on the cyst wall have been used for the detection of cysts in faecal and environmental samples (Riggs *et al*, 1983; Sauch, 1985; Sterling *et al*, 1988; Gilmour, 1990).

1.3.7. Treatment

There are a variety of drugs in general use for the treatment of giardiasis, but the drug most commonly prescribed in the U.K. is metronidazole and in the U.S.A. is quinacrine (Adam, 1991). The parasite shows some degree of resistence to these drugs (Davidson, 1984).

1.3.8 Waterborne outbreaks of Giardiasis

The first waterborne outbreak of giardiasis in the U.K. occurred in Bristol between July and August 1985 (Jephcott *et al,* 1986). During this period of time there were 108 laboratory confirmed cases. Waterborne transmission of the parasite was postulated to explain the peak in cases seen in an area served by the same water supply. Of the proven 108 cases, 80 % lived or worked in the area supplied by the same water source. The water was treated by microstraining, slow sand filtration and superchlorination. No cysts were detected in the surface or potable water when it was sampled. The source of contamination was never actually established. However, it was thought that post treatment contamination may have occurred at a site on the main distribution line which was under maintenance at the time. No cysts were detected in water and soil samples taken from nearbythis exposed site.

In June 1990 the second waterborne outbreak of giardiasis occurred (Bell *et al,* 1991) amongst residents of a multistorey block of flats in Edinburgh. At least 8 residents contracted giardiasis within a five week period. The water supply was held in two water tanks situated on the roof of the building. This was considered to be the source of contaminated water, as a results of faecal contamination, presumed to be deliberate. Bacterial organisms indicative of faecal contamination were also detected in the water supply, as well as cysts. Faecal matter in which cysts were detected was recovered from the base of the water storage tank.

The first waterborne outbreak of giardiasis was reported in Tokyo, Japan (Davies and Ritchie, 1948). It was the result of sewage contamination of the water supply and 116 people from the same apartment complex were affected. One hundred and six waterborne outbreaks of giardiasis were reported in the U.S.A. up until 1988 (Craun, 1988). Shaw *et al,* (1977) reported the largest outbreak of waterborne giardiasis which occurred in Rome, New York in 1974/75. Epidemiological information suggested that 5300 people may have been affected in this incident, although the figure from laboratory proven cases was 350 cases. Waterborne giardiasis has also been reported in Sweden (Neringer *et al,* 1987; Anderson and de Jong, 1988). The waterborne route has also been implicated in travellers returning from the U.S.S.R. (Jokipii and Jokipii, 1974; Brodsky *et al,* 1974; Wilson, 1981).

1.3.9. Recovery and detection of *Giardia* in water.

Methods for the recovery and detection of *Giardia* cysts from water were developed in the late 1970's (Jakubowski *et al*, 1978; Jakubowski and Ericksen, 1979). These were later applied to the recovery and detection of *Cryptosporidium* oocysts and are described in section 1.2.13. As for *Cryptosporidium,* these methods have been modified at the S.P.D.L. (Gilmour *et al*, 1989) and have been accepted as the standard method for detection of cysts in water by the Department of the Environment, Standing Committee of Analysts (SCA).

1.3.10. Occurrence and removal of *Giardia* cysts in water.

1.3.10.1. Surface water.

Rose *et al*, (1991) detected cysts in 16 % of a variety of surface water samples (181) taken throughout the U.S.A. at a range of 0.04 - 1.4 cysts/L. The occurrence and concentration of cysts was greater in polluted water when compared to water which was considered to be pristine. LeChevallier *et al*, (1991a) reported concentrations of cysts ranging from 0.04 - 66 cysts/L in 81.2 % of 85 samples of surface water tested. These waters were highly polluted. Ongerth (1989) detected cysts in 43 % of 222 samples taken from three rivers at a range of 0.5 - 1 cyst/L. Payment (1992) recently reported cyst concentrations of up to 38 cysts/L in samples of surface water in Canada.

1.3.10.2. Potable water.

Ongerth *et al*, (1987) detected *Giardia* cysts in 7 out of 9 (77.7 %) samples taken from water following conventional treatment. In samples of water taken after pressure filtration, 7 out of 13 (53.84 %) contained cysts and 1 out of 3 (33.3 %) of samples taken following diatomaceous earth filtration contained cysts. LeChevallier *et al*, (1991b) detected cysts in 14 out of 82 (17.1 %) of samples of treated drinking water at a mean concentration of 0.0445 cysts/L. Cysts were also detected in 20 out of 33 (61 %) of samples of water following granulated activated carbon filtration (GAC) at a mean concentration or 5.68 cysts/L, 4 out of 23 (17 %) of mixed media filters (mean concentration 0.76 cysts/L), 25 % (3 out of 12) samples after dual media filtration (mean concentration 2.46 cysts/L) and in 5 out of 14 (36 %) of samples taken after rapid sand filtration at a mean concentration of 3.74 cysts/L.

Schuler *et al*, (1991) reported greater than 99.9 % removal of *Giardia* cysts by slow sand filtration and diatomaceous earth filtration. Payment (1992) detected cysts in 9 out of 14 (64.28 %) of treated water samples after settlement at a mean concentration of 0.038 cyst/L, 1

out of 5 (20 %, mean concentration 0.001 cyst/L) of samples after filtration, and 1 out of 9 (11 %, mean concentration 0.007 cyst/L) of final water samples. The overall removal efficiency quoted was 99.9981 %.

1.3.10.3 Waste water.

Levels of cysts detected in waste water vary depending on the size of population served by the sewage treatment works, the rate of attack in the population and the size of the treatment works. Removal of *Giardia* cysts by conventional sewage treatment has been quoted as lying between 40 and 100 % (Sykora *et al*, 1988). Rose *et al*, (1986) reported 51 cysts/L in raw sewage compared to 1 cyst/L in treated effluent, giving an estimated removal of 98.04 %. In 1989, Rose *et al* reported detecting *Giardia* cysts in 29 - 50 % of raw sewage samples tested and the concentration in the treated effluent ranged from 0.01 - 4.25 cysts/L. Sykora *et al*, (1991) detected cysts in 11 out of 11 samples from waste water treatment works, ranging in concentration from 4 - 14 000 cysts/L. They found that activated sludge was more efficient at cyst removal, providing 90 - 100 % removal, than trickling filter processes, which removed between 40 and 60 % cysts. Gilmour *et al*, (1990) detected cysts in 9 out of 9 samples of treated waste water effluent at a mean concentration of 358.6 cysts/L.

1.3.11. Disinfection.

Commonly used disinfectants in water and their concentrations are chlorine 1mg/l, chloramines 0.1 mg/l, chlorine dioxide 0.3 mg/l, ozone 0.4 mg/l and ultra violet irradiation 16,000 $\mu W\text{-}s/cm^2$.

1.3.11.1. Chlorine.

Jarroll *et al*, (1981) found that 1.5 mg/l chlorine for 10 min at 25°C (pH 6, 7, 8) was required to give > 2 log reduction in excystation of *G. muris* cysts. Rice *et al*, (1981) reported similar results. Hibler *et al*, (1987), reported that Ct' values of 185 - 342 mg/l.min at 0.5°C to 146 - 280 mg/l.min at 5°C (pH 6-7) gave 3-4 log inactivation, determined by animal infectivity. Hoff (1986) quoted Ct' values for 99 % inactivation of *G. intestinalis* cysts which ranged from 65 - 142 mg/l.min at 3°C to 5°C (pH 6.5 - 7) to between 9 - 12 mg/l.min at 25°C (pH 6 - 8) compared with those required to attain a similar inactivation level of *G. muris* cysts which ranged from 68 - 360 mg/l.min at 3°C to 5°C (pH 6.5 - 7) to 29 - 206 mg/l.min at 25°C (pH 5 - 9). Smith *et al*, (1988) reported free chlorine concentrations of 3 - 10 mg/l for 10 min at 20°C and 5°C (pH 7) were required to inactivate 100 % of *G. intestinalis* cysts.

1.3.11.2. Ozone.

Wickramanayake *et al*, (1984) reported C^t values for 99 % inactivation of *G. lamblia* cysts of 0.17 mg/l.min at 25°C and 0.53 mg/l.min at 5°C at pH 7. Labatiuk *et al*, (1991) found that C^t values ranging from 0.5 mg/l.min to 6.5 mg/l.min were required to give inactivation of 2.7 to 3 logs at pH 6.7 and 22°C. These C^t values are within the achievable levels of ozone commonly used in water treatment (e.g. 0.4 mg/l for 6 min) (Peeters *et al*, 1989). Levels quoted for 99 % inactivation of Polio virus 1 (Roy *et al*, 1982) range from 0.08 - 0.22 mg/l.min (20°C and 5°C) and for *E. coli* is 0.02 mg/l.min (1°C) at pH 7.2 (Wickramanayake *et al*, 1984).

The mechanism of inactivation reported by Sproul (Singer, 1990) suggested that the cyst wall was attacked by ozone, permitting attack on the plasma membrane and ultrastructural components of the organism.

1.3.11.3. Other disinfectants.

Chlorine dioxide was reported by Hoff (1986) to be more effective than chlorine with a C^t value of 11.2 mg/l.min required to achieve >99 % inactivation. Chloramines were reported to require higher C^t values than chlorine for cyst inactivation (Rubin, 1988; Meyer *et al*, 1988).

Rice and Hoff (1981) reported a maximum dose of ultra violet irradiation of 63,000 $\mu W\text{-}s/cm^2$ gave less than 1 log inactivation of *Giardia* cysts. For *E. coli* 99.9 % inactivation was achieved with U.V. level of 3,000 $\mu W\text{-}s/cm^2$. This lies well within the U. S. minimum dose of 16,000 $\mu W\text{-}s/cm^2$.

CHAPTER 2. MATERIALS AND METHODS.

2.1. Source of organisms.

C. parvum oocysts used in this study were obtained from the following sources:
1) Purified bovine oocysts, purchased from the Moredun Research Institute (M.R.I.), Edinburgh. This isolate was passaged in calves at the M.R.I.
2) Human oocysts in faecal samples, isolated from human faecal samples submitted for routine examination to the Scottish Parasite Diagnostic Laboratory (SPDL), Stobhill Hospital.
3) Purified cervine/ovine oocysts (c/o oocysts); purchased from the M.R.I., Edinburgh. This strain, originally isolated from deer faeces, was passaged in sheep at the M.R.I.

2.2. Purification of oocysts.

2.2.1. Detection of positive samples.

Human stool samples were concentrated by the formol-ether technique (Allen and Ridley 1970). For the detection of *C. parvum* oocysts, a drop of the concentrate was placed on a microscope slide, air dried, fixed in methanol for three minutes and then stained using the auramine-phenol (Diachem) technique described by Casemore *et al* (1985a) and in section 2.3.1.

2.2.2. Purification of oocysts from faecal samples.

Purification of oocysts from positive faecal samples from symptomatic patients was performed as follows. The stool was diluted with reverse osmosis (RO) water and filtered through a series of sieves (Endecotts, London) ranging from 500μm to 50μm to remove the larger particulate matter. The filtrate was then decanted into 50 ml centrifuge tubes, centrifuged at 900 x ***g*** for 5 min and the supernatant aspirated. A further 50 ml of RO water was added, the pellet resuspended, centrifuged at 900 x ***g*** for 5 min and the supernatant aspirated. This procedure was repeated until the supernatant was clear. The pellet was then resuspended in 10 ml distilled water and overlaid with an equal volume of diethyl ether, mixed thoroughly and centrifuged at 900 x ***g*** for 5 min. The supernatant and the fatty plug were discarded and the pellet was washed thoroughly in distilled water and centrifuged at 900 x ***g*** for 5 min, aspirated and resuspended in 10 ml of RO water. This was underlaid with a sucrose solution of specific gravity 1.18, taking care not to mix the two solutions and then centrifuged at 900 x ***g*** for 15 min. The interface between the two solutions was carefully collected by drawing it up using a wide gauge needle and syringe, depositing it in a 50 ml centrifuge tube and the excess sucrose removed by dilution

in RO water. If the oocyst suspension was judged to contained too much contaminating matter, a further sucrose density gradient was performed either using the same specific gravity, or a discontinuous density gradient (specific gravity 1.02, 1.09 and 1.18, oocysts being retained at the interface of the 1.09 and 1.18 layers).

Purified oocysts were stored in distilled water at 4°C. Counts were made using a haemocytometer.

2.2.3. M.R.I. oocysts.

Oocysts purchased from the M.R.I. had been purified using a semi-automated method. This method involved incubation in 1 % sodium dodecyl sulphate (SDS) and both acid sedimentation and sucrose flotation (S. Wright, pers. comm.). The oocysts were received suspended in phosphate buffered saline (PBS) pH 7.2, containing 100 U penicillin and 100µg streptomycin per ml.

2.3. Staining methods for oocyst detection.

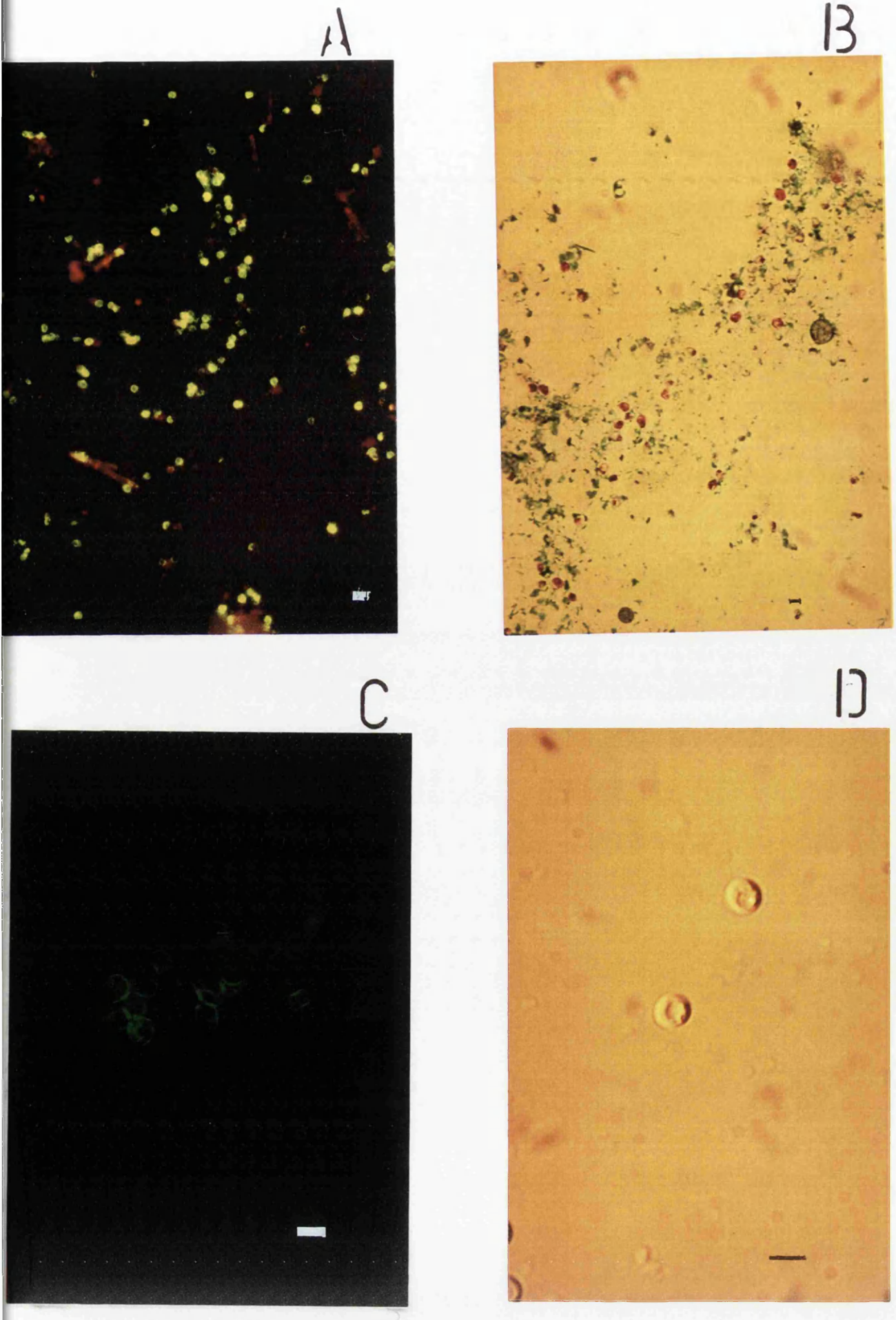
2.3.1. Auramine phenol.

A drop of the sample was placed on a microscope slide, allowed to air dry (or gently warmed using a fan heater) and fixed in methanol for 5 min. The slide was allowed to dry and was then submerged in a staining trough containing auramine phenol (Diachem) for 5 min, washed in water and counterstained by submerging in carbol fuchsin for 10 sec. The sample was allowed to air dry before being examined under the blue filter block of a fluorescence microscope (section 2.5.). Oocysts were seen as green fluorescent discs with the characteristic "doughnut" like shape (photograph 2.1.).

2.3.2. Modified Ziehl-Nielsen.

A drop of sample (either following formol-ether concentration or simply a drop of faecal sample diluted with RO water) was applied to a microscope slide, allowed to air dry and fixed in methanol for 3 min. The slide was submerged in carbol fuchsin for 15 min, decolourised in a 2-3 % acid/alcohol solution for 10 to 15sec and counterstained in 0.3 % malachite green for 30 sec. Slides were rinsed well and allowed to dry. Oocysts were seen as red spheres about 5 µm in diameter on a blue/green background. Internal contents sometimes stained red (photograph 2.1.).

Photograph 2.1. Oocysts stained with (A) Auramine Phenol (x 400), (B) Ziehl Nielsen (x 400), (C) Monoclonal Antibody (x 400) and under (D) Nomarski Differential Interference Contrast microscopy (x 1000). Bar = 5 μm.


A
B
C
D

2.3.4. Monoclonal antibody (MAb).

This stain was used predominantly on water samples and samples where low numbers of oocysts might be present. MAbs were murine IgG, raised against epitopes expressed on the surface of the oocysts wall. 25µl of sample were applied to each well of a four welled microscope slide (Hendley, Essex), air dried, or dried gently in front of a fan heater and fixed in methanol for 5 min. After drying, 25µl of MAb with a fluoroscein-isothiocyanate (FITC) conjugate (Northumbria Biologicals, UK) were applied to each of the four wells containing the sample. The slide was placed in a moisture chamber and incubated in the dark at 37°C for 30 min. The slide was then washed 3 times in PBS for 5 min. When the slide was dry a small drop (approximately 10µl) of mounting medium was applied to each well and a 50 x 22 mm coverslip was placed on top. Oocysts appeared as "bright apple green fluorescent" objects when examined using a fluorescence microscope (photograph 2.1.). See section 2.5 for microscopy.

Latterly 4',6-diamidino-2-phenylindole (DAPI) (5 µl, 1:10,000) was applied after the MAb was washed off, incubated at 37°C, washed, dried and examined as described. This aided in the identification of possible oocysts detected in environmental samples. The nuclei were highlighted by the dye. (Grimason *et al*, in press).

2.4. Viability.

2.4.1. Fluorogenic vital dyes.

The viability of oocysts was assessed using the inclusion or exclusion of the fluorogenic vital dyes 4'6-diamidino-2-phenyl indole (DAPI) and propidium iodide (PI), according to the method of Campbell *et al* (1992). 100µl suspensions of oocysts in Hanks' Balanced Salt Solution (HBSS) (Gibco, UK) without phenol red were incubated for 2h with 10µl working solution of DAPI and 10µl working solution of PI at 37°C in the dark. Monoclonal antibody, reactive with the oocyst wall, was added at optimal dilution to aid identification. Oocysts were washed three times in HBSS by centrifugation and aspiration of the supernatant and the final supernatant volume reduced to 100 µl before viewing by fluorescence microscopy. If *in vitro* excystation was to be performed thereafter, oocysts were washed twice in HBSS and once in Eagle's minimum essential medium containing Hanks' salts (HMEM) but without phenol red (Sigma Ltd., UK). The pellet was resuspended, a 10µl aliquot was pipetted onto a glass microscope slide and a coverslip was applied and sealed with nail varnish. The slide was examined using fluorescence microscopy.

2.4.2. Excystation protocol.

After the dyes were washed out of the oocyst suspension, the oocysts were resuspended in 100 µl HMEM. Fresh solutions of bovine bile (10mg bile/ml HMEM) and sodium hydrogen carbonate (11mg $NaHCO_3$/2.5ml RO water) were made up and 200µl of the bile solution and 50µl of the $NaHCO_3$ solution were added to the oocyst suspension which was mixed well before being incubated at 37°C for 4 h. Partially and fully excysted and oocysts were counted and expressed as a percentage of partially and fully excysted plus unexcysted oocysts.

2.5. Microscopy.

Slides were examined under both fluorescence and Nomarski Differential Interference Contrast (DIC) microscopy using an Olympus BH-2 epifluorescence microscope, equipped with an ultra-violet filter block for DAPI (excitation 335 nm; emission 450 nm), a green filter block for PI (excitation 488 nm; emission 610 nm) and a blue filter block for FITC (excitation 490 nm; emission 510 nm).

DIC microscopy was used to analyse oocysts for the presence or absence of internal contents, ie. whether they were 'ghosts' (empty shells) or not, (and to indicate their viability status when dyes were not used). Ghost oocysts are non-refractile apart from the residual body.
PI+ oocysts fluoresced bright red under the green filter block. Oocysts were considered to be DAPI+ only if the nuclei of the sporozoites fluoresced a distinctive sky-blue under the UV filter block, and were not PI+. Those oocysts which were not PI+ and showed either an even fluorescence , a rim of fluorescence or an absence of fluorescence when viewed under the UV block were considered to be DAPI-.

2.6. Water sampling, filter processing and sample analysis.

Sampling, processing and analysis of the influent, effluent, raw and filter back-wash waters was performed according to the method described in the DoE publication for the " Isolation and Identification of *Giardia* Cysts, *Cryptosporidium* Oocysts and Free Living Pathogenic Amoebae in Water etc. 1989 " (Anon, 1990b).

2.6.1. Sampling sites.

Water treatment works typical of coagulation, sedimentation , rapid sand filtration and slow sand filtration were identified with help from the staff of Strathclyde Regional Council (SRC) Water Department.

2.6.2. Sampling.

Water was sampled using a submersible pump (12 V, Beresford, UK.), driven by a petrol generator (Honda), which pumped the water sample through a D-PPPY polypropylene microwynd depth filter (Cuno) of 1 μm nominal pore size. The filter was held in a cartridge housing (Cuno) and the pump was connected to this by hose piping. The cartridge outflow was equipped with a flow restrictor and a water flow meter (Kent) was attached to this with more tubing (photograph 2.2.). The flow of the water sample through the filter was adjusted to 1 L per min when sampling started, although depending on the quality of the water this often reduced further as the volume sampled increased. Where possible, a large volume of water was sampled, usually between 500 and 1000 L for final water and 100 to 500 L for sewage effluent and raw water.

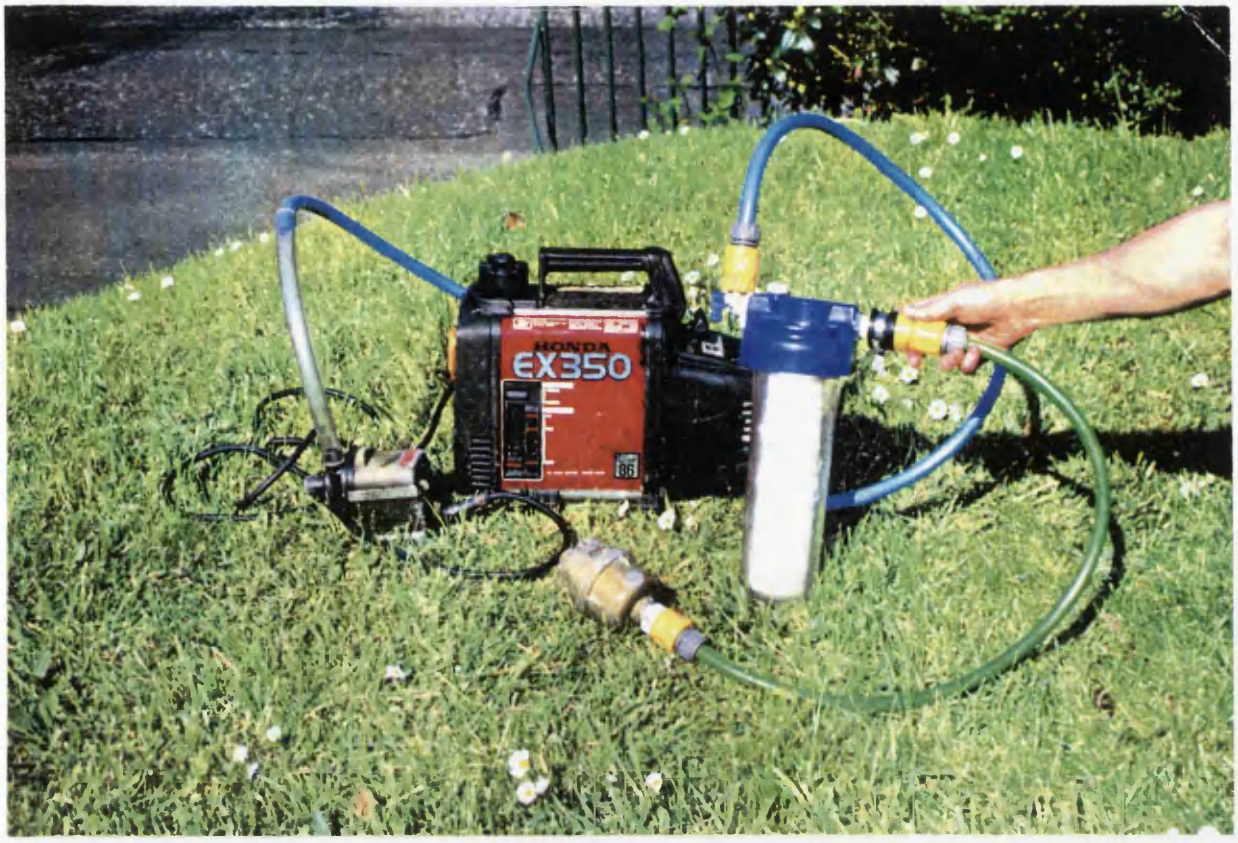
2.6.3. Filter processing.

The filter was cut lengthwise , divided into thirds and each third was washed (photograph 2.3.) in 0.1% Tween 80 (Sigma, UK). The total washings, amounting to approximately 4 L were centrifuged at 900 x *g* for 15 min in 1 L centrifuge bottles. The supernatant was discarded by aspiration, the pellet resuspended and decanted into four 250 ml conical centrifuge bottles (Corning) and then centrifuged for 15 min at 900 x *g*. The supernatant was again aspirated, the pellet resuspended and transferred into four 50 ml conical centrifuge tubes and centrifuged at 900 x *g* for 5 min. The supernatant was aspirated, the pellets resuspended and pooled into either one or two 50 ml centrifuge tubes , made up to 50 ml with RO water and centrifuged as before. The supernatant was aspirated, resuspended in 10 ml of RO water, underlaid with a sucrose solution of 1.18 g/ml and centrifuged at 900 x *g* for 15 min. The interface was carefully removed and transferred to a clean 50 ml centrifuge tube, diluted with RO water and centrifuged at 900 x *g* for 5 min. The supernatant was aspirated, the pellet resuspended and transferred to a 1.5 ml eppendorf microfuge tube and microfuged at 12,500 x *g* for 1 min, following which the supernatant was aspirated and the pellet resuspended in 0.5 ml RO water.

2.6.4. Analysis.

Analysis of the final pellet for *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts was carried out by staining slides with FITC conjugated MAb specific to antigens on the outer walls of each of the parasites. The staining method is described in section 2.3.4. above. The MAb most commonly used for the detection of *Cryptosporidium* spp. oocysts was supplied by Northumbria Biologicals Ltd., UK., although on occasions those from Cellab (Australia) and Meridian Diagnostics (USA) were also used (both supplied by Bradsure Biologicals, UK).

Photograph 2.2. (A) Sampling equipment, showing the petrol generator (g), the pump (p), filter and housing (f) and water flow meter (w). (B) seeding equipment, showing oocyst injection points (arrowed).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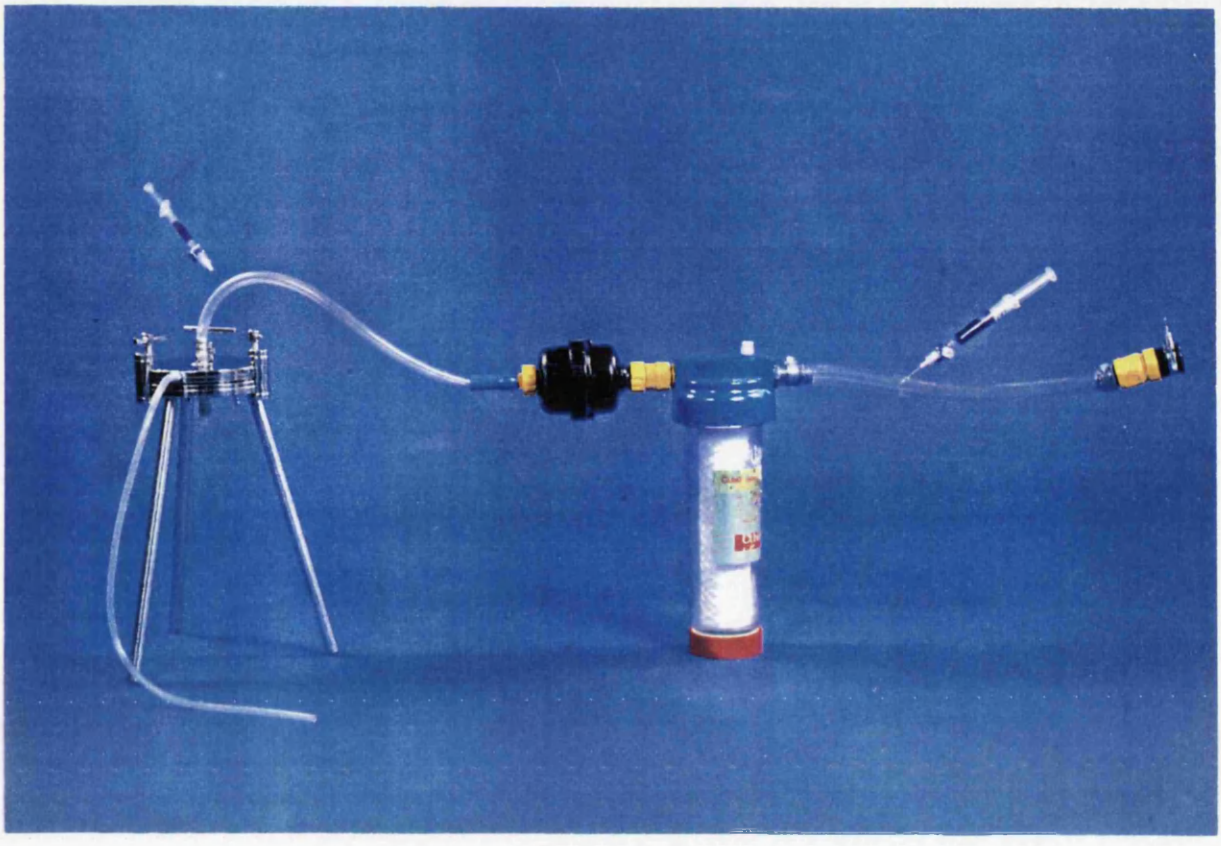


Photograph 2.3.Filter processing method.

(A) Filter cut lengthwise.

↓

(B) Core removed.

↓

(C) Filter divided into inner, middle and outer thirds.

↓

(D) and (E) Each third was teased apart.

↓

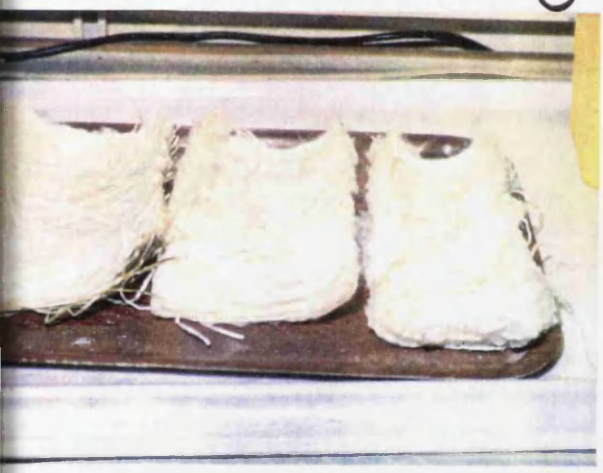
(F) Third of a filter washed in 1L 0.1 % Tween 80

in SPDL washing machine.

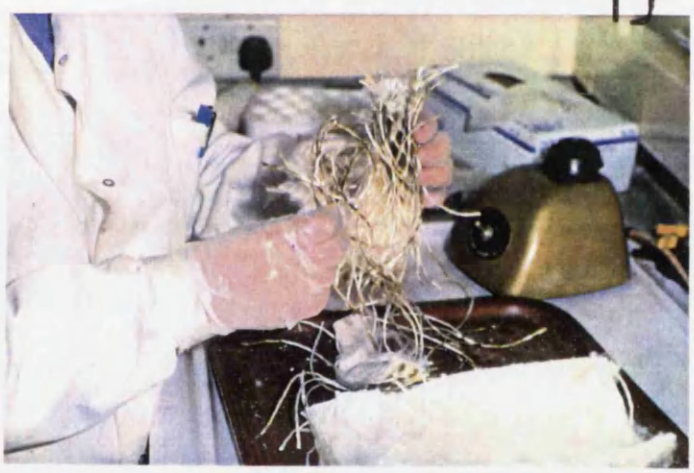
A

B

C



D



E

F

MAbs used for the detection of *Giardia* spp. cysts were supplied by Meridian Diagnostics (USA) and Cellabs (Australia), through Bradsure Biologicals, UK. The labelling procedures were the same for both organisms.

Meridian MAb. A 1 in 20 dilution of the primary antibody was made up and 25µl applied to each well of the slide, which was then incubated for 30 min at 37°C, in a humidified chamber. The slide was washed 3 times in PBS, allowed to dry and 25µl of a 1 in 20 dilution of the labelling reagent (secondary antibody with FITC conjugate) applied to each well of the slide. The slide was incubated in the dark at 37°C for 30 min, then washed 3 times in PBS and allowed to dry. Mounting medium (PBS/glycerol) was applied to the slide and a coverslip overlaid. The slide was then scanned at x 20 and x 40 objective (x 10 eyepiece) magnifications.

Cellab MAb. Staining with this MAb was carried out in the same way as for the Northumbria MAb. There was no need to reconstitute the MAb because it was supplied in solution.

2.6.5. Sand filter core samples.

Samples of sand were taken from slow sand filter beds using a core sampler. 500 g of sand were weighed out and placed in a 5 L beaker with 1 L of 0.1 % Tween 80 solution in RO water. The sand was washed/agitated for 10 min using the SPDL washing machine and then allowed to stand for a few min to allow the sand and larger particulate matter to settle. The wash water was transferred to a 1 L centrifuge bottle and centrifuged at 900 x *g* for 15 min, the supernatant aspirated and the pellet resuspended and transferred to a 250 ml centrifuge tube. At this stage the sample was processed as described for a water sample in section 2.6.3. above.

2.7. Seeding experiments to assess recoveries from Cuno filters.

Sampling apparatus was set up, with the inlet hose attached to a tap as for sampling final distribution water (photograph 2.2). Water was allowed to run through the filter at 1 L/min. After at least 10 L had passed through, a known number of oocysts were injected into the tube from the tap to the influent port of the filter cartridge housing, using an *in situ* needle with a two way luer lock valve (disco fix 2000). 100 L of water was allowed to flow through the filter after which the filter was removed from the cartridge housing and processed as described for normal water samples. Known numbers of oocysts were also seeded into 1 L centrifuge buckets, 250 ml conical centrifuge tubes, 50 ml centrifuge tubes before and after the sucrose gradient and into the 1.5 ml microfuge tubes. The method for sample processing was then followed from each stage of seeding to the endpoint, stained using the MAb and the number of oocysts recovered was counted.

2.8. Magnetic particles for immunomagnetic separation.

Immunomagnetic separation was used for the separation of oocysts from water samples. This method uses polystyrene beads with an iron based core. Two types of beads were assessed for their efficiency in the recovery of oocysts; M-450 (uncoated) and M-280 (streptavidin coated). M-450 beads were coated with anti-*Cryptosporidium* MAb and used to recover unlabelled oocysts. The M-280 beads, coated in streptavidin by the manufacturers were labelled with a MAb against FITC. The oocysts were labelled with FITC conjugated MAb. The methods for MAb purification, labelling of beads and recovery of oocysts are described below.

Suspensions of Dynabeads (Dynal U.K.) M-280 containing 6-7 x 10^8 beads/ml (10 mg/ml) in PBS with 0.1 % bovine serum albumin (BSA) and 0.02 % sodium azide (NaN_3) were purchased from Dynal UK. M-450 beads have a diameter of 4.5 μm, specific gravity 1.5 g/cm^3, magnetic susceptibility approximately 10^{-2} cgs units and surface area 3-5 m^2/g.The M-280 beads have a diameter of 2.8 μm, specific gravity 1.3 g/cm^3, magnetic susceptibility 8+/- 2 x 10^{-3} cgs units and surface area 3-8 m^2/g

2.8.1. Preparation of beads.

The vial containing the beads was mixed well to ensure that all the beads were well dispersed. The volume of bead suspension containing the desired concentration of beads was pipetted into a glass tube and washed in PBS. The tube was placed in a magnetic particle concentrator (MPC) for 60 sec after which the fluid was pipetted off and the required volume of PBS was added to give the desired concentration of beads.

2.8.2. Purification of anti-FITC.

Anti-FITC ascites fluid was supplied by Public Health Laboratory Service (PHLS), Colindale Avenue, London. Purification of the IgG was performed using caprylic acid precipitation (Steinbuch and Audran, 1969 and Russ *et al*, 1983).

1 ml of ascites was measured out and transferred to a beaker, a stirring rod added and placed on a magnetic stirrer. 2 volumes (ie. 2ml) of 60 mM sodium acetate buffer (pH 4) were added which gave a pH of ~4.8. A volume of 0.04 ml (for mouse Ab, 0.4 ml per 10 ml original volume) caprylic acid (octanoic acid) was added slowly to the ascites solution with vigorous stirring for 30 min. at room temperature. This was centrifuged at 5000 x *g* for 10 min, carefully decanted and the supernatant saved. The supernatant was transferred to dialysis tubing and dialysed versus 3 changes of PBS overnight.

2.8.3. Preparation of dialysis tubing.

Dialysis tubing was cut to the appropriate length (10 - 20 cm) and placed in a large volume (2L) of 5 mM ethylene-diamine-tetra-acetic acid (EDTA), and 200 mM sodium bicarbonate and boiled for 5 min. This was poured off, the tubing rinsed in deionised water and boiled again in an EDTA/bicarbonate wash. This was again discarded and the dialysis tubing was removed and washed in a stream of RO water. It was then ready for use.

2.8.4. Ammonium sulphate precipitation.

Following initial purification using caprylic acid (section 2.8.2.) and dialysis (section 2.8.3.), ammonium sulphate precipitation was used to further purify the sample (Harlow and Lane, 1988).

The dialysed antibody was transferred to a beaker, a stirring bar added and placed on a magnetic stirrer. While the antibody was stirring gently, sufficient saturated ammonium sulphate solution was added to bring the concentration to 50 % saturation. The solution was adjusted to pH 7 (with HCl or NaOH) at room temperature. This was transferred to 4°C for 6 h and the precipitate was centrifuged at 3000 x *g* for 30 min at 4°C. The supernatant was carefully removed and discarded and the pellet was resuspended in 0.3 - 0.5 volumes PBS of the starting volume. (Care was taken to avoid bubbles and frothing). The antibody solution was transferred to dialysis tubing and dialysed against 3 changes of PBS overnight, then removed and stored in a clean glass tube. A protein assay was performed to determine the concentration of protein present using a Lowry reagent protein assay kit (Sigma, UK).

2.8.5. Biotinylation of antibodies.

The protein was dialysed against 0.1 M $NaHCO_3$, pH approximately 8.2 - 8.6, with no azide or other preservative and the concentration was adjusted to 1.0 mg ml^{-1}. The biotinamidocaproate N-hydroxy-succinimide ester (Sigma, UK) was weighed out and dissolved in dimethylsulphoxide (DMSO) immediately before use to give a concentration of 1 mg/ml. 120 µl was added per ml of protein to the antibody, mixed and rotated at room temperature for 4 h. The biotinylated antibody was dialysed against PBS overnight. The final solution of biotinylated anti-FITC antibody was divided into 50µl aliquots and stored at -20°C, until used.

2.8.6. Labelling magnetic particles.

2.8.6.1. M-450 beads.

The MAb was adsorbed onto the surface of the particles (beads). Dynabeads M-450 were incubated with anti-*Cryptosporidium* MAb (supplied by PHLS, Colindale) for 30 min with end over end rotation at room temperature. They were washed 3 times in PBS in the MPC and resuspended in PBS to give the required concentration.

Beads were added to a suspension of oocysts in a glass test tube and the suspension mixed for 30 min with end over end rotation. Bound beads and oocysts were separated by placing the tube in the MPC for 1 min and aspirating the supernatant. Beads and oocysts were resuspended in 0.5 ml PBS and the recovery assessed using a haemocytometer.

2.8.6.2. M-280 beads.

Three methods were used to label the beads. The first method involved labelling the beads with an anti-FITC MAb (initial concentration 1 mg ml^{-1}) and the oocysts with the anti-*Cryptosporidium* MAb separately and then mixing the labelled beads and labelled oocysts together. The second method involved labelling the beads with anti-FITC MAb and then adding the anti-*Cryptosporidium* monoclonal to the beads before mixing with unlabelled oocysts. In the third method anti-FITC MAb and anti-*Cryptosporidium* MAb were added to the beads simultaneously, and the labelled beads were then added to unlabelled oocysts.

1) The volume containing the required number of beads was pipetted into a 1.5 ml eppendorf microfuge tube and resuspended in 0.5 ml PBS. 2µl anti-FITC MAb were added to the suspension of beads and mixed for 30 min at room temperature with end over end rotation. The beads were washed by placing the tube in the MPC for between 30 and 60 sec and pipetting off the supernatant. The beads were washed at least two more times in PBS. Stock solutions of 4 x 10^7 labelled beads per ml, from which dilutions could be made, were prepared in this way.

2) Beads were labelled with anti-FITC MAb as described above. After the beads were washed, they were resuspended in approximately 0.5 ml PBS and 100µl anti-*Cryptosporidium* MAb were added. This was incubated for 30 min in the dark. The beads, now labelled with anti-FITC MAb and with anti-*Cryptosporidium* MAb, were then washed in PBS as described in (1) above. Stock solutions of 4 x 10^7 labelled beads per ml, from which dilutions could be made, were prepared in this way.

3) Beads, anti-FITC MAb and anti-*Cryptosporidium* MAb were mixed in a 1.5 ml eppendorf tube, incubated at 37°C for 30 min and washed as described above.

2.8.7. Labelling oocysts for separation with beads.

Oocysts were suspended in a 1.5 ml eppendorf tube in PBS and the volume reduced to as near to 100µl as possible. If an environmental sample was being analysed, it was washed in HBSS. Pure suspensions of oocysts were easily reduced to 100µl, while environmental samples on occasions could only be reduced to 500µl. 100µl of anti-*Cryptosporidium* MAb were added to the oocysts, mixed thoroughly and incubated at 37°C in the dark for 30 min. The excess MAb was washed out 3 times with PBS.

2.8.8. Separation of oocysts with beads.

1) Oocysts labelled with MAb were separated as follows. The oocysts were resuspended to 100 µl and 100µl of the suspension of beads were added to the eppendorf containing the oocysts. The volume was made up to 0.5 ml to increase the efficiency of mixing and the tube was mixed for 30 min at room temperature on a vortex (Scotlab, UK). After mixing the tube(s) was placed in the MPC-E (Dynal, UK), designed to hold 6 x 1.5 ml eppendorf tubes for 60 sec and the supernatant was removed very carefully by pipette. The beads/oocyst mixture could be washed again with PBS if required, or resuspended in PBS in preparation for analysis. In experimental situations, when the recovery of large numbers of oocysts was to be calculated, the beads/oocyst mixture was resuspended in a known volume (0.5 ml or 1 ml) and counts were performed using a haemocytometer, to assess the recovery efficiency of the method. When the assay was used in sample analysis, or in the experimental recovery of low numbers of oocysts the mixture was resuspended in as small a volume as possible (approximately 10 µl) applied to a microscope slide, covered with a coverslip, sealed with nail varnish and the number of oocysts counted (diagrams 2.1 and 2.2.).

2) Unlabelled oocysts (i.e. no MAb) were separated from samples as follows. Suspensions of oocysts were prepared to the same concentrations and volumes as described above, except that they were unlabelled. The 'cocktail' of beads/anti-FITC MAb/anti-*Cryptosporidium* MAb was treated in the same way as the beads/anti-FITC MAb combination and mixed with the oocysts as described above. Separation of oocysts from samples and assessment of the recovery efficiency of the method followed the same protocol as 1) above.

2.8.9. Experimental Design.

2.8.9.1. Bead ratio and titration of reagents.

Bead to oocyst ratio.
Suspensions of 10^5 oocysts were prepared and labelled with MAb or left untreated as described above. Beads with the appropriate MAbs were added to the labelled or unlabelled oocysts in ratios of 4:1, 20:1 and 40:1 and separation was performed. Numbers were assessed by counting on a haemocytometer (Neubauer counting chamber).

Titration of reagents.
Oocysts were plated out onto a multispot microscope slide, air dried, fixed and labelled with 10 µl anti-*Cryptosporidium* MAb. The excess MAb was washed off, the slide allowed to dry and the oocysts were labelled with serial dilutions (1/100, 1/200, 1/400, 1/800 and 1/1600) of the anti-FITC MAb. After 30 min the slide was washed, allowed to air dry and 10µl extravidin tetramethylrhodamine B isothiocyanate (extravidin TRITC) was applied to each well (1/50 dilution). The slide was washed, allowed to dry and examined under fluorescence (filter as for PI, section 2.5.). This also served as a control to check that biotinylation of the anti-FITC MAb had been successful.

2.8.9.2. Recovery from seeded samples of PBS, pond water and diluted faeces.

Samples of PBS, pond water and diluted faeces (faecal water) were seeded with either NBI or Cellab MAb labelled oocysts to give concentrations of 1, 5, 10, 50 and 100 oocysts/ml. 5 x 1 ml aliquots of each water type and oocyst concentration were placed in 1.5 ml eppendorf tubes to which 10^6 or 10^4 beads were added. Mixing and separation proceeded as described above (diagram 2.1.). Beads and oocysts were resuspended in 10 - 20 µl PBS and the sample placed on one well of a multispot slide, allowed to air dry, a coverslip applied and the number of oocysts recovered were counted. Each experiment was repeated 5 times, using 2 MAbs, 2 concentrations of beads, 5 concentrations of oocysts and 3 different types of water.

The number of oocysts actually present in the theoretical dilutions of 1, 5, 10, 50 and 100 oocysts/ml was assessed by concentrating 1 ml of sample to 100 µl, resuspending in PBS and applying 4 x 25 µl aliquots to each well of a 4 well slide and counting the number of oocysts present.

Controls were processed according to the normal method used for assessing viability of oocysts in environmental samples. 1ml of sample was concentrated to 100 µl by microfugation and

Diagram 2.1. (this page) Flow diagram of the immunomagnetic separation technique.
Diagram 2.2. (facing page) Pictorial diagram of the immunomagnetic separation technique (from Dynal technical information).

Oocysts + MAb

↓

PBS, pond or faecal water

(to give concentration of 1, 5, 10, 50 and 100 oocysts/ml)

↓

Add magnetic beads (10^6 or 10^4)

↓

Mix for 30 min

↓

Separate in magnetic particle concentrator 1-2min

↓

Remove supernatant

↓

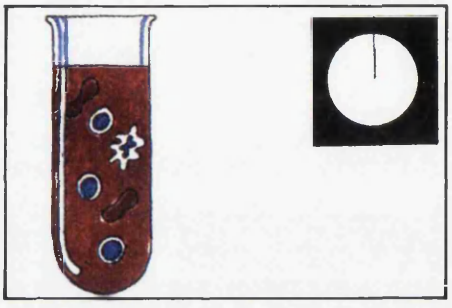
Resuspend in 10 - 20 µl PBS

↓

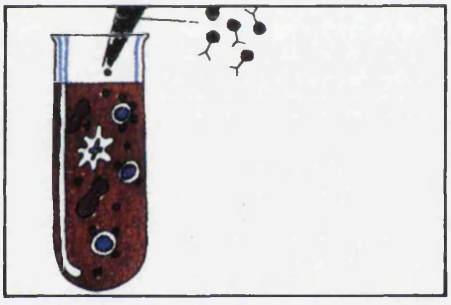
Apply to slide and count oocysts

THE TECHNIQUE

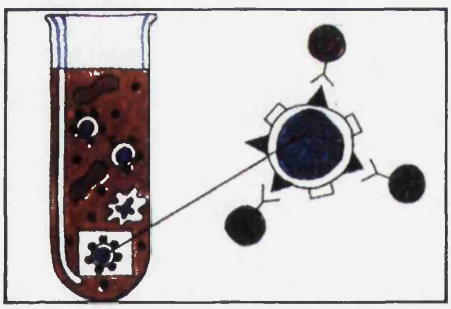
The procedure for carrying out ImmunoMagnetic Separation (IMS) is simple, rapid and reliable.



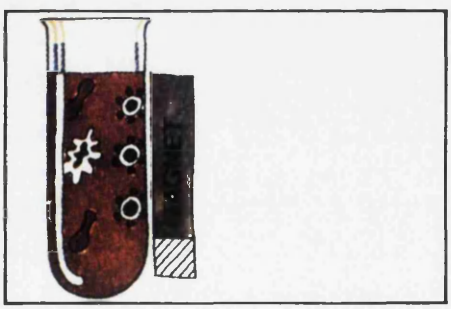
1 Prepare sample suspension:-Blood, tissue extract, culture supernatant or any other biological fluid.



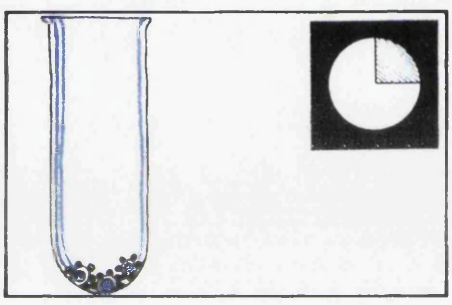
2 Add DYNABEADS® coated with antibody/protein specific to target (cells, microorganisms, DNA or proteins).



3 Incubate to allow DYNABEADS® to bind to the target. In general this incubation will be between 5 and 60 minutes at 4°C with continuous gentle mixing.



4 Apply magnet, which immobilises DYNABEADS®/TARGET complex to the side of tube. Aspirate supernatant. The unique magnetisable properties of DYNABEADS® allow complete recovery of bead/rosettes in 60 seconds using DYNAL'S Magnetic Particle Concentrator (MPC®).



5 Wash & resuspend isolated cells. This is accomplished using an MPC.® 3 washes take approximately 5 minutes.

resuspended. 10 µl of sample was applied to one well of a multispot slide, allowed to air dry, mounting medium and a coverslip applied and the number of oocysts present counted.

2.8.9.3. Recovery of oocysts from seeded sludge.

Oocyst free sludge (1 ml) was seeded with known numbers of oocysts and serial dilutions were made to give from 1 to 10^5 oocysts per sample, in multiples of 10. Subsamples were applied to four welled microscope slides and stained as described for environmental samples to check the correlation between the theoretical dilution and the actual number of oocysts detected in the samples. Beads (4×10^6) were added to the sample, mixed thoroughly and separated as before (section 2.8.8, method 1). The pellet was resuspended in approximately 10µl and applied to a microscope slide and scanned for the presence oocysts.

2.9. Ozone.

2.9.1. Ozone production.

Ozone was generated using a Labo ozone generator (photograph 2.4.), supplied by Ozotech Ltd., U.K. Oil free compressed air was fed to the LABO at 6-7 bar gauge pressure. The compressed air was dried to better than -50°C dewpoint in a twin-cell heatless (pressure swing) adsorption dryer. After drying, the air was expanded by means of a pressure regulator. In its expanded state, the dry air flowed through the ozone production cell, in which it was subjected to a high tension silent electrical discharge, causing a proportion of the two-atom molecules of oxygen in the air to be converted into the tri-atomic state of molecular ozone. The ozone production cell is water cooled, to remove the surplus heat which is produced by the electrical discharge. After passing through a flowmeter, with regulating valve, ozonised air was available either from a saturation tank equipped with a pump to recirculate the water, or from the sampling tube.

2.9.2. Determination of ozone in air.

In order to test the efficieny of the ozone production cell it was necessary to measure ozone production on a regular basis. The following iodimetric method was used.
A 1 L aspirator bottle with a tubulure bottom outlet was connected to the sampling tube. 1-2g of potassium iodide (KI) were added to the bottle and it was completely filled with RO water. The bottle was inverted, the sampled cock opened to allow ozonised air to flow out and the stopcock on the tubulure bottom opened. The stopcock in the neck of the bottle was then opened slightly to allow 825 ml of water to drain out into a measuring cylinder, being replaced

as it did so by by ozonised air from the sample tube. On completion of sample collection, the stopcocks were closed in reverse order to which they were opened and the apparatus disconnected from the sample tube. The aspirator bottle was shaken vigorously for 1 min. The sample was transferred to a conical flask, acidified with 2 ml sulphuric acid (25% v/v) and titrated against 0.1N sodium thiosulphate ($Na_2S_2O_3$) solution. A small inaccuracy associated with the loss of a little iodine in the water which was drained off during sampling could be accounted for by a 3% correction factor.

Given that 1 L of 1 N sodium thiosulphate solution is equivalent to 24g of ozone, the concentration of ozone can be calculated as follows:

$$[O_3]\ mg/l = \frac{\text{vol.(ml) 0.1 N thiosulphate x 2.4 x 103}}{\text{0.825 (sample vol. in L) x 100}}$$

2.9.3. Production of residual ozone in water.

The sample tube was connected to a Drechsel gas bottle (photograph 2.4.) containing 500 ml of RO water through which ozone was bubbled. This bottle was connected to another bottle containing 500 ml of water and approximately 5g KI, which destroyed excess ozone. In addition this bottle was connected to a destructor (heated to approximately 300°C) to ensure total destruction of excess ozone. The ozone production cell was switched on (air flow 300 l/h, pressure 0.55 bar, power 160 W) and allowed to run, bubbling ozone through the sample water for a period of time, which was dependent on the concentration of ozone required.

2.9.4. Measurement of residual ozone in water.

2.9.4.1. Iodimetric technique.

After the water was ozonated the ozone production cell was switched off and 100 ml of ozonised water was measured out into a measuring cylinder containing 3g KI (BDH, UK) and 3g iodine indicator (Fisons, UK). This resulted in the formation of a deep purple colour. The solution was transferred to a beaker containing 2 ml sulphuric acid (25% v/v) and titrated against 0.01N sodium thiosulphate (Fisons, UK). The volume of thiosulphate used was measured and the concentration of residual ozone was calculated using the following equation:

$$[O_3]\ mg/l = \frac{\text{vol.(ml) 0.01N thiosulphate x 240}}{100}$$

Photograph 2.4. Ozone generator (A) and drechsel bottles (arrowed).


A

2.9.5. Oocyst exposure to ozone.

Three different experimental systems were used to expose oocysts to ozone. These are described below.

Method 1.

The sampling tube was attached to the Drechsel bottles as described in the production of ozone residual in water above. The RO water in the first bottle, which was to be used for experimental and measurement purposes, was held at 5°C or 20°C. After the water had been ozonated for the required time, 100 ml were used to measure residual ozone and a series of 10 ml aliquots were decanted into 25 ml sterile glass universals, to which 200µl of oocyst suspension (5 x 10^6/ml) were added. After incubation at the relevant temperature for contact times between 2.5 and 30 min, the ozone was neutralised by the addition of 3 drops of 0.1 N sodium thiosulphate. At this point a further 100 ml of water was removed from the Drechsel bottle and the residual ozone was measured as described. The universal was vortexed and 2 x 2 ml aliquots of the sample were removed, washed 3 times in HBSS and viability was assessed using the assay described in section 2.4.1. above. Controls consisted of oocysts helds in RO water at the two temperatures tested and oocysts in RO water with 3 drops of 0.1 N sodium thiosulphate.

Method 2

Ozonated RO water at 5°C and 20°C was produced in a Drechsel gas bottle as described in method 1 above. 100 ml was removed and used to measure ozone residual. Immediately after the time zero (T=0) ozone concentration measurement was made, 5x10^6 purified oocysts of bovine origin were added to the ozonated water. At times 2 min, 4 min, 6 min and 10 min, 50 ml of the ozonated water were decanted into a 50 ml centrifuge tube containing sodium thiosulphate to neutralise the ozone. The tubes were centrifuged at 900 x ***g***, aspirated to approximately 1 ml, resuspended, decanted into a 1.5 ml microfuge tube, centrifuged at 12,500 x ***g*** for 30 sec and washed in HBSS. Viability was assessed as previously described. At the final time point, 10 min, a further 100 ml was measured out and residual ozone was measured.

Method 3

The column reservoir of a laboratory contactor (Ozotech Ltd., UK) was filled with chlorine free water and ice, which was allowed to melt to cool the water to less than 1°C. The columns were filled and the ice/water circulated to cool the system. The ozone generator was set up to give the required concentration of ozone and air flow. Chlorine free water was cooled with chlorine free ice to make 7 L at approximately 2°C, and placed in the column reservoir. The cooling water was drained out rapidly to prevent the system warming. The water in the

reservoir was inoculated with 7 x 10^6 oocysts, the column filled, the sample circulated and the ozone supply turned on. Ozone doses of 1, 3 and 5 mg/L were tested at 5 and 10 min contact times. At the end of the contact time the ozone supply to the columns was turned off, a 250 ml sample was taken for residual analysis, and the water was filtered through a 1.2μm cellulose nitrate membrane filter (Sartorius GmbH, Germany) in order to recover the oocysts. The filter was cut into strips and washed by agitation in a 50 ml centrifuge tube containing sodium thiosulphate (to neutralise the ozone) and a solution of 0.1% Tween 80. The supernatant was decanted into a 50 ml tube and concentrated by centrifugation as described for method 2. Oocyst viability was assessed using the assay described before in section 2.4.1. Controls consisted of stock oocysts and oocysts which were circulated around the contactor system under the same conditions at which the experiments were carried out, with the exception that the water was not ozonated.

2.10. Destruction of oocysts by sand and chlorine.

The destructive effects of sand on oocysts and subsequent chlorination were assessed by shaking oocysts with sand. Viability was assessed after agitation of oocysts in sand and again after subsequent chlorination. The experimental design and methods used are described below.

Sand (grain diameter ranged from 0.58 - 0.63 mm) was obtained from a slow sand filter and tested for the presence of *Cryptosporidium* spp. oocysts. A large quantity of sand was washed in RO water by agitation and then allowed to settle for a few seconds to remove the smaller particulate matter, before discarding the wash water. This was repeated several times until the wash water was clear. The sand was then washed in 10 % sodium hypochlorite (Charles Tennant, Blantyre, UK) to sterilise it and washed a further 4 to 5 times in RO water to dilute out the hypochlorite. 10g aliquots of sand were weighed out into 50 ml centrifuge tubes and washed again in RO water immediately before starting the experiments.

2.10.1. Shaking of oocysts with sand.

A stock solution of purified oocysts (3.4 x 10^6 oocysts/ml) of bovine origin was prepared. Two ml of RO water and 100μl of the oocyst stock solution were added to each tube. Tubes were placed on a horizontal shaker and rotated at 128 rpm, for 5, 30, 60, 90 min and 2 h. Controls consisted of oocysts sampled directly from the stock solution, oocysts left in unshaken sand, and oocysts shaken in RO water and sampled at the above time points.

At each time point a tube was removed from the shaker, the sand was allowed to settle and 2 ml of the fluid pipetted into a 2 ml microfuge tube (Sarstedt (U.K.), Leicester). This was

centrifuged at 12,500 x *g* for 15 sec and the pellet washed in 2 ml of Hanks' Balanced Salt Solution (HBSS) without phenol red. Oocyst viability was then assessed.

2.10.2. Chlorine disinfection of oocysts shaken with sand.

Oocysts were treated with chlorine after shaking with sand for 5 min. After allowing the sand to settle, 2 ml of fluid were removed, added to a 50 ml centrifuge tube containing 25 ml of 1 mg/l free chlorine and allowed to stand for 5 min at room temperature. Sodium thiosulphate was added to neutralise the chlorine and the tube was centrifuged at 900 x *g*. The supernatant was aspirated, leaving approximately 1 ml and the pellet resuspended, decanted into a 2 ml microfuge tube and washed in HBSS, as above. Oocyst viability was then assessed (section 2.4.1.).

2.10.3. Preparation of chlorine solutions.

Dilutions of sodium hypochlorite containing 14-15 % available chlorine were made with chlorine demand free RO water to the required concentration (as indicated below, section 2.10.3.1.).

2.10.3.1. Determination of calibration curve for measurement of chlorine concentration.

A buffer solution was made by dissolving 2.4g disodium hydrogen phosphate and 4.6g potassium dihydrogen phosphate in RO water, 10 ml of 8 g/l EDTA were added and the mixture diluted to 100 ml with water.

A 0.0891 g/l solution of potassium permanganate was prepared and 0.0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 ml volumes were measured out into a series of 100 ml volumetric flasks and made up to the mark with water. These solutions were equivalent to 0.0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l of chlorine respectively when reacted and measured with diethyl-*p*-phenylene diamine (DPD). 5 ml of DPD and 5 ml of buffer solution were mixed in a 250 ml conical flask and 100 ml of the first potassium permanganate standard (0.5 ml) equivalent to 0.5 mg/l chlorine were added, the solution mixed thoroughly and the absorbance was measured immediately at 550 nm in a glass cell, with 10 mm light path, in a spectrophotometer (Cecil). The reference cell contained water. The same procedure was repeated for the other standards. This gave a calibration curve for determination of chlorine concentration in solutions.

2.10.3.2. Determination of free available chlorine.

Free available chlorine was measured using the diethyl-*p*-phenylene diamine (DPD) (Wallace and Tiernan, Kent, U.K.) method (Palin, 1967 and 1978) described above for producing a calibration curve using potassium permanganate. The chlorine solutions to be tested were used in place of the potassium permanaganate solutions, the absorbance measured and the concentration was obtained by extrapolation of the optical density (O.D.) reading with concentration of potassium permanganate on the calibration curve (graph not shown).

Free chlorine was also determined by the DPD method, using Residosol A and B test reagent solutions (Wallace and Tiernan, Kent, UK). For the determination of free chlorine up to 1 mg/l, 2 drops of Residosol A and three drops of Residosol B were added to a 10 ml measuring cylinder and made up to 10 ml with the sample water and mixed thoroughly. The absorbance at 550 nm was measured on spectrophotometer, as before (section 2.10.3.1.). Sample water was used as a blank. If the chlorine residual in the sample under test was expected to exceed 1 mg/l then 4 drops of Residosol A and 6 drops of Residosol B were used. If the chlorine residual was expected to exceed 4 mg/l, a dilution of a portion of the original sample was made to bring it within the range of the assay before proceeding with the determination of chlorine. A standard calibration curve was obtained using the same method, with Residosol solutions A and B, and potassium permanganate.

2.11. Superoxide dismutase.

Several methods were used to assay for the antioxidant anzyme superoxide dismutase (SOD), including spectrophotometry and electrophoresis.

2.11.1. Spectrophotometrical measurement of superoxide dismutase activity.

2.11.1.1. 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium reduction assay.

This method employed xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase activity was then measured spectrophotometrically (550nm) by the degree of inhibition of this reaction. Reagents were purchased from Randox, N. Ireland.

Sample preparation

Purified suspensions of oocysts (bovine, c/o and lamb isolates) were washed and resuspended to 1 ml in RO water and snap freeze thawed between 1 and 3 times in liquid nitrogen. Each suspension was centrifuged at 12,500 x *g* for 60 sec and the supernatant used in the assay, the pellet being discarded. Oocyst concentrations are given in chapter 8, section 8.3.

Reagents

Mixed substrate was made up containing xanthine and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) at initial concentrations of 0.05 mmol/l and 0.025 mmol/l respectively. A buffer of CAPS (3-[cyclohexamylamino]-2-hydroxyl-1-propanesulfonic acid) and EDTA at initial concentrations of 50 mmol/l (pH 10.2) and 0.94 mmol/l respectively, was made up.

Xanthine oxidase was used at an initial concentration of 80 U/l. A series of superoxide dismutase standards was made by diluting a solution with an initial concentration of 3.75 U/ml in a 10 mM phosphate buffer pH 7.0.

All reagents were warmed to 37°C and reaction mixtures were incubated at 37°C. Readings were made at room temperature. A reagent blank was made up by pipetting 0.05 ml phosphate buffer and 1.7 ml mixed substrate into a cuvette with a 1 cm path length and mixing well. 0.25 ml xanthine oxidase was added to the mixture and the initial absorbance read after 30 sec on a spectrophotometer (CE 292, Cecil, Cambridge, UK) at 505 nm wavelength. The final absorbance was measured 3 min later, the cuvette having been incubated at 37°C for the intervening period. Measurements were made against air.

The standard curve was produced by measuring the absorbance of the dilutions of the known standard against air as described for the reagent blank above, using 0.05 ml of the standard to be measured on each occasion instead of phosphate buffer. Similarly, the absorbance of the sample was measured and recorded as described using 0.05 ml of sample supernatant in place of the phosphate buffer.

Calculation

The change in absorbance per minute (ΔA/min) of the standard or sample was calculated by subtracting the final absorbance reading A2 from the initial absorbance reading A1 and dividing by 3 min.

$$\frac{A2 - A1}{3} = \Delta A/\text{min of standard or sample.}$$

The reagent blank rate is the rate of the uninhibited reaction, which = 100 %. All standard rates and diluted sample rates were converted into percentages of the blank rate and subtracted from 100 % to give the percentage inhibition.

$$100 - \frac{(\Delta A_{\text{std or sample/min}} \times 100)}{(\Delta A_{\text{blank/min}} \times 100)} = \% \text{ inhibition}$$

The percentage inhibition for each standard was plotted against Log_{10} (concentration of the standard in SOD units/ml) and the percentage inhibition of the sample was used to obtain units of SOD from the standard curve.

2.11.1.2. Ferricytochrome *c* reduction assay.

The reduction rate of cytochrome *c* by superoxide radicals can be monitored spectrophotometrically (550 nm) using xanthine and xanthine oxidase as the source of superoxide ions. SOD competes for superoxide and decreases the reduction rate of cytochrome *c*, similar to the system described above (McCord and Fridovich, 1969; Flohé and Ötting, 1984).

Sample preparation

Suspensions of oocysts were prepared as described for the previous assay (section 2.11.1.1.).

Reagents

Potassium dihydrogen phosphate
Disodium hydrogen phosphate
Ethylenediaminetetraacetic acid disodium salt (EDTA)
Xanthine, crystalline (99 - 100 %)
Xanthine oxidase
Cytochrome *c*

Solution A. 0.76 mg (5 μmol) xanthine in 10 ml 0.001 N sodium hydroxide and 24.8 mg (2 μ mol) cytochrome *c* were admixed with 100 ml 50 mM phosphate buffer pH 7.8 containing 0.1 mM EDTA. This solution is stable for 3 days at 4°C, although it was kept at 25°C during experimental procedures.

Solution B. A freshly prepared solution of xanthine oxidase, approximately 0.2 U/ml, in 0.1 mM EDTA and kept on ice.

Procedure

2.9 ml of solution A were pipetted into a test tube, 50 µl of sample (as in the previous assay this was substituted with either water, SOD standards or unknowns) and the reaction started with 50 µl of solution B. The solution was mixed, transferred to a cuvette and the absorbance change per min ($1/\Delta E$ min^{-1}) was measured at 550 nm. This was plotted against the concentration of SOD standards and the concentration of SOD in unknown samples was obtained by extrapolation (graph not shown).

2.11.2. Electrophoretic detection of superoxide dismutase.

2.11.2.1. Horizontal agarose gel electrophoresis.

Methyl thiazolyl tetrazolium (MTT) is reduced by electron donors to form a dark blue-purple insoluble formazan. The reaction proceeds rapidly in the presence of phenazine methosulphate (PMS) which acts as an intermediary catalyst. After exposure to light the enzyme can be seen as pale zones on the dark background formed by the formazan.

Sample preparation

Oocysts suspensions were prepared as described in section 2.11.1.1.

Gel preparation

Homogenous gels containing 1 % agarose were prepared by dissolving 0.3 g of agarose in 27 ml of heated gel buffer (0.01 M phosphate buffer, pH7), which was poured on to a clamped mould and cooling tray, removing all air bubbles and allowed to set. The gel was placed on a horizontal gel bed (Pharmacia Flat Bed apparatus, FBE 3000) and connected to the electrodes with saturated ultrawicks. The electrode buffer used at the cathode was 1 M sodium hydroxide (300 ml) and at the anode was 0.05 M sulphuric acid (300 ml). Small pieces of filter paper (2mm x 3 mm, Pharmacia Ltd., UK, Cat No. 17-0651-01) were soaked in wells of a microtitre plate containing 10µl of sample for 30 s following which a further 10 µl were applied to the well. After the filter strips were soaked for a further 30 s, they were placed on the gel which was then run at 12 V/cm for 4 h (1000 V for 45 - 60 min), with cooling.

Following electrophoresis, the gel was removed from the apparatus, transferred to a clean, level glass plate, clamped and sealed at the edges with 2 % agarose sealing solution. 5 mg of methyl

thiazoyl tetrazolium (MTT) in 1 ml of water, 5 mg of phenazine methosulphate (PMS) in 1 ml water were mixed with 25 ml 0.05 M Tris/HCl buffer pH 8.0 and 25 ml 2 % agarose solution.This solution was poured onto the glass plate holding the clamped, sealed gel, exposed to light for several minutes and then incubated at 37°C. The enzyme, if present, is seen as pale zones on a dark background.

2.11.2.2. Polyacrylamide gel electrophoresis.

Nitro blue tetrazolium (NBT) is reduced by transfer of electrons from photoreduced riboflavin, resulting in the formation on the gel of a blue colour. Where SOD was present, pale zones were visible in contrast to the blue colour (Beauchamp and Fridovich, 1971).

Sample preparation

Suspensions of oocysts were prepared as described above (section 2.11.1.1.). 50 µl of the supernatant were decanted into a clean eppendorf tube, to which 50 µl of loading/sample buffer were also added. Control A consisted of 50 µl of a 1 mg/ml SOD solution containing 3570 SOD U/mg and 50 µl of loading/sample buffer. Control B was 50 µl of a solution containing 3.75 SOD U/ml and 50 µl of loading/sample buffer.

Reagents

Precast native polyacrylamide gels were purchased from Biorad (UK).

Loading/sample buffer - 5 ml TRIS pH 7.5 were mixed with 10 ml glycerol and 2 ml 0.2 % bromophenol blue.

Running buffer (5 x concentrate) - 43.2 g of glycine and 3.0 g of TRIS were made up to 600 ml with RO water. For final use, 100 ml of the concentrated running buffer were mixed with 400 ml of RO water.

Nitro blue tetrazolium, 2.45×10^{-3} M

Tetramethylenediamine (TEMED), 0.028 M

Riboflavin, 2.8×10^{-5} M

Potassium phosphate pH 7.8, 0.036 M

Procedure

The gel was submerged in running buffer and 20 µl of sample and 10 µl each of controls A and B were loaded onto the gel and run for approximately 1 h at 200 V and 100 mA. The gel was removed and SOD was localised by soaking in 2.45×10^{-3} M NBT for 20 min, followed by immersion in a solution containing 0.028 M TEMED, 2.8×10^{-5} M riboflavin and 0.036 M potassium phosphate (pH 7.8), with subsequent illumination for 5 to 15 min on a

transiluminator. The gels became uniformly blue during illumination, except at positions where SOD was present, where there was an achromatic zone (Beauchamp and Fridovich, 1971).

2.12. Statistical analyses.

In order to compare the occurrence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts detected in samples of water from different types of treatment, or the seasonal occurrence, Chi squared test and Fischer's exact probability test were performed. To compare the concentrations of oocysts and cysts detected in different types of water, between seasons or in samples of water from different types of treatment, the Mann-Whitney U test was used.

Two-way analysis of variance was used to test for significant differences in oocyst recoveries dependent on the number of beads used, the MAb used and to assess the differences in recoveries from different types of water.

Analysis of covariance was used to compare the effects of different parameters on the viability of oocysts exposed to ozone. Chi squared test was used to test for significance between the two methods used for ozonation.

One way analysis of variance (ANOVAR) was used to test for significant differences in oocyst viability between controls and treatment with sand and chlorine. Following arc-sin transformation of percentage values, a two tailed unpaired t-test was used to locate the significance within ANOVAR (i.e. whether there was a significant difference between treatment with sand and treatment with sand and chlorine).

Chi squared, Mann-Whitney U test, analysis of variance and analysis of covariance were performed using a Minitab statistical analysis package. Fischer's exact probability test was performed manually.

CHAPTER 3. THE OCCURRENCE OF *CRYPTOSPORIDIUM* SPP. OOCYSTS AND *GIARDIA* SPP. CYSTS IN WATER.

3.1. Introduction.

Cryptosporidium and *Giardia* are known to cause diarrhoeal disease in man throughout the world. Infection is established when the oocyst or cyst stage is ingested either directly or indirectly, via the faecal-oral route. The minimum infectious dose for *Cryptosporidium* is, as yet, not fully known, although studies in lambs indicate that it could be as low as 5 oocysts (Blewett *et al*, 1992) and 1 oocyst has been shown to cause infection in mice (Kwa *et al*, 1992). Infected livestock can excrete up to 10^{10} infective oocysts during the course of infection (Blewett, 1989). The infectious dose for *Giardia* is considered to lie between 10 and 100 organisms (Rendtorff, 1954).

Concern has, more recently, been focused on water as a medium for transporting the infectious stages of these parasites. There have been numerous reports over the last 30 years of waterborne outbreaks of giardiasis (Craun, 1988 and 1991) and within the last six years cryptosporidiosis has also been documented as a waterborne disease, with several outbreaks being reported in the United States (D'Antonio *et al* 1985, Hayes *et al* 1987, Rose *et al* 1987). The first reported outbreak of cryptosporidiosis in the United Kingdom occurred in 1988 (Smith *et al* 1989) and was a result of post-treatment contamination. Other reports, notably one which occurred in the Swindon/Oxford area in 1989 (Richardson *et al* 1991), have been the result of contamination of potable water prior to treatment; *in situ* treatment had not removed or inactivated the oocysts. Agricultural runoff and sewage effluent have been named as possible sources of contamination of surface water used for abstraction as potable water.

Methods developed for the isolation and detection of protozoan parasites in water (Anon, 1990b) enable detection of the causative organism where water contamination is indicated or disease is reported. Furthermore, methods are available to detect these parasites in environmental samples, thus providing an indication of the extent and level of environmental contamination Studies undertaken primarily in the USA and also in the UK (Smith *et al*, 1990; Gilmour *et al*, 1990; Smith and Rose, 1990) have investigated the occurrence and levels of *Cryptosporidium* and *Giardia* in a variety of water types. Their results indicate that these parasites are ubiquitous in the environment.

The number of documented cases of diarrhoeal disease being caused by the consumption of drinking water contaminated by these parasites has demonstrated the importance of the waterborne route of transmission. It is important that drinking water should be free of these

parasites, especially as the minimum infectious dose for both *Cryptosporidium* and *Giardia* is low. Assessment of the numbers of these organisms present in surface water used for abstraction as drinking water and also in distribution or passing through conventional water treatment into distribution is therefore essential.

A survey has been undertaken in which samples of a variety of waters were examined for the presence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts. This section reports their occurrence and concentration in surface water and treated water in the U.K. Data are also presented on the presence of these parasites in raw sewage and sewage effluent which is of concern as a possible route of contamination of water courses.

3.2. Materials and Methods.

The materials and methods are described in chapter 2, section 2.6.

3.3. Results.

3.3.1. Surface Water.

3.3.1.1. Occurrence.

A total of 196 samples were tested for *Cryptosporidium* spp. oocysts and 155 for *Giardia* spp. cysts over a 17 month period, the results of which are shown in table 3.1. and figure 3.1. *Cryptosporidium* oocysts were detected in 77 (39.28%) of the samples tested, at a range of 0.006 - 15.6 oocysts/L and *Giardia* cysts were detected in 61 (39.35%) of the samples tested at a range of 0.009 - 2.1 cysts/L. The mean number of oocysts detected (0.67 oocyst/L) was 3.6 times greater than the mean number of cysts detected (0.19 cyst/L), while the median concentration of oocysts (0.102 oocyst/L) was 1.9 times that detected for *Giardia* cysts (median concentration 0.053 cyst/L). At least one of the parasites was detected in 84 (54.19%) of the samples analysed, while both were detected concurrently in 34 (21.93%) of the samples (table 3.2. and figure 3.2.). No significant difference was obtained between the occurrence of *Cryptosporidium* oocysts and *Giardia* cysts, although the concentration of oocysts detected was significantly greater than the concentration of cysts detected ($p<0.001$).

Of the 196 samples tested, information on 155 was obtained from the relevant water authorities on whether they were exposed to agricultural pollution, domestic animal pollution, natural wildlife pollution and/or sewage effluent. The results are shown in table 3.4. and figures 3.9. and 3.10.

The occurrence of oocysts and cysts in waters which received treated sewage effluent (from municipal sewage treatment works) was significantly greater than the occurrence in waters which did not receive such discharges (X^2 = 18.61 and 13.51 for *Cryptosporidium* and *Giardia* respectively, $p<0.001$).

The monthly occurrence and mean and median concentrations of oocysts and cysts are shown in table 3.1. and figures 3.3. and 3.4. The maximum mean concentration of oocysts was detected in May 1990 and the maximum median concentration in April 1989. For *Giardia* these maximum values occurred in April 1990 (mean) and March 1990 (median). The minimum mean concentration of oocysts was detected in March 1989 and the minimum median in December 1989. For *Giardia* both the minimum mean and median concentrations occurred in May 1989. No significance was detected in the occurrence or concentration of either parasite between the months or when the months were grouped into seasons (eg. spring = March, April, May. etc.). These data are presented in figure 3.11.

3.3.1.2. Type of water.

Reservoir water

Most of the samples analysed were samples of reservoir water (115 for *Cryptosporidium* and 108 for *Giardia*), of which oocysts were detected in 44 (38.26%) of the samples and cysts in 44 (40.74%) of the samples (table 3.4. and figures 3.9. and 3.10.) *Cryptosporidium* (60%) and *Giardia* (68.7%) occurred significantly more frequently in reservoir water which received discharges of sewage effluent when compared to those which did not receive such discharges, where *Cryptosporidium* oocysts were detected in 28.75% and *Giardia* cysts in 28.94% of the samples. (*Cryptosporidium* X^2 = 10.7, $p<0.01$; *Giardia* X^2 = 14.78, $p<0.001$). No significant difference was observed in the concentration of oocysts (0.008 - 2.3 oocysts/L) or cysts (0.01 - 0.181 cyst/L) detected in reservoirs receiving discharges of sewage effluent compared to the concentration of oocysts (0.006 - 2.1 oocysts/L) and cysts (0.009 - 0.27 cyst/L) detected in reservoirs which received no discharge. The mean concentration of oocysts (0.201 oocyst/L) detected in reservoirs with sewage contamination was 1.36 times higher than the mean (0.147 oocyst/L) detected in non-contaminated reservoirs, while the median concentrations were the same (0.04 oocyst/L). The mean concentration of cysts detected in reservoir water was the same (0.07 cyst/L) whether there was sewage contamination or not, while the median number of cysts detected in reservoirs with contamination (0.067 cyst/L) was 2.06 times greater than the median concentration of cysts (0.032 cyst/L) detected in samples of reservoir water which received no sewage effluent discharge.

Of the 32 samples which were analysed for both parasites, one or other was detected in 24 (75%) of the samples taken from water receiving discharge and 35 out of 76 (46%) which did not receive any sewage effluent discharge. Both parasites were detected concurrently in 12 out of 32 (37.5%) samples from reservoirs receiving a discharge and 11 out of 76 (14.5%) of those not receiving a discharge.

River water

A total of 40 river samples were examined for *Cryptosporidium* oocysts, of which 27 were also examined for *Giardia* cysts (table 3.4. and figures 3.9. and 3.10.). Oocysts were detected in 27 out of the 40 samples (67.5%) and cysts in 16 of the 27 samples (59.26%). These were further divided into rivers which either did or did not receive discharges of sewage effluent. Oocysts occurred in 75% of those receiving a discharge at a range of 0.1 - 15.6 oocysts/L (mean 1.646 oocysts/L, median 0.8 oocyst/L) and in 50% of those not receiving a discharge at a range of 0.02 - 0.07 oocyst/L (mean 0.039 oocyst/L, median 0.035 oocyst/L). There was no significant difference in the occurrence of oocysts in samples of river water whether they received contamination from sewage effluent or not, but there concentration of oocysts was significantly greater in river water receiving a discharge compared to river water not receiving a discharge ($p<0.005$). *Giardia* cysts were detected in 60% of waters receiving sewage at a range of 0.02 - 2.1 cysts/L (mean 0.61 cyst/L, median 0.1 cyst/L) and 58.33% of those not receiving sewage effluent at at range of 0.014 - 0.125 cyst/L (mean 0.037cyst/L, median 0.027 cyst/L). There was no significant difference in the occurrence of cysts between the two river types, but significantly greater concentrations of cysts were detected in those receiving sewage effluent compared to those not receiving sewage effluent ($p<0.05$). The mean and median concentrations of oocysts detected in rivers with sewage effluent were 42.2 and 22.86 times the mean and median concentrations of oocysts in river water not receiving sewage effluent discharge. The mean and median concentrations of cysts in water receiving a sewage effluent discharge were 16.48 and 3.7 times the mean and median concentrations respectively in water not receiving a sewage discharge.

In water receiving a sewage effluent discharge oocysts and cysts were detected concurrently in 9 out of 15 (60%) samples, while 11 out of 15 (73.3%) contained either one or both parasites. In rivers not receiving sewage effluent 4 out of 12 (25%) samples contained both and 9 out of 12 (75%) contained either one or both.

Recreational water

Twenty one samples were taken from surface waters used for recreational purposes (eg. water sports such as wind surfing), and *Cryptosporidium* oocysts were detected in 4 (19%) and *Giardia* cysts in 100 % (table 3.4. and figures 3.9. and 3.10.). Some of these areas received sewage effluent discharges, while the rest received no apparent contamination of this sort. Oocysts were detected in 2 of 15 (13.3%) samples of water taken from those receiving sewage effluent at a range of concentrations from 0.66 - 1 oocyst/L (mean and median 0.83/L) and in 2 of 6 (33.3%) not receiving any sewage discharge at a range of 0.33- 0.67 oocyst/L (mean and median 0.5/L). The mean and median values were 1.66 times higher in water which received sewage effluent discharge than in those which did not. *Giardia* cysts were detected in all samples regardless of whether they received sewage effluent or not. In waters with sewage effluent the concentration of cysts ranged from 0.31 - 3.85 cysts/L (mean 1.48 cysts/L, median 1.25 cysts/L) and in those not receiving sewage effluent, the concentrations ranged from 0.05 - 2.67 cysts/L (mean 0.79 cyst/L, median 0.5 cyst/L). The mean and median concentrations of cysts in water receiving sewage effluent compared to water not receiving sewage effluent were 1.89 and 2.5 times greater respectively than the mean and median values in water with no discharge. There was no significant difference in the occurrence or concentration of either parasite, independent of whether it received sewage discharge or not.

3.3.2. Waste water.

3.3.2.1.Municipal treatment.

Raw sewage

Six samples of raw sewage influents were analysed for the presence of *Cryptosporidium* and *Giardia*, all of which were positive for both parasites (table 3.5.). A mean of 29.58 oocysts/L was detected (range 2.5 - 75 oocysts/L) and a mean of 478.33 cysts/L was detected (range 242.5 - 792.5 cysts/L).

Treated effluent

Seventy samples of secondarily treated sewage effluents from ten sewage treatment works in Scotland were analysed for the presence of oocysts and cysts (table 3.5.). Oocysts were detected in 25 of the 70 samples (35.71%) at a range of 0.024 - 26.5 oocysts/L (mean 1.76 oocysts/L) and cysts were detected in 58 of the 70 samples (82.86%) at a range of 0.095 - 361.7 cysts/L (mean 25.07 cysts/L). *Giardia* cysts occurred with greater frequency (X^2 =

32.226, d.f. = 1 and $p < 0.0001$) and at a significantly greater concentration ($p < 0.0001$) than *Cryptosporidium* oocysts.

3.3.2.2. Private treatment.

Oocysts and cysts were detected concurrently in 1 of 3 private treated sewage effluents at a concentration of 0.013 organisms/L (table 3.5.)

3.3.2.3. Industrial sewage.

Raw sewage

Cryptosporidium (92.5 - 149,100 oocysts/L) and *Giardia* (10 - 42.36 cysts/L) were detected concurrently in animal slaughterhouse effluents (table 3.5). *Cryptosporidium* oocysts were detected in untreated poultry slaughterhouse effluent at a concentration of 292.3 oocysts/L, but *Giardia* cysts were not detected.

3.3.3. Removal of oocysts and cysts by sewage treatment.

In the case of one sewage treatment plant, 5 samples of raw influent sewage and 5 samples of treated effluent were analysed and both parasites were detected in all 10 samples. These results are presented in table 3.5.). *Cryptosporidium* oocysts were detected at a concentration range of 2.5 - 75 oocysts/L (mean 29.5 oocysts/L, median 30 oocysts/L) and *Giardia* cysts were detected at a concentration range of 242.5 - 792.5 cysts/L (mean 489 cysts/L, median 497.5 cysts/L) in the raw sewage. In the treated sewage oocysts were detected at a concentration range of 0.42 - 26.5 oocysts/L (mean 6.03 oocysts/L, median 0.7 oocysts/L) and cysts were detected at a concentration range of 0.65 - 10.9 cysts/L (mean 5.39 cysts/L, median 3.4 cysts/L).

For *Cryptosporidium* the mean and median values in raw sewage were 4.89 and 42.86 times greater, respectively, than in the treated, showing a statistically significantly lower concentration of oocysts in the sewage after treatment ($p < 0.05$). The mean and median concentrations of *Giardia* cystswere 90.72 and 146.32 times greater, respectively, in raw than in treated sewage. A significant difference was seen between the concentrations before and after treatment ($p < 0.05$). The concentration of *Giardia* cysts in raw sewage was significantly greater than the concentration of *Cryptosporidium* oocysts ($p < 0.05$), while in treated sewage there was no significant difference between the concentration of the two parasites.

The removal of oocysts by sewage treatment, based on mean concentrations was 79.57 % and based on median concentrations was 97.67 %. The removal of cysts, based on mean concentrations was 98.9 % and based on median concentrations was 99.32 %.

The sewage treatment plant from which these samples were taken had a dry flow rate of 10.224×10^6 L/day and wet flow rate of 13.632×10^6 L/day. The mean concentration of *Cryptosporidium* oocysts detected in the effluent was 6.03 oocysts/L and *Giardia* cysts was 5.39 cysts/L. Consequently, the number of oocysts and cysts discharged in the effluent under dry flow conditions would be 6.2×10^7 oocysts/day and 5.5×10^7 cysts/day and under wet flow conditions would be 8.2×10^7 oocysts/day and 7.3×10^7 cysts/day for *Cryptosporidium* and *Giardia* respectively.

3.3.4. Treated water.

3.3.4.1. Occurrence.

Over the 17 month period, a total of 209 treated waters were sampled for *Cryptosporidium* spp. oocysts and 199 for *Giardia* spp. cysts. *Cryptosporidium* oocysts were detected in 78 (37%) of those samples and *Giardia* cysts were detected in 33 (16%) of samples. The mean and median numbers of oocysts and cysts detected per litre were 0.075 and 0.03 (range 0.008-1.36) oocysts/L and 0.037 and 0.016 (0.007-0.34) cysts/L, respectively (table 3.6. and figures 3.5. and 3.6.). *Cryptosporidium* oocysts occurred with both significantly greater frequency ($\chi^2 = 22.14$, $p < 0.001$) and at a significantly higher concentration than *Giardia* cysts ($0.01 < p < 0.05$) in the samples tested.

Oocysts were detected in treated water in every month of the survey except in January when only one sample was tested and in February sampling was not conducted (table 3.6. and figure 3.2.). *Giardia* cysts were detected in 12 out of the 17 months. In February 1989 and May 1990 samples were not examined for *Giardia*. In January 1989, as for *Cryptosporidium,* only one sample was taken. In both May and November 1989, although 14 samples were examined for *Giardia* cysts, none was detected.

For both parasites, the highest percentage of positive samples were found in December. The highest mean number of oocysts per litre was detected in November 1989 and the highest number of cysts per litre was detected in March 1989 (figures 3.11. and 3.12.). No statistically significant differences were observed between seasons for levels detected or frequency of occurrence for either parasite.

Of 199 samples analysed for both *Cryptosporidium* oocysts and *Giardia* cysts, in 99 (49.7%) either one or both organisms were detected (table 3.3 and 3.8.), at a mean concentration of 0.08 (oo)cyst/L (range 0.007 - 1.36 organisms/L). In 11 of the samples (5.5%) both parasites were detected together, at a mean concentration of 0.059 organism per litre (range 0.007 - 0.16 organism/L).

Comparison of the total mean concentration of organisms detected per litre during the course of the survey, revealed that nearly four times the number of oocysts as cysts were detected; in individual months this ranged from between 1 and 17 times more oocysts than cysts. In those samples where both cysts and oocysts were detected, the mean concentration of oocysts per litre was approximately 2.5 times the number of cysts present.

3.3.4.2. Water treatment.

Of the 209 water samples examined, the details of treatment were available for 161 of the samples examined for *Cryptosporidium* and for 140 of those examined for *Giardia*. Based upon this information the samples were divided into treatment groups. The data are presented in table 3.7.

Those waters receiving minimal treatment, such as microstraining, screening or no treatment demonstrated the highest occurrence of oocysts 41% - 67% (mean 0.072 oocyst/L, median 0.026 oocyst/L, range 0.008 - 0.72 oocyst/L) and cysts 21% - 67% (mean 0.013 cyst/L, median 0.03 cyst/L, range 0.009 - 0.019 cyst/L). Of the samples taken following slow sand filtration, 33% contained oocysts (mean 0.049 oocyst/L, range 0.014 - 0.16 oocyst/L) and 2.9% contained *Giardia* cysts (1 sample, 0.034 cyst/L). Of the samples taken after rapid gravity filtration between 20 and 50% contained *Cryptosporidium* oocysts (mean 0.033 oocyst/L, median 0.03 oocyst/L; range 0.01 - 0.14 oocyst/L) and 50% contained cysts (mean 0.065 cyst/L, median 0.065 cyst/L, range 0.04 - 0.09 cyst/L). Oocysts were detected in 31.8% of water samples taken after pressure filtration (mean 0.063 oocyst/L, median 0.038 oocyst/L, range 0.008 - 0.24 oocyst/L) and cysts were detected in 10.5 - 50 % of these samples (mean 0.02 cyst/L, median 0.028 cyst/L, range 0.007 - 0.048 cyst/L). Where two step treatment was performed (eg. coagulation, flocculation, rapid gravity filtration and chlorination), relatively low incidences of oocysts and cysts were detected.

As the amount of data for individual treatment types is small, the data were grouped together into microstraining, screening and untreated final, and these were compared to results from those undergoing rapid or slow sand filtration and pressure filtration. No statistical difference

in the levels or frequency of occurrence of *Cryptosporidium* oocysts in any particular final water after treatment were found and similarly no significant difference was observed in the level of *Giardia* cysts detected in different water types. However *Giardia* was detected with greater frequency ($0.01 < p < 0.05$) in those waters receiving less treatment compared to those undergoing rapid gravity filtration, slow sand filtration or pressure filtration.

3.3.4.3. Removal of oocysts and cysts by water treatment processes.

Conventional treatment

In one treatment works (table 5.8. and figures 5.13. and 5.14.), where water underwent conventional treatment (coagulation, sedimentation, rapid gravity filtration and chlorination), oocysts and cysts were detected in the 12.5% and 50% of the samples of untreated reservoir water respectively. In the treated water 12.5% of the samples contained oocysts and none contained cysts. No significance could be demonstrated in the level of organisms detected or the frequency of detection for either organism.

Slow sand filtration

In another location where slow sand filtration was in place, 29% of water samples taken prior to filtration contained oocysts, while oocysts were detected in 38% of treated water samples (table 3.8 and figure 3.13). However, the mean number of oocysts detected in untreated and treated waters were 0.39 and 0.031 oocysts per litre respectively; a reduction in the concentration of oocysts of approximately 92%. However, statistical analysis revealed no significant difference in the level of oocysts detected or in the frequency of occurrence. Examination of core samples from the sand filter beds, did not reveal the presence of oocysts.

In this treatment works, *Giardia* cysts were detected only once in the untreated water and were not detected at all in the treated water. Indeed, throughout the entire survey period, *Giardia* cysts were detected in only one sample after slow sand filtration and as there was no corresponding pretreatment sample, removal efficencies could not be calculated.

Pressure filtration

At a further works where the treament was coagulation, pressure filtration and chlorination, oocysts were detected in 25% of both untreated and treated water samples and *Giardia* cysts were detected in 50% of the untreated water samples, but were not detected in any of the samples of treated water.

Microstraining

In a works where water was treated by microstraining and chlorination, 56% (18/32) of water samples prior to treatment contained oocysts, at a mean concentration of 0.213 oocysts/L (table 3.8. and figures 3.13. and 3.14.). Two distribution sites were tested and oocysts were present in 45% (47% and 44%) of the samples tested (64) at a mean concentration of 0.082 (0.05 and 0.117) oocysts/L; approximately 62% removal efficiency. No statistical difference in the level of oocysts detected or the frequency of occurrence was observed.

Giardia cysts were detected in 78% of the samples of untreated water at a mean concentration of 0.065 cyst/L and in 20% of the combined treated waters at a concentration of 0.012 (0.013 and 0.011) cyst/L (table 3.8. and figures 3.13. and 3.14.); this indicates a removal efficiency of 81% . The higher concentration of cysts detected before treatment was statistically significant ($p = 0.02$, C.I. = 95.3%, median = 0.035), compared to the concentration detected after microstraining (median = 0.016). *Giardia* cysts were also detected more often before, compared to after treatment ($p< 0.001$). Faecal coliforms (data not shown) were not reported in either of the two treated waters although they were present in the untreated water on numerous occasions.

Although in the accumulation of the data reported here, samples of untreated and treated waters were taken from the same sites on each occasion and at the same time, information was not obtained on the flow rate of water through the particular works.

3.3.5. Recovery efficiency.

The mean recovery of oocysts from filters ranged from 7.5 % to 17.9 % depending on the number of oocysts inoculated (table 3.9.). A mean of 80.3 % of oocysts were retained by the filters, with 19.7 % of oocysts passing through the Cuno filter and being retained on the Sartorius filter (table 3.10.). The mean oocyst recovery from Sartorius filters was 90.34 % (table 3.11.). The recovery of oocysts from the different centrifugation stages (1 L $\rightarrow$ 250 ml $\rightarrow$ 50 ml) ranged from 30.3 % to 91.5 % (table 3.11.). After sucrose density flotation the mean oocyst recovery was 78.3 % of the number of oocysts seeded (table 3.11.).

The recovery of *Giardia* spp. cysts from water samples was 27 $\pm$ 6 % according to Gilmour (1990).

3.4. Discussion.

Throughout the survey *Cryptosporidium* oocysts and *Giardia* cysts were detected in a variety of surface waters tested, which were abstracted for use as potable water and were also used in water associated leisure activities. The frequency of occurrence and the concentration at which the parasites were present agrees, in general, with the results of other studies conducted in the USA. Rose (1991) detected oocysts in 55% of samples (n = 181) at a concentration range of 0.0025 - 44 oocyst/L and cysts in 16 % of samples at a concentration of 0.02 - 1.4 cysts/L in a range of surface waters which were exposed to a variety of different forms of contamination. The occurrence and concentration of oocysts and cysts was greater in water which was polluted, compared to pristine waters. LeChevalier *et al* (1991a) conducted a study in the North East USA looking at the occurrence and concentration of oocysts and cysts in heavily polluted water. They found 87% contained oocysts at a concentration of 0.04 - 484 oocysts/L and cysts in 81.2% at a concentration range of 0.04 - 66 cysts/L (n = 85). Hansen and Ongerth (1991) detected oocysts at a concentration of 0.18 - 63.5 oocysts/L in 34/35 (97.1%) of samples of surface water taken from two rivers in the Pacific Northwest (USA). In two previous studies (Ongerth and Stibbs 1987; Ongerth 1989) *Cryptosporidium* oocysts were detected at a concentration of 2 - 112 oocysts/L in water samples from 6 rivers (n = 11) and *Giardia* cysts were detected in 43% of samples from 3 pristine rivers at a concentration of 0.5 - 1 cyst/L. Payment (1992) in a study recently carried out in Canada reported ≤ 63 oocysts/L and ≤ 38 cysts/L.

Human wastewater can contain varying numbers of protozoan parasites, which depends on the size of the population served and the prevalence of infection in that community (Smith and Rose 1990). In other studies where the concurrence of oocysts and cysts in raw and treated wastewaters was assessed (DeLeon *et al*, 1988; Rose *et al*, 1988), higher concentrations of oocysts were detected (521 - 5,100 oocysts) in raw wastewater than were detected in this study, although similar concentrations of cysts were detected this study compared with those carried out in America which were detected at a concentration range of 3.7 - 725/L. In this study *Giardia* occurred with significantly greater frequency and at significantly higher concentrations in treated wastewater than *Cryptosporidium* did. The American studies used large volume filtration to sample the raw sewage, while in this study 2 L grab samples were taken because of the problems associated with the size of the final pellet volume when using large scale centrifugation.

In a select number of raw (n = 5) and treated (n = 5) sewage samples (taken at the same sewage treatment works, on different dates) all of which were positive for *Cryptosporidium* oocysts and *Giardia* cysts, both parasites were detected at significantly higher concentrations

in raw sewage compared to the treated sewage. There was also a significantly higher concentration of *Giardia* cysts in the raw sewage compared to the concentration of *Cryptosporidium* oocysts in raw sewage. These data suggest either that there is more infection with *Giardia* in the communities served by this sewage treatment works and that *Giardia* cysts are less efficiently removed or destroyed by the treatment processes in operation. The Communicable Diseases and Environmental Health (Scotland) weekly bulletin reports much lower numbers of *Giardia* infections that infections with *Cryptosporidium*, so the fact that greater concentrations of cysts are detected in raw sewage may be a consequence of undiagnosed giardiasis, asymptomatic giardiasis and possibly chronic giardiasis. Removal efficiency data calculated from the mean concentrations of organisms detected in the 5 samples of raw and treated sewage suggested that treatment removed approximately 80 % of *Cryptosporidium* oocysts and 99 % of *Giardia* cysts. These results are similar to removal efficencies of sewage treatment reported in other studies, which ranged from 74 % to 99.8 % removal of *Cryptosporidium* oocysts (Madore *et al*, 1987) and from 40 % to 100 % removal of *Giardia* cysts (Rose *et al*, 1986; Sykora *et al*, 1988). The greater concentration of cysts compared to oocysts in raw sewage is probably responsible for the greater concentration of cysts compared to oocysts in treated sewage.

Sewage effluents were, in many cases, discharged into river or reservoir water (either directly, or indirectly by tributaries) which was used for abstraction as potable water and/or for the pursuit of water related leisure and sporting activities. The occurrence and concentration of oocysts and cysts in waters either receiving or not receiving treated sewage effluent was assessed. *Cryptosporidium* oocysts and *Giardia* cysts were detected at a significantly greater concentration in river water receiving sewage effluent compared to river water not receiving sewage effluent. In reservoir water receiving sewage effluent oocysts and cysts were detected with significantly greater frequency than in reservoir water not receiving sewage effluent.

Cryptosporidium oocysts and *Giardia* cysts have also been shown to be present in water used by people for recreation and this constitutes a health risk to those people. Many of the waters used receive some sort of contamination, such as raw and treated sewage, agricultural runoff, industrial effluents and so on. Considering the low infectious doses of both parasites, an individual would be at risk of ingesting a sufficient number of organisms during participation in water sports, especially those which involve submersion, to establish infection.

The results described here show that both *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts may be present in drinking water after conventional treatment. Oocysts and cysts were present throughout most of the 17 months of the survey and were detected in most of the water samples taken post-treatment.

In other investigations on the occurrence and distribution of these parasites in the environment (LeChevallier *et al*, 1991a; Rose *et al*, 1990) *Cryptosporidium* oocysts tended to be found more often than *Giardia* cysts. When *Giardia* cysts were detected, however, the number of organisms per litre has been reported to be significantly greater than the numbers of *Cryptosporidium* oocysts found. In treated water the mean number of *Cryptosporidium* oocysts was over two times higher than the number of *Giardia* cysts, whereas in samples of untreated water there were just over 3 times the number of oocysts to cysts. LeChevallier *et al* (1991a) reported 1.5 times the number of oocysts to cysts in raw surface water. In some cases where the sampling of water was conducted both pre- and post-treatment it was observed that a proportion of both organisms are capable of passing through water treatment without removal.

A study by LeChevallier *et al* (1991b) obtained similar prevalence results to those obtained in this study; LeChevallier reported that out of 82 samples of finished drinking water, 26.8% contained *Cryptosporidium* oocysts and 17.1% contained *Giardia* and one or both organisms were detected in 39% of samples. LeChevallier *et al* (1991b) also obtained data about the concentrations of oocysts and cysts in water and they reported geometric means for *Cryptosporidium* at 1.52 oocysts/100 litres, with *Giardia* about three times that at 4.45 cysts/100 litres. Other studies (Ongerth and Stibbs, 1987; Hibler, 1988; Rose, 1988) which investigated the concentrations of these parasites in surface waters reported levels of 0.006 - 66 cysts/l and 0.009 - 484 oocysts/l. In this study arithmetic means of 0.075 oocysts/L (median 0.03 oocyst/L) and 0.037 cysts/L (median 0.016 cyst/L) were reported. Statistical analysis showed that *Cryptosporidium* oocysts occurred with significantly greater frequency and at higher concentrations than *Giardia* cysts. In water prior to treatment there was also an overall greater occurrence and concentration of oocysts to cysts.

Data on the prevalence and concentration of *Cryptosporidium* oocysts in drinking water in the U.S.A. has been reported by Rose *et al* (1988) and Smith & Rose (1990) and is similar to data obtained in this study. Rose *et al* (1990) reported the presence of oocysts in 2 out of ten filtered water samples at a mean concentration of 0.001 oocyst/L and in 2 out of 4 unfiltered potable water supplies at 0.006 oocyst/L, and

Smith and Rose (1990) quoted a range of 0.04 - 0.026 oocyst/L (36 samples) in drinking water in the USA and 0.006 - 0.26 (65 samples) in the UK. Neither of these data sets were associated with waterborne outbreaks of cryptosporidiosis. When contamination of water has been implicated in an outbreak of cryptopsoridiosis, retrospective sampling has detected 0.42 - 2.2 oocysts/L (20 samples) and 0.04 - 4.8 oocysts/L (7 samples) in the USA and UK respectively (Smith & Rose, 1990). In an outbreak in Carrollton, Georgia, USA (Hayes *et al*,1987) an

average of 0.63 oocyst/L was detected within distribution, with the highest level of 2.2 oocysts/L as quoted above.

Although *Cryptosporidium* and *Giardia* were detected in all the types of treated water tested, the waters receiving little or no treatment (microstraining, screening, untreated final) were positive more often and in up to 67% of the samples both cysts and oocysts were detected. These figures are higher than those found by Hibler (1988) who reported 6.6% of 1,214 unfiltered potable water samples positive for *Giardia*. However, Rose (1988) reported *Cryptosporidium* in 50% of samples from unfiltered potable water.

Filtration is obviously effective at removing a proportion, at least, of the oocysts and cysts present in the raw supply. Research by Tanner and Ongerth, (1990) on removal of *Giardia* spp. cysts by slow sand filtration involved studies at 3 slow sand filtration treatment works. In two of the works cysts were detected in the raw water, but only in one of these were cysts detected in the final water. In the works where cysts were detected both before and after slow sand filtration the concentration was reduced from 0.09 cysts/L to 0.01 cysts/L. In this study cysts were detected in only 1/35 samples at a concentration of 0.034 cyst/L. As a corresponding sample of raw water was not available, removal efficiency could not be calculated. Oocysts, however, were detected 33% of the samples examined following slow sand filtration. It is possible that oocysts might become trapped in the filter over time or following a high challenge of oocysts on the filter and subsequently trickle through over time. Results from examination of waters receiving conventional treatment were similar to those reported by Rose (1988), with 20% positive and at a concentration of 0.031 oocyst/L. However, results concerned with *Giardia* were dissimilar from those reported by Ongerth *et al*, (1987), who detected *Giardia* cysts in 7 out of 9 conventionally treated waters, while this survey reported none. However, *Giardia* were detected in 2 out of 4 samples which underwent only rapid gravity filtration and chlorination (0.065 cysts/L). This suggests that sedimentation may be of importance in oocyst and cyst removal (0.031 oocyst/L in conventional; 0.14 oocyst/L in rapid gravity filtration and chlorination only). In one outbreak, problems with sedimentation may have had some part in the passage of oocysts through the treatment system and into the drinking water supply (Hayes *et al*, 1989).

Between 10-50% of samples examined following pressure filtration were found to contain *Giardia* cysts; a similar result to that reported by Ongerth *et al*, (1987). *Cryptosporidium* oocysts were detected in 31.8% of the samples in this study, but there are no figures from similar studies for comparison. However, as *Giardia* cysts are not always removed by pressure filtration, it is perhaps unsurprising that oocysts are not always removed either.

A number of the results in this survey were obtained from final water samples for which a raw water sample was also taken, thus allowing removal efficiencies of the treatments to be calculated. *Cryptosporidium* oocysts were detected in 29% (mean concentration 0.39 oocyst/L) of samples before slow sand filtration, compared to 38% (mean concentration 0.031 oocyst/L) afterwards. Although no statistical significance was observed, the levels after treatment were approximately 92% less than before. A peak of oocysts was detected in the raw water (2.1 oocysts/L) whereafter oocysts were detected over a period of time at lower levels in the treated water. Oocyst concentration in the raw water decreased and then ceased while detection in the final water continued. Again, this situation may be a result of oocysts becoming trapped in the sand filter and then being released slowly, explaining the high initial load on the filter, with the subsequent 'trickle' of oocysts into the final water, and the higher frequency of detection.

After only microstraining and chlorination 45% of samples were positive for *Cryptosporidium* (mean concentration 0.082 oocyst/L) and 20% for *Giardia* (mean concentration 0.012 cyst/L), while their respective results in the raw water were 56% (mean 0.213 oocyst/L) and 78% (mean 0.065 cyst/L). No significance was obtained for the levels of *Cryptosporidium* detected, although there was a significant decrease in the frequency of detection and concentration of *Giardia* cysts in the final water. This decrease is likely to be a combination of the larger size of the cysts, along with possible clumping of cysts, association with other matter (turbidity removal) and a build up of matter on the microstrainer reducing the pore size. The water quality at this site was A1 and recovery of both organisms was almost certainly very low during sampling. In water undergoing conventional treatment oocysts and cysts were detected in water before and after treatment (12.5% and 50% respectively). After treatment oocysts were still detected, but no cysts were detected.

Although only low numbers of oocysts and cysts were detected in the final waters, the minimum infectious dose for establishment of *Giardia* and *Cryptosporidum* infection is also considered to be small (Rentdorff, 1954; Miller *et al*, 1986, 1990; Blewett *et al*, 1992; Kwa *et al*, 1992)) and the numbers of oocysts/cysts detected in final water might be sufficient to cause infection. If oocysts and cysts are not removed by water-treatment processes, unless they are killed or rendered non-infective, they are a considerable risk to public health. DeRegnier *et al* (1989) reported complete loss of viability in *Giardia muris* cysts exposed to tap water for 14 days, with most cysts becoming non-viable in as little as 3 days, however in river and lake water cysts survived for considerably longer periods. If cysts have been present in the environment they may be more vulnerable by the time they reach water treatment and therefore more susceptible to water treatment and consequently survive for less than three days in tap water. Water treatment itself may stress the cysts sufficiently to reduce their longevity considerably and allow them to survive for a shorter duration in tap water. Robertson *et al*

(1992a) reported survival of oocysts in river and tap water for over 176 days (and in sea water for over 35 days). In addition the authors presented data on the effects of some water treatment parameters which affected the survival of oocysts. It is possible that for oocysts, ageing in the environment and progress through water treatment may have a detrimental effect on their survival. Oocysts which have not been present in the environment for long (perhaps from cattle faeces deposited in rivers close to abstraction points, or from sewage effluent) may be more robust and able to withstand the pressures of water treatment, thus constituting a risk to the health of water consumers.

In this study no cases of disease were reported in connection with the waterborne route, although both *Giardia* or *Cryptosporidium* were detected in final water. However, a possible association with disease was reported for the finding of oocysts in water after microstraining, but this is as yet unconfirmed and work is continuing to investigate further the possibility of a waterborne outbreak of cryptosporidiosis. This suggests that low level occurrence of these organisms in drinking water does not necessarily present a health risk to the population served by these water supplies. This could be because the oocysts and cysts distributed in the water were non-viable, possibly inactivated by the water treatment processes. In the absence of a reliable viability assay for individual cysts and oocysts data on the viability of the organisms was not collected in this study. LeChevallier *et al* (1991b) found that 13.3% of the 46 *Giardia* cysts detected in drinking water samples had a viable type morphology whereas only 2 out of 23 *Cryptosporidium* oocysts found in potable water were considered probably viable, while one third of the oocysts seen in raw water contained sporozoites and were considered to be viable.

Work perfomed at the SPDL (Parker and Smith, 1993) on the effects of sand from (slow) sand filtration water treatment showed during the course of time and with increased agitation, extreme disruption and deformation of oocysts and a very low viability according to the inclusion or exclusion of fluorogenic vital dyes. In addition to this, the intensity of fluorescence was reduced, making detection and identification difficult. This suggests possible destruction of the surface epitope. Chlorination subsequent to (sand) filtration may then inactivate most oocysts.

The results presented in this survey demonstrate that *Cryptosporidium* oocysts and *Giardia* cysts, both present in the aquatic environment, commonly penetrate water treatment and are present in drinking water at concentrations within the range of concentrations detected in waterborne outbreaks of disease. None of the types of treatment was entirely successful in removing both parasites. What could not be addressed during the study period was the viability of individual organisms detected and this is an important factor to consider. It may be that the

organisms detected in treated drinking water on a regular basis have been rendered non-viable for a variety of reasons, including the combined effects of different stages of water treatment.

The method for recovering oocysts from filters is time consuming, labour intensive and from the results quoted in section 3.3.5. can be seen to be inefficient at recovering oocysts. This suggests that the number of oocysts or cysts present in a volume of water may be many times higher than the concentration quoted. This means that the risk to public health posed by the presence of oocysts in water may well be greater as a result. Methods are being investigated to improve the method, both in terms of the increasing the recovery efficiency and reducing the time required for sample processing (chapter 5).

Disclaimer: This work was carried out in conjuction with Dr. A. M. Grimason, Scottish Parasite Diagnostic Laboratory.

Photograph 3.1. *Cryptosporidium* sp. oocyst (C) and *Giardia* sp. cyst (G) labelled with MAb in a water sample. Bar = 5μm.

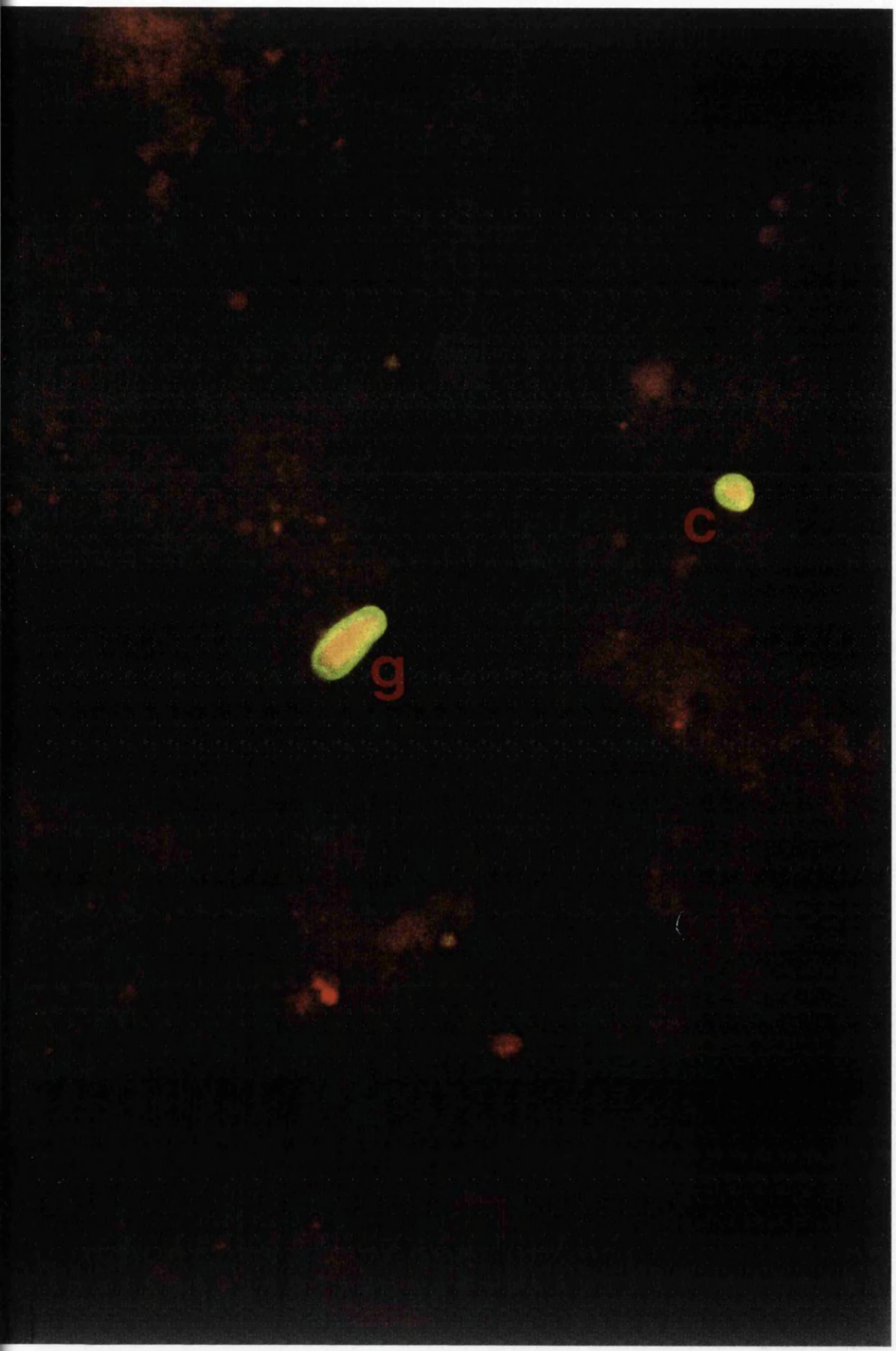

c
g

Table 3.1. Monthly Occurrence of *Cryptosporidium* spp. Oocysts and *Giardia* spp. Cysts Detected in United Kingdom Surface Waters.

Protozoan Parasite	*Cryptosporidium* sp. Oocysts						*Giardia* sp. Cysts					
Month/Year	Number of samples	Number of samples positive	Percent of samples positive (%)	Range (oocysts/L)	Mean* Conc n (oocysts/L)	Median Conc n (oocysts/L)	Number of samples	Number of samples positive	Percentage of samples positive (%)	Range (cysts/L)	Mean* Conc n (cysts/L)	Median Conc n (oocysts/L)
1989												
January	1	0	0	-	-	-	1	0	0	-	-	-
February	-	-	-	-	-	-	-	-	-	-	-	-
March	11	2	18.2	0.006 - 0.029	0.018	0.018	11	3	27.3	0.013 - 0.029	0.023	0.026
April	9	2	22.2	0.14 - 2.5	1.32	1.32	9	4	44.4	0.09 - 0.25	0.094	0.055
May	3	2	66.7	0.017 - 0.183	0.1	0.1	3	1	33.3	0.008	0.008	0.008
June	10	1	10	0.021	0.021	0.021	10	4	40	0.014 - 0.181	0.1	0.103
July	5	2	40	0.027 - 2.3	1.164	1.164	5	4	80	0.01 - 0.123	0.048	0.029
August	3	0	0	-	-	-	3	2	66.6	0.097 - 0.169	0.133	0.133
September	5	3	60	0.054 - 0.256	0.143	0.12	4	2	50	0.024 - 0.12	0.072	0.072
October	5	2	40	0.033 - 0.042	0.037	0.037	5	3	60	0.033 - 0.265	0.133	0.1
November	13	5	38.5	0.02 - 1.04	0.324	0.17	13	3	23.1	0.017 - 0.038	0.024	0.018
December	6	5	83.3	0.009 - 0.04	0.018	0.012	6	4	66.6	0.0118 - 0.68	0.22	0.095
1990												
January	7	4	57.1	0.016 - 0.16	0.074	0.06	7	4	57.1	0.048 - 0.09	0.068	0.067
February	20	8	40	0.009 - 2.1	0.32	0.046	20	7	36	0.0147 - 0.11	0.044	0.027
March	47	15	32	0.014 - 1.83	0.367	0.07	47	13	27.6	0.014 - 1.52	0.339	0.145
April	19	10	52.6	0.019 - 2.48	0.74	0.455	11	7	63.6	0.009 - 2.1	0.513	0.06
May	32	16	50	0.007 - 15.6	1.784	0.861	-	-	-	-	-	-
Total	196	77	39.28	0.006 - 15.6	0.672	0.102	155	61	39.35	0.009 - 2.1	0.188	0.053

* Arithmetic mean based upon total number of positive samples.

Table 3.2. Occurrence of *Cryptosporidium* spp. Oocysts and / or *Giardia* spp. Cysts in United Kingdom Surface Waters.

Protozoan Parasite	Number of samples	Number of samples positive	Percentage of samples positive (%)	Range (organisms/L)	Mean * Concentration (organisms/L)	Median Concentration (organisms/L)
Cryptosporidium spp.	196	77	39.3	0.006 - 15.6	0.672	0.102
Giardia spp.	155	61	39.4	0.009 - 2.1	0.188	0.053
Cryptosporidium spp. and *Giardia* spp.	155	34	21.9	-	-	-
Cryptosporidium spp. and / or *Giardia* spp.	155	84	54.2	-	-	-

* Arithmetic mean based upon total number of positive samples.

Table 3.3. Occurrence of *Cryptosporidium* sp. Oocysts and / or *Giardia* sp. Cysts in United Kingdom Treated Water.

Protozoan Parasite	Number of samples	Number of samples positive	Percentage of samples positive (%)	Range (organisms/L)	Mean * Concentration (organisms/L)	Median Concentration ([oo]cysts/L)
Cryptosporidium spp.	209	78	37.3	0.008 - 1.36	0.075	0.03
Giardia spp.	199	33	16.5	0.007 - 0.34	0.035	0.016
Cryptosporidium spp. and *Giardia* spp.	199	11	5.5	-	-	-
Cryptosporidium spp. and / or *Giardia* spp.	199	99	49.8	-	-	-

* Arithmetic mean based upon total number of positive samples.

Table 3.4. Occurrence of *Cryptosporidium* spp. Oocysts and *Giardia* spp. Cysts in United Kingdom Surface Water and Water- related Samples.

Protozoan Parasite	*Cryptosporidium* spp. Oocysts						*Giardia* spp. Cysts					
Water Type	Number of samples	Number of samples positive	% +ve	Range (oocysts/L)	Mean* Conc n (oocysts/L)	Median Conc n (oocysts/L)	Number of samples	Number of samples positive	% +ve	Range (cysts/L)	Mean* Conc n (cysts/L)	Median Conc n (cysts/L)
Reservoirs												
(-) Sewage Effluent	80	23	28.8	0.006 - 2.1	0.147	0.04	76	22	28.9	0.009 - 0.27	0.076	0.033
(+) Sewage Effluent	35	21	60	0.008 - 2.3	0.201	0.042	32	22	68.8	0.01 - 0.181	0.07	0.067
Rivers												
(-) Sewage Effluent	12	6	50	0.021 - 0.07	0.039	0.035	12	7	58.3	0.014- 0.125	0.037	0.027
(+) Sewage Effluent	28	21	75	0.1 - 15.6	1.646	0.8	15	9	60	0.02 - 2.1	0.61	0.1
Recreational Waters												
(-) Sewage Effluent	6	2	33.3	0.33 - 0.67	0.5	0.5	6	6	100	0.05 - 2.67	0.786	0.5
(+) Sewage Effluent	15	2	13.3	0.66 - 1.0	0.83	0.83	15	15	100	0.31 - 3.85	1.483	1.25
Agricultural Waters	3	0	0	-	-	-	3	3	100	0.012 - 14.1	5.298	-

* Arithmetic mean based upon total number of positive samples.

Table 3.5. Occurrence of *Cryptosporidium* spp. Oocysts and *Giardia* spp. Cysts in United Kingdom Domestic and Industrial Waste-water Samples.

Protozoan Parasite	*Cryptosporidium* spp. Oocysts					*Giardia* spp. Cysts				
Waste-water Type	Number of samples	Number of samples positive	Percentage of samples positive (%)	Concentration range (oocysts/L)	Mean* Conc n (oocysts/L)	Number of samples	Number of samples positive	Percentage of samples positive (%)	Concentration range (cysts/L)	Mean* Conc n (cysts/L)
Municipal Raw	6	6	100	2.5 - 75	29.58	6	6	100	242.5 - 792.5	478.33
Industrial Raw										
Animal Slaughterhouse	1	1	100	92.5	92.5	1	1	100	42.36	42.36
Animal Lairage	2	2	100	1.17×10^5 -1.49×10^5	1.33×10^5	2	2	100	10 - 20	15
Poultry Slaughterhouse	1	1	100	292.3	292.3	1	0	0	-	-
Private Treated	3	1	33.33	0.013	0.013	3	1	33.3	0.013	0.013
Municipal Treated:	70	25	35.71	0.024 - 26.5	1.76	70	58	82.86	0.095 - 361.7	25.07
Industrial Treated:										
Poultry Treated	1	1	100	1.76	1.76	1	0	0	-	-
Poultry Offal Cake	1	1	100	232/g	232/g	1	0	0	-	-
Cattle Slurry	2	0	0	-	-	2	1	50	3.33/g	3.33/g

* Arithmetic mean based upon total number of positive samples.

Table 3.6. Monthly Occurrence of *Cryptosporidium* spp. Oocysts and *Giardia* spp. Cysts Detected in United Kingdom Treated Waters.

Protozoan Parasite	*Cryptosporidium* spp. Oocysts						*Giardia* spp. Cysts					
Month/Year	Number of samples	Number of samples positive	Percentage of samples positive (%)	Concentration range (oocysts/L)	Mean* Conc n (oocysts/L)	Median Conc n (oocysts/L)	Number of samples	Number of samples positive	Percentage of samples positive (%)	Concentration range (cysts/L)	Mean* Conc n (cysts/L)	Median Conc n (cysts/L)
1989												
January	1	0	0	-	-	-	1	0	0	-	-	-
February	-	-	-	-	-	-	-	-	-	-	-	-
March	35	13	37.1	0.008 - 0.14	0.051	0.04	35	11	31.4	0.007 - 0.34	0.063	0.018
April	17	6	35.3	0.08 - 0.26	0.124	0.093	17	4	23.5	0.025 - 0.048	0.05	0.043
May	14	6	42.9	0.008 - 0.03	0.019	0.019	14	0	0	-	-	-
June	14	5	35.7	0.008 - 0.16	0.057	0.042	14	3	21.4	0.019 - 0.034	0.027	0.028
July	6	3	50	0.013 - 0.041	0.029	0.032	6	2	33.3	0.012 - 0.013	0.013	0.013
August	7	3	42.8	0.036 - 0.4	0.15	0.036	7	2	28.6	0.01 - 0.012	0.011	0.011
September	5	1	20	0.008	0.008	0.008	5	1	20	0.009	0.009	0.009
October	9	4	44.4	0.009 - 0.068	0.029	0.019	9	1	11.1	0.017	0.017	0.017
November	14	4	28.6	0.03 - 0.75	0.254	0.118	14	0	0	-	-	-
December	5	4	80	0.009 - 0.1	0.039	0.023	5	2	40	0.009 - 0.016	0.013	0.013
1990												
January	21	7	33.3	0.009 - 0.1	0.038	0.027	21	2	9.52	0.01 - 0.015	0.013	0.013
February	18	9	50	0.007 - 0.087	0.031	0.02	18	2	11.1	0.014 - 0.016	0.015	0.015
March	26	8	30.8	0.008 - 1.36	0.195	0.019	26	1	3.84	0.018	0.018	0.018
April	7	2	28.6	0.009 - 0.01	0.01	0.01	7	2	28.6	0.009 - 0.01	0.01	0.01
May	10	3	30	0.008 - 0.085	0.042	0.032	-	-	-	-	-	-
Total	209	78	37.3	0.008 - 1.36	0.075	0.03	199	33	16.5	0.007 - 0.34	0.035	0.016

* Arithmetic mean based upon total number of positive samples.

Table 3.7. Occurrence and concentration of *Cryptosporidium* spp. Oocysts and *Giardia* spp. Cysts Detected in United Kingdom Waters After Treatment.

Protozoan Parasite	*Cryptosporidium* spp. Oocysts					*Giardia* spp. Cysts				
Type of Water Treatment	Number of samples	Number of positive samples	Percent of positive samples (%)	Conc n range (oocysts/L)	Mean* Conc n (oocysts/L)	Number of samples	Number of positive samples	Percentage of positive samples (%)	Conc n range (cysts/L)	Mean* Conc n (cysts/L)
Coag., PF, Cl	22	7	31.8	0.008-0.247	0.063	19	2	10.5	0.007-0.048	0.028
Coag., floc., PF, Cl	2	0	-	-	-	2	1	50	0.01	0.01
SSF, Cl	36	12	33.3	0.014- 0.167	0.049	35	1	2.9	0.034	0.034
RGF, Cl	4	1	25	0.14	0.14	4	2	50	0.04-0.09	0.065
Coag.,sed., RGF, Cl	10	2	20	0.024-0.038	0.031	6	0	0	-	-
Coag., pulsator, clarifier, RGF, Cl	4	2	50	0.01-0.029	0.02	4	0	0	-	-
Microstrainer, Cl	75	31	41.3	0.008-0.72	0.077	62	13	21	0.009-0.019	0.013
Screening, Cl	4	2	50	0.02-0.1	0.06	4	0	0	-	-
Cl	1	0	-	-	-	1	0	0	-	-
Untreated final	3	2	66.7	0.012-0.013	0.013	3	2	66.7	0.012-0.013	0.012
TOTAL	161	59	36.6	0.008 - 0.72	0.064	140	21	15	0.007 - 0.048	0.02

Cl = chlorination, coag. = coagulation, PF = pressure filtration, floc. = floculation, SSF = slow sand filtration, RGF = rapid gravity filtration, sed. = sedimentation.
* Arithmetic mean based upon total number of positive samples.

Table 3.8. Occurrence of *Cryptosporidium* and *Giardia* in water before and after treatment.

	% positive		mean oocyst/L		% positive		mean cyst/L	
Treatment	pre	post	pre	post	pre	post	pre	post
ssf	29	38	0.39	0.031				
ms + Cl	56	45	0.213	0.082	78	20	0.065	0.012
coag., sed., rgf, Cl.	12.5	12.5	0.219	0.038	50	0	0.135	0

Table 3.9. Recovery of *Cryptosporidium* spp. oocysts from Cuno filters.

No. oocysts seeded	Mean oocysts recovered (%)	Range of oocysts recovered (%)
10^3	7.5 ± 3.4	3.4 - 12.5
10^4	11.2 ± 4.1	5.7 - 16.8
10^5	16.1 ± 6.6	9.9 - 26.3
10^6	17.9 ± 8.1	6.1 - 28.4

Table 3.10. Retention of *Cryptosporidium* spp. oocysts by Cuno filters.

No. oocysts seeded x 10^6	% oocysts recovered	% oocysts retained
5.4	19.6	80.4
7.3	29.2	70.8
13.1	22.4	77.6
118.7	7.4	92.6
Mean	19.7 ± 9.1	80.3 ± 9.1

Table 3.11. Mean recoveries of *Cryptosporidium* spp. oocysts from centrifugation, sucrose flotation and Sartorius filters.

No. oocysts	Centifugation stage	Oocysts recovered (%)	Range recovered (%)
10^6	50 ml	86.7 ± 12.1	77.01 - 104
10^6	250 → 50 ml	91.5 ± 6.8	86.6 - 101.3
10^6	1L→250ml→50ml	30.3 ± 5.1	22.7 - 40.6
4.15 x 10^5	sucrose flotation	78.3 ± 11.0	66.5 - 90.5
9.63 x 10^4	sartorius filter	90.3 ± 4.8	85.6 - 96.1

Figure 3.1. Monthly occurrence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in surface water.

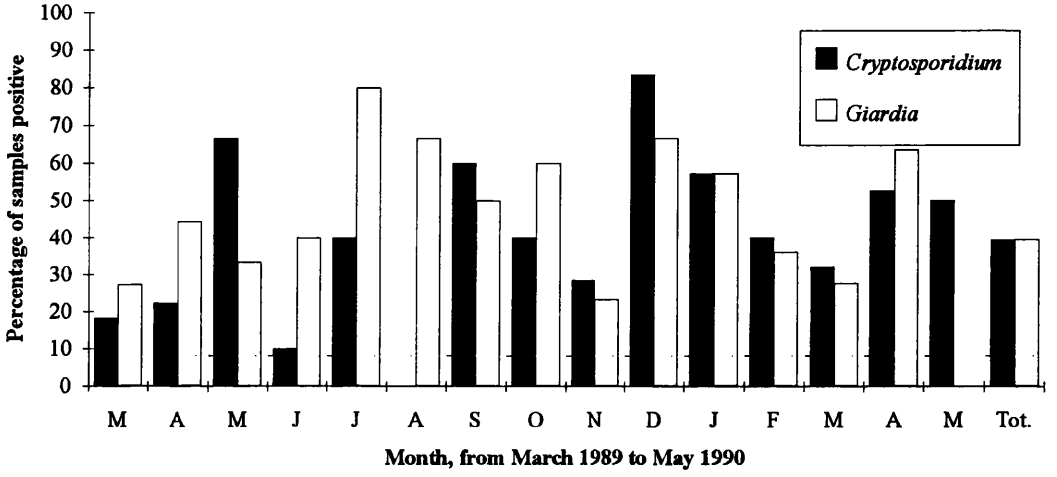


Figure 3.2. Monthly occurrence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in treated water.

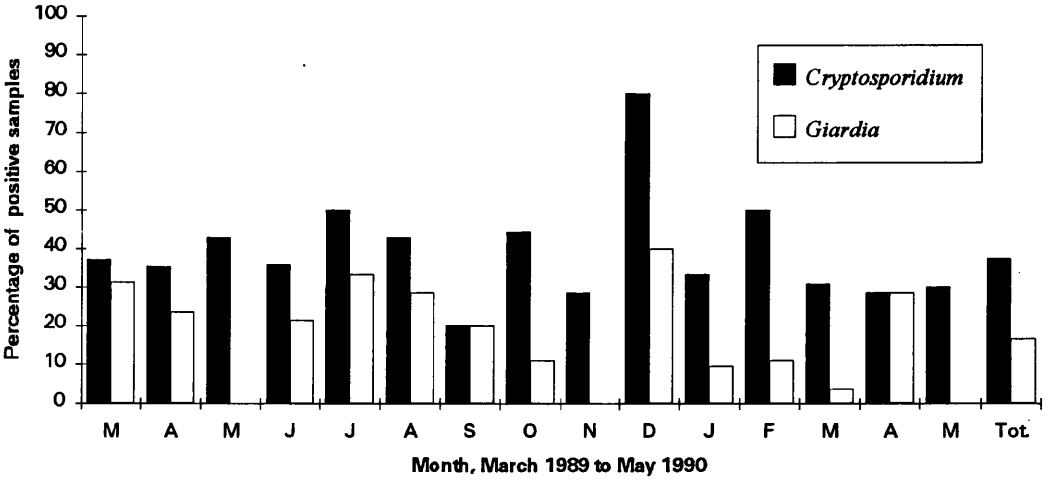


Figure 3.3. Monthly mean and median concentrations of *Cryptosporidium* spp. oocysts in surface water.

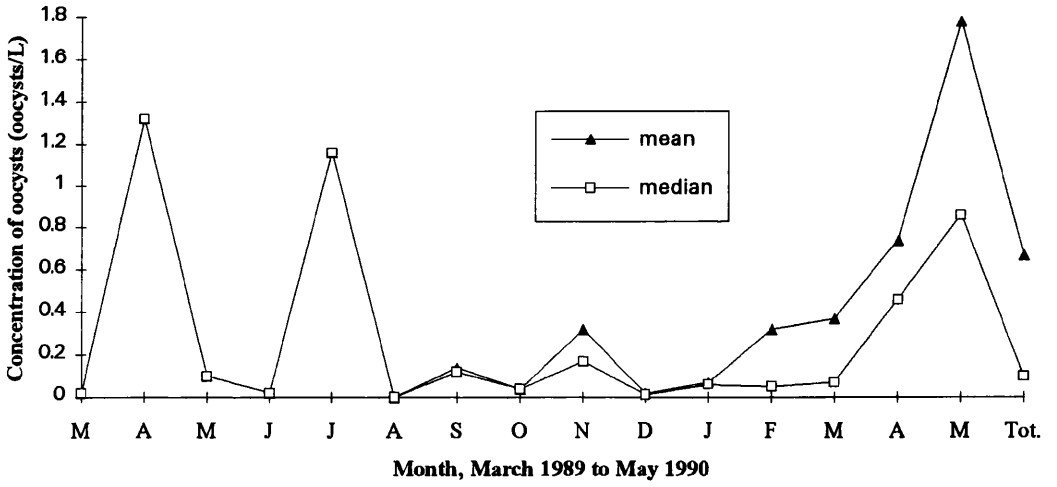


Figure 3.4. Monthly mean and median concentration of *Giardia* spp. cysts in surface water.

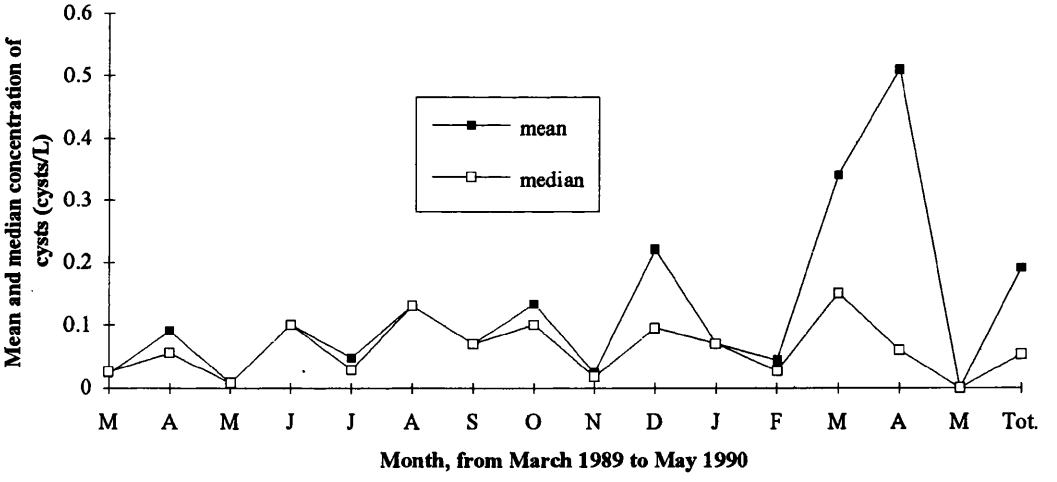


Figure 3.5. Monthly mean and median concentrations of *Cryptosporidium* spp. oocysts in treated water.

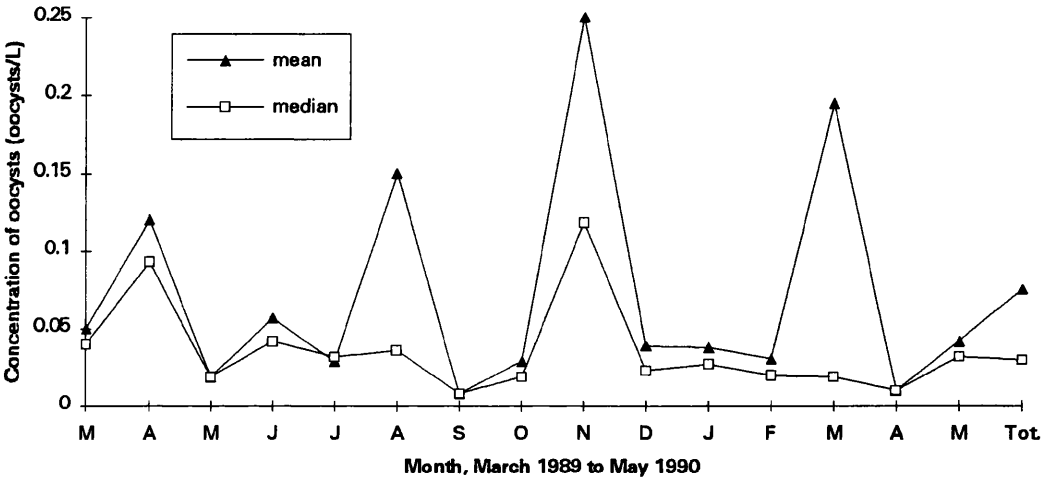


Figure 3.6. Monthly mean and median concentrations of *Giardia* spp. cysts in treated water.

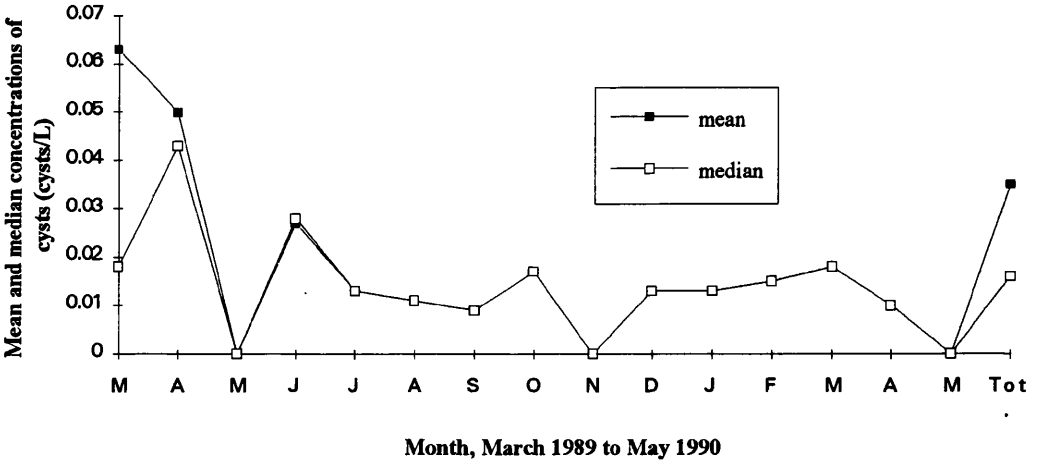


Figure 3.7. Occurrence and concurrence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in surface water.

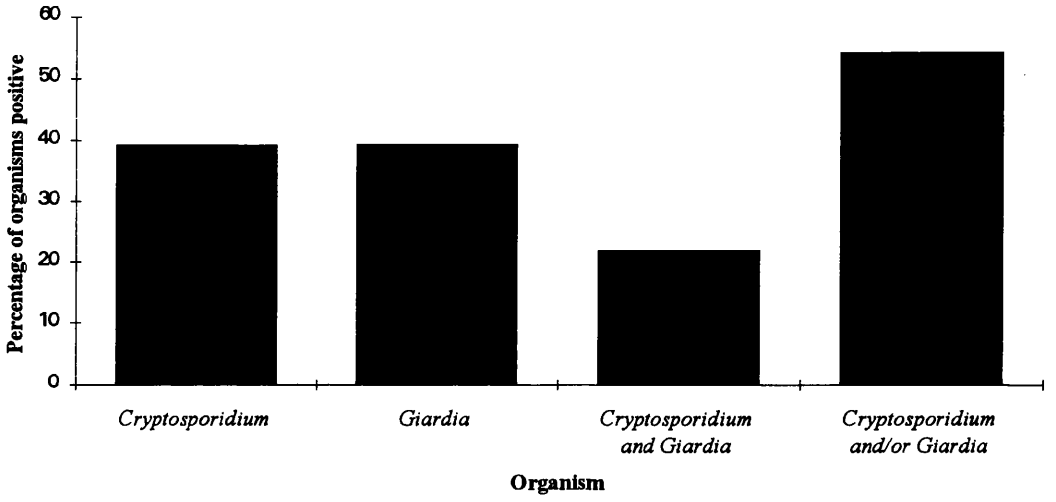


Figure 3.8. Occurrence and concurrence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in treated water.

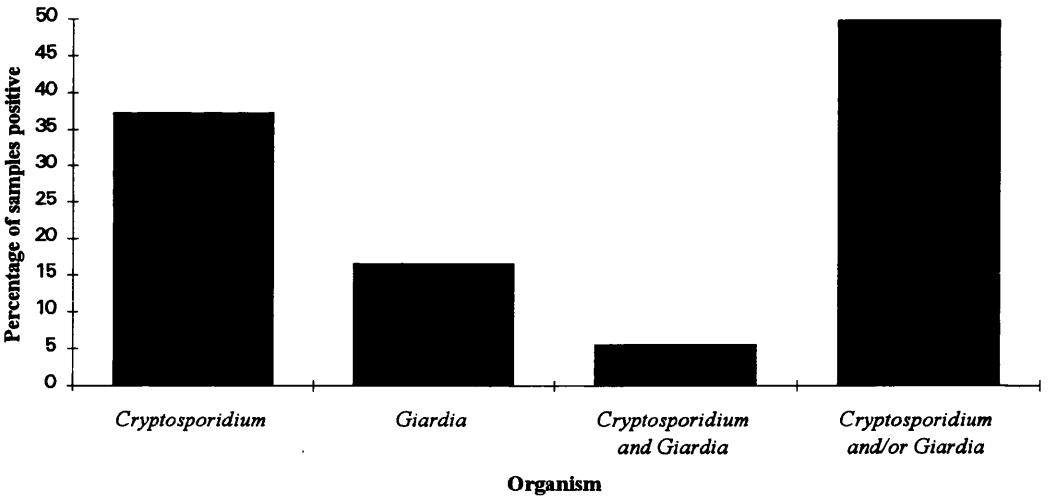


Figure 3.9. Occurrence of *Cryptosporidium* spp. oocysts in surface water receiving/not receiving sewage effluent.

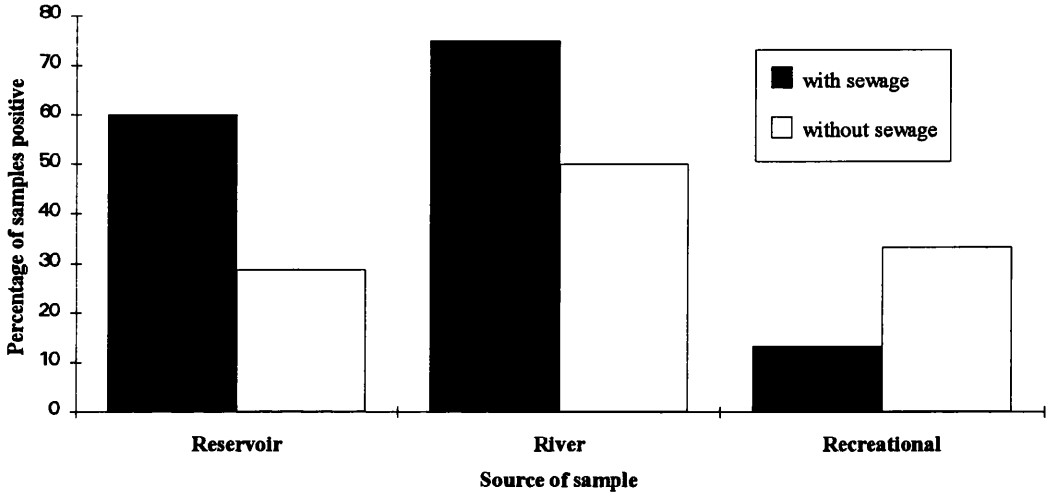


Figure 3.10. Occurrence of *Giardia* spp. cysts in surface water receiving/not receiving sewage effluent.

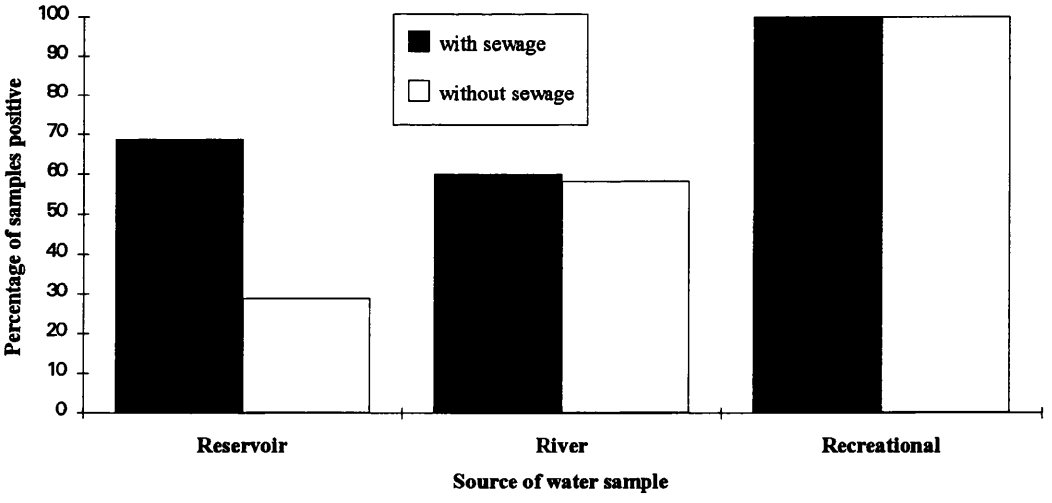


Figure 3.11. Seasonal occurrence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in surface water.

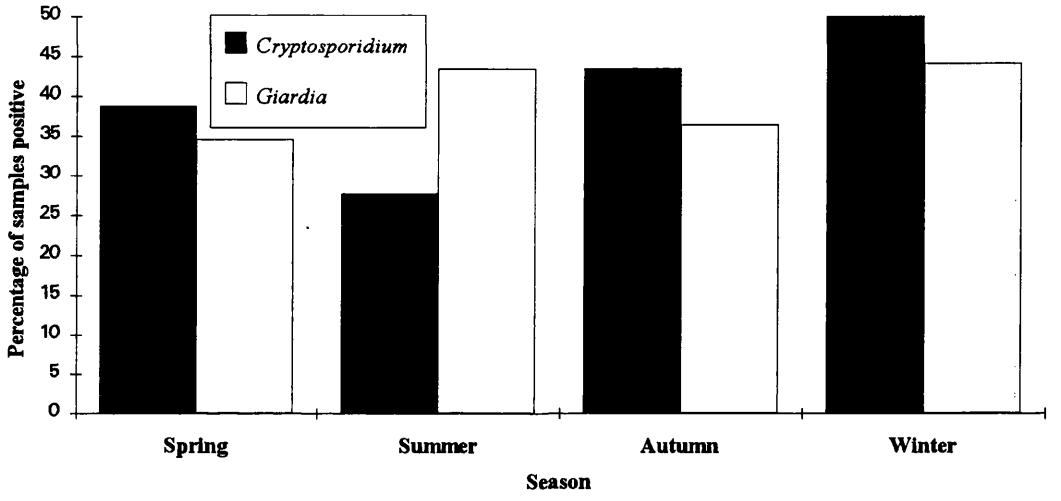


Figure 3.12. Seasonal occurrence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in treated water.

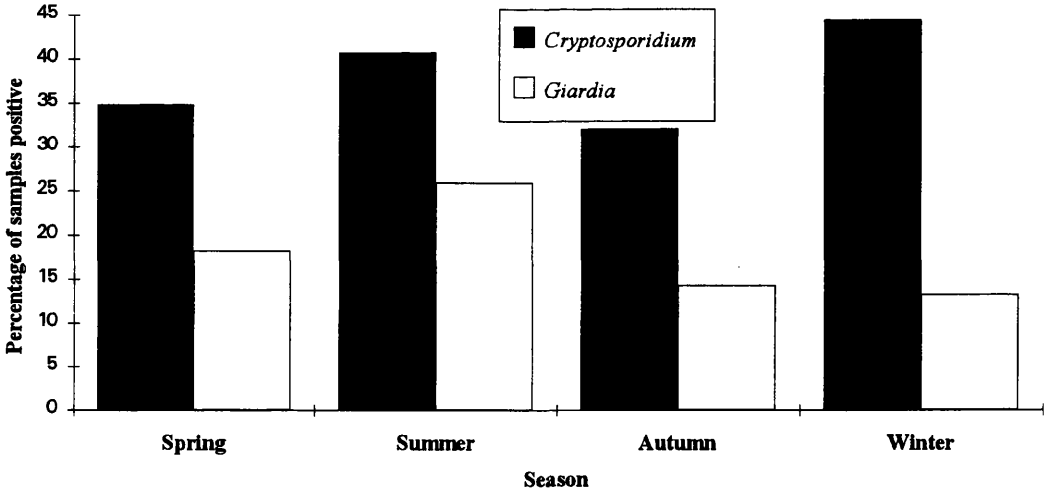


Figure 3.13. Mean concentration of *Cryptosporidium* spp. oocysts before and after treatment.

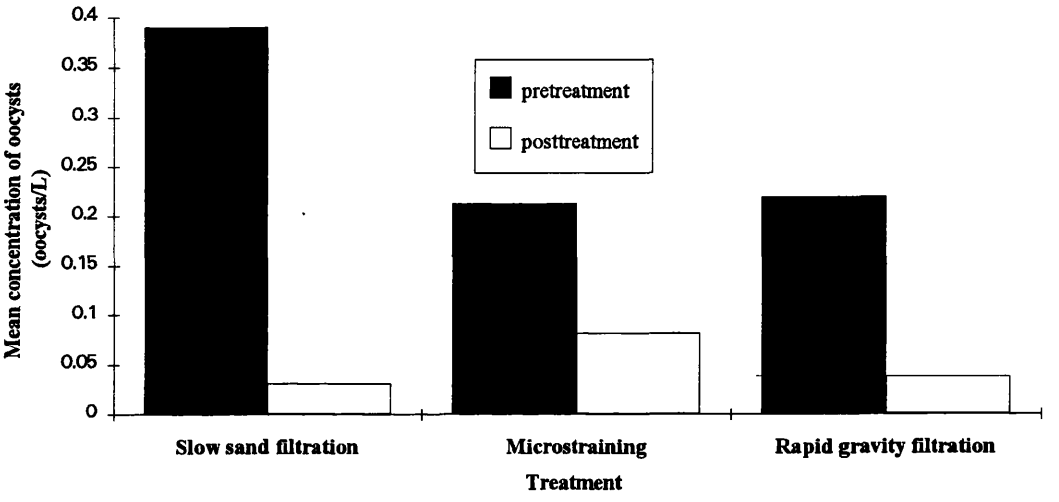
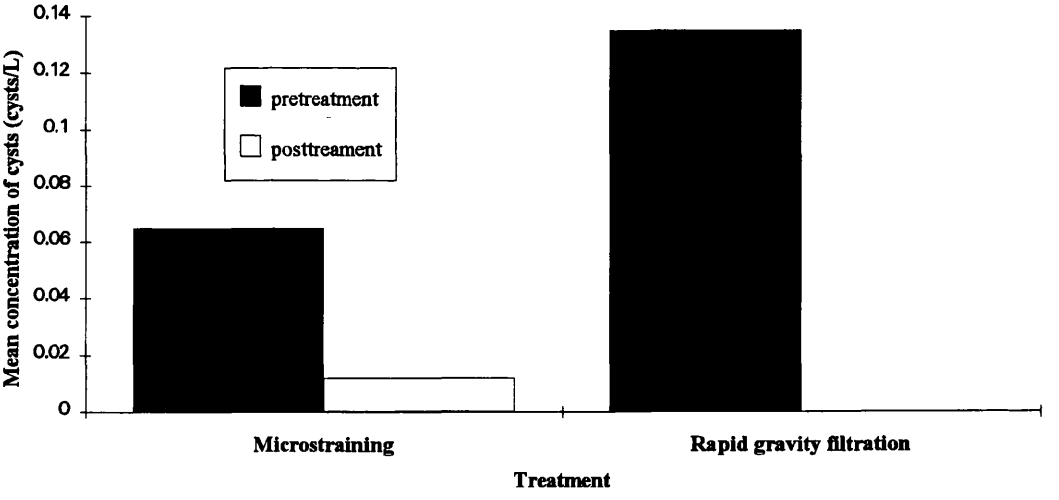


Figure 3.14. Mean concentration of *Giardia* spp. cysts before and after treatment.



CHAPTER 4. SURVEY OF AN UPLAND WATER SUPPLY TO ASSESS THE OCCURRENCE AND PREVALENCE OF *CRYPTOSPORIDIUM* SPP. OOCYSTS AND THEIR LIKELY IMPACT ON HUMAN HEALTH.

4.1. Introduction.

Cryptosporidium spp. oocysts are ubiquitous in the aquatic environment. A number of surveys have demonstrated the presence of oocysts in a variety of water types (Smith *et al*, 1990; Smith and Rose, 1990; LeChevallier *et al*, 1991 a and b;). In the last ten years there have been several outbreaks of cryptosporidiosis which have been a result of consumption of water contaminated with oocysts, either before or after water treatment (Hayes *et al*, 1987; Smith *et al*, 1989; Richardson *et al*, 1991). Table 4.1 below summarises the documented outbreaks of waterborne cryptosporidiosis in the UK and USA, including the source of contamination and whether it was pre- or post-treatment.

Table 4.1. Waterborne outbreaks of cryptosporidiosis in the UK and the USA.

Waterborne Outbreak	Number Affected	Source of Contamination	Oocysts Detected
Ayrshire, Scotland. 1988.	27	Cattle Slurry	Treated Water 0.04 - 4.8 / L Backwash Water 300 / L Sludge 1,000 / L Sludge Cake 3 / g
Swindon/ Oxfordshire, England. 1989.	516	Cattle Slurry and / or muck	Raw Water 0.002 - 14 /L Treated Water 0.002 - 77 /L Backwash Water 1,000 / L Reservoir Sediment 100 /g
Loch Lomond, Scotland. 1989.	244	Unknown	Raw Water 0.01 - 2.3 / L Treated Water 0.008 - 0.4 / L
North Humberside, England. 1990.	470	Unknown	Raw Water 0.11 - 2 / L Treated Water positive (no data)
Isle of Thanet, England. 1991.	47+	Unknown	oocysts not detected
Texas, USA. 1984.	79	Sewerage contamination of well	oocysts not detected
Carrollton, USA. 1987.	13,000	Operational irregularities	Sewage 1.2 - 34/L Raw water 0.08/L Treated water 0.63/L

In 1989 an increase in the number of cases of cryptosporidiosis in Scotland was detected and a possible association was made between those suffering from cryptosporidiosis and the water supplied to them from Loch Lomond (Anon, 1990a). During the period January to June 1989, 442 cases of cryptosporidiosis were reported in Scotland. An investigation was carried out by the health board areas of Argyll and Clyde, Lanarkshire and Lothian, where 244 cases were reported. Of these, 206 follow up questionnaires were returned which showed that 54 % were under 10 years old, and 41 % of those were in the age group 1 to 4 years. Twenty-two percent were admitted to hospital during the course of illness. The onset of symptoms was mostly in April or May. The outbreak tailed off after May, with the reported incidence of disease returning to background levels. In Lothian the outbreak appeared to have begun earlier and continued for longer.

Of the total number of patients with stools positive for *Cryptosporidium*, 39 % received water from Loch Lomond. It was reported that there was a statistically higher incidence of cryptosporidiosis in people supplied with water from Loch Lomond, compared to those in the same areas who received water from different supply sources. The range of incidence rates in different districts all with the same Loch Lomond water supply indicated that other factors may also be important.

In the two weeks preceding the onset of illness, 37 % of the patients had had contact with people with similar symptoms, though these might not necessarily have been diagnosed as suffering from cryptosporidiosis. This suggests a high incidence of person to person transmission. Thirty-two percent had travelled away from their home town, although only 2 people had been abroad. Contact with farm animals or pets was reported in 49 % of people. Few cases of illness, however, were reported amongst animals with which there had been contact.

Water is abstracted from Loch Lomond at Ross Priory. It is then pumped through two mains, one to Blairlinnans water treatment works for distribution to the north bank of the River Clyde and Renfrewshire. The other main takes water to Balmore water treatment works from where it is distributed to Lanarkshire and West Lothian.

Abstracted water is passed through coarse and mechanically operated fine screens before being chlorinated and pumped to the treatment works. There it is passed through stainless steel microstrainers with a nominal pore size of 23 μm. The pH is raised to reduce plumbosolvency and a further dose of chlorine is added (booster chlorination).

Analysis of water was instigated in April 1989 and oocysts were detected in the raw water at Ross Priory (0.01 - 2.3 oocysts/l) and in the treated water from Old Kilpatrick and Gowanbank storage reservoirs (0.008 - 0.4 oocyst/l) on sporadic occasions thereafter.

The investigators concluded from the results that an outbreak had occurred in April and May 1989 amongst people living in the area supplied with water from Loch Lomond. The finding of low numbers of oocysts in both raw and treated loch water, taken in conjunction with the epidemiological evidence suggested an association between consumption of Loch Lomond water and disease. However, the evidence was not conclusive as to the source of infection contributing to the occurrence and spread of cryptosporidiosis, and other factors such as post treatment contamination cannot be ruled out (Anon, 1990a).

Subsequent to the information gathered and reported above further analysis was performed on information obtained from 298 diagnosed cases of cryptosporidiosis in Strathclyde region from the same episode (Robertson, L.J., personal communication).

Of the 298 cases diagnosed, 151 were males and 147 were females. The age of patients ranged from less than 1 month old to over 88 years old. The age distribution is shown in table 4.2. below.

Table 4.2. Age distribution of diagnosed cases of cryptosporidiosis.

Age category	Percentage of cases in each category
< 1 month - 1 year 0 months	10.2 %
1 year 1 month - 4 years 0 months	38.2 %
4 years 1 month - 12 years 0 months	19.4 %
12 years 1 month - 70 years 0 months	29.0 %
> 70 years 1 month	3.0 %

Hospital admission was required for 18 % (54) of the 298 diagnosed cases of cryptosporidiosis. The age distribution of those cases is shown in table 4.3 below.

Table 4.3. Age distribution of cases which required hospital admission.

Age category	Percentage of cases in each category
< 1 month - 1 year 0 months	24.1 %
1 year 1 month - 4 years 0 months	40.7 %
4 years 1 month - 12 years 0 months	14.8 %
12 years 1 month - 70 years 0 months	13.0 %
> 70 years 1 month	7.4 %

Out of the 298 diagnosed cases, all were divided into age categories (as shown in table 4.2) and the percentage of patients within each of those age categories requiring hospital admission was calculated as shown in table 4.4 below.

Table 4.4. Age distribution of hospital admissions, calculated from total number of cases (298), i.e. not only from those requiring hospitalisation..

Age category	Percentage of cases in each category
< 1 month - 1 year 0 months	44.8 %
1 year 1 month - 4 years 0 months	20.4 %
4 years 1 month - 12 years 0 months	14.6 %
12 years 1 month - 70 years 0 months	8.5 %
> 70 years 1 month	44.4 %

It can be seen from table 4.4 above that the patients most likely to require hospitalisation were in the youngest and oldest age ranges, while patients in the intervening categories were less likely to require hospitalisation.

Patients were asked which of four categories described their symptoms. These categories were diarrhoea, abdominal pain, vomiting and fever. Table 4.5 below shows the percentage of patients who described these symptoms. 86 % reported experiencing at least 2 of the symptoms and 20 % experienced all four.

Table 4.5. Symptoms reported by patients.

Symptom	Percent of patients reporting symptom
diarrhoea	94 %
abdominal pain	69 %
vomiting	58 %
fever	40 %

In addition to tracing the source of water used by the patients, information was gathered regarding foreign travel, contact of patients with others experiencing similar symptoms and contact with farm animals or pets. Of the 298 cases, 43.6 % were known to be supplied with Loch Lomond water at home, work or school. The remaining 54.6 % were not known to be associated with a Loch Lomond supply.

Of the 130 patients known to receive water from Loch Lomond, 66 % (86/130) were reported to have been abroad, have had contact with others with similar symptoms, have had contact with animals or a combination of these.

Of the 168 patients not on a Lomond supply, 75 % (126/168) had been abroad, had contact with others with similar symptoms, had contact with animals or a combination of these.

It was concluded that, from the data collected, there was no definitive case for associating any of the cases of cryptosporidiosis with ingesting water supplied from Loch Lomond. There were problems with the manner in which data was compiled, with a variety of questionnaire formats in use and different interview patterns used by the environmental health officers involved. In addition the investigating environmental health officers varied from being fully trained to 3rd year students.

As a result of the finding of oocysts in raw and treated loch water and the possible association with an outbreak of cryptosporidiosis this epidemiological study was set up. The aim of the study was to assess the seasonal occurrence of oocysts entering water treatment by long term sampling at the abstraction point and to assess the occurrence of human and animal cryptosporidiosis in the Lomond southern catchment area. In addition the source of oocysts penetrating the aquatic environment in the area was to be assessed and the viability of all waterborne oocysts was to be determined. The occurrence of human cryptosporidiosis in the population supplied with Lomond water was to be monitored. These data would suggest whether any cases, clusters of cases or outbreaks of cryptosporidiosis were waterborne and also the possible source of contamination.

4.2. Materials and Methods

Materials and methods are described in detail in chapter 2. This section contains those methods which were used primarily in this section of the work, or which require additional explanation for their application to this work.

4.2.1. Water sampling, filter processing and sample analysis

Sampling, processing and analysis of the raw water and sewage effluent was performed according to the method described in the DoE publication for the " Isolation and Identification of *Giardia* Cysts, *Cryptosporidium* Oocysts and Free Living Pathogenic Amoebae in Water etc. 1989 " (Anon, 1990b).

4.2.1.1. Sampling sites.

4.2.1.1.1. Raw water sampling at Ross Priory.

A filter cartridge housing (Cuno) was fitted on line at the abstraction point at Ross Priory in order to sample raw water. The water was sampled constantly, with filters being changed after 48 h, 48 h and 72 h, providing three samples per week over the two year period. The flow rate was kept low, at approximately 1 l/min. Filters were changed by Central Scotland Water Development Board (CSWDB) staff and delivered to the SPDL for processing and analysis.

4.2.1.1.2. Distribution water.

Samples of final distribution water were taken and analysed by Strathclyde Regional Council (SRC) Water Department staff from the Blairlinnans and Dalmacoulter distribution lines. Samples of final distribution water were taken from the Gowanbank distribution line (Gowanbank, Preston, Humbie and Eastcraigs) and processed and analysed by Lothian Regional Council (LRC) water department staff.

4.2.1.1.3. Sewage effluents.

Sewage treatment works discharging effluent into the Rivers Blane and Endrick, and into the Loch Lomond itself were identified with the help of Central Regional Council (CRC) Department of Water and Drainage, CSWDB and Clyde River Purification Board. Effluents from the following sewage treatment works were sampled.

Table 4.2.1. Sewage treatment work effluents sampled and the river into which outfall passes.

Receiving River	Sewage treatment works	Receiving River	Sewage treatment works
Endrick Water	Fintry Balfron Croftamie Drymen Gartocharn	Blane Water	Strathblane Killearn

Effluent from Croftamie treatment works was discharged into the Catter Burn which flows into Endrick Water and effluent from Gartocharn was discharged into Blairennich Burn which also flows into Endrick Water. Samples were also taken on occasion from Drymen Bridge, a point before which most effluents have been discharged into the river. Figures 4.1 and 4.2 show the locations of the sewage treatment works on the two rivers and figure 4.3 shows Loch Lomond, with the abstraction point, Ross Priory, and the point where the Endrick flows into the loch.

4.2.1.1.4. Slurry and faecal specimens.

Samples of slurry and faeces (bovine) were obtained from farms and veterinary practices in the southern catchment area of Loch Lomond through Mr. W. Reilly of the Environmental Health (Scotland) Unit (EH(S)U). Further samples of faeces of bovine, equine and ovine origin were collected from the banks of the Endrick and Blane Waters in the vicinity of the sewage treatment works which were sampled.

4.2.1.1.5. Fish.

Minnows and three spined sticklebacks were caught in the Endrick using a fishing net and transported back to the laboratory.

4.2.1.2. Sampling technique - filtration and filter processing.

4.2.1.2.1. Sampling.

See chapter 2, section 2.6.2.

4.2.1.2.2. Filter processing.

See chapter 2, section 2.6.3.

4.2.1.2.3. Analysis.

See chapter 2, section 2.6.4.

4.2.2. Processing of faecal and slurry samples.

Faecal and slurry samples were concentrated by the formol-ether technique (Allen and Ridley 1970). For the detection of *Cryptosporidium parvum* oocysts, a drop of the concentrate was placed on a microscope slide, air dried, fixed in methanol for three minutes and then stained using the auramine-phenol (Diachem) technique described by Casemore *et al* (1985), see also chapter 2, section 2.3.1.

4.2.3. Processing of fish.

Minnows and three spined sticklebacks were caught in the River Endrick using a net. They were pithed on arrival at SPDL. The fish were immersed in 10 % sodium hypochlorite for 5 min. to remove possible oocysts from the surface and were then washed in 3 changes of RO water. The fish were then homogenised and centrifuged at 900 ***x g*** in a 50 ml centrifuge tube. The supernatant was aspirated and the pellet resuspended in 10 ml RO water. Sucrose density flotation was performed on the pellet as for water samples, including the washing stages (chapter 2, section 2.6.3.) and detection by MAb labelling and fluorescence microscopy (chapter 2, sections 2.3.4, 2.5 and 2.6.4).

4.2.4. Staining methods for oocyst detection.

Staining methods for the detection of oocysts in this section included auramine phenol staining and labelling with MAb. These are described in chapter 2, sections 2.3.1 and 2.3.4.

4.2.5. Viability.

Viability of the oocysts was assessed using the inclusion or exclusion of the fluorogenic vital dyes 4'6-diamidino-2-phenyl indole (DAPI) and propidium iodide (PI), according to the method of Campbell *et al* (1992). This is described in chapter 2, section 2.4.1.

4.2.6. Beads.

Streptavidin coated M-280 magnetisable particles (Dynal, UK) were labelled with a biotinylated anti-FITC MAb (received as ascites fluid from PHLS, Collindale, and purified and biotinylated at SPDL). These were added to final pellets to which the vital dyes and anti-*Cryptosporidium* MAb had been added, with the excess washed out, as for viability analysis. Beads and oocysts were mixed for 30 min at room temperature and separation was performed for 60 sec on the magnetic particle concentrator. At the end of this period the supernatant was removed and saved and the remaining pellet (beads and oocysts) resuspended in 10 µl PBS. The sample was applied onto a microscope slide covered with a coverslip, sealed with nail varnish and scanned for the presence of oocysts. Viability was assessed by the inclusion or exclusion of the vital dyes as described above and in chapter 2, section 2.4.1.

Further information on the methods used for immunomagnetic separation is presented in chapter 2, section 2.8 and in chapter 5.

4.2.7. Microscopy.

See chapter 2, section 2.5.

4.2.8. Statistical analysis.

See chapter 2, section 2.12.

A MINITAB statistical package was used to perform Chi-squared and Mann-Whitney U tests for significance in occurrence and concentration of oocysts in water and water related samples. Differences were considered to be statistically significant when $p \leq 0.05$.

4.3. Results.

4.3.1. Occurrence of oocysts in water and water related samples.

4.3.1.1. Occurrence of oocysts in raw water at Ross Priory.

Oocysts were detected in 32 out of a total of 279 samples processed from Ross Priory (11.5 %), with a range of concentrations from 0.0019 - 0.12 oocyst/l (median = 0.01 oocyst/l, mean = 0.018 oocyst/l, standard deviation = 0.025 oocyst/l). These data are presented in table 4.3.1. and figure 4.4.

Faecal coliforms were detected on many occasions in the raw water at Ross Priory. There was no association between the presence of coliforms, or their levels, and the detection of oocysts.

The results obtained from the constant monitoring of raw water, abstracted from Loch Lomond at Ross Priory, indicated that there were oocysts present in the raw water on sporadic occasions between March 1991 and March 1993. Detection of oocysts was mostly sporadic, although there was a small cluster of positive samples in May 1992 and a larger cluster of positive samples, the detection of which continued between November 1992 and January 1993. In November 1991 and January 1992 there were a few samples in which low concentrations of oocysts were detected.

Seasonal comparisons revealed no statistical difference between Spring, Summer, Autumn and Winter 1991/2. For the same seasons in 1992/3 there was a significantly greater occurrence of oocysts in raw water in winter 1992/3 (December 1992, January 1993 and February 1993) compared to the other 3 seasons. There was no significant difference between any of the other seasons. A statistically significant difference was detected in the occurrence of oocysts in winter 1991/2 compared to winter 1992/3; oocysts were detected with greater frequency in the latter. There were no significant differences in the concentration of oocysts detected in any one month compared to the others.

The high occurrence of oocysts in raw water samples corresponded roughly with the increase in cases of cryptosporidiosis seen from the beginning of November 1992. The cases were recorded with onset dates beginning mid-November, while oocysts were initially detected on 23/11/92 in Ross Priory (the last positive sample was on 16/09/92). In distribution, oocysts were detected first on 9/11/92 (Lenzie) and 1/12/92 (Port Glasgow, the area in which most cases occurred).

There was a statistically significant lower occurrence and concentration of oocysts in water abstracted at Ross Priory compared to the occurrence and concentration of oocysts in sewage effluent. There was a significantly greater occurrence of oocysts in raw water from Ross Priory compared to distribution water sampled by SRC from the Blairlinnans distribution line. There was no significant difference in the occurrence of oocysts in raw water compared to distribution water from West Lothian, sampled by LRC. There was no significant difference in the concentration of oocysts detected in raw water compared to distribution water.

In comparison to data collected from sampling water abstracted at Ross Priory during the period April 1989 to July 1990, the occurrence and concentration of oocysts detected in raw water in the 1989 to 1990 period was significantly greater than in the 1991 to 1993 period.

Table 4.3.1. Samples of Loch Lomond water positive for *Cryptosporidium* spp. oocysts and their viability status.

Sample	Date	Oocysts/l	PI	DAPI+	DAPI-	Ghosts
5	3/4/91	0.12	81%	14%	5%	-
6	5/4/91	0.06	81%	14%	5%	-
28	28/5/91	0.012	-	-	-	-
33	10/6/91	0.012	-	-	-	-
52	28/8/91	0.085	-	-	-	-
77	4/11/91	0.0047	-	-	-	-
82	15/11/91	0.017	1 oocyst	-	-	-
84	20/11/91	0.006	-	-	-	-
104	6/1/92	0.0023	-	-	-	-
106	10/1/92	0.0092	-	-	-	-
111	22/1/92	0.006	-	-	-	-
158	15/5/92	0.012	1 oocyst	-	-	-
159	18/5/92	0.0045	-	-	-	1 oocyst
160	20/5/92	0.024	3 oocysts	2 oocysts	2 oocysts	3 oocysts
164	29/5/92	0.023	n.d.	n.d.	n.d.	n.d.
197	17/08/92	0.012	-	-	-	-
210	16/09/92	0.0019	-	-	-	2 oocysts
239	23/11/92	0.006	1 oocyst	-	-	-
241	27/11/92	0.01	2 oocysts	-	-	-
244	04/12/92	0.0085	1 oocyst	-	-	1 oocyst
245	07/12/92	0.014	-	-	-	-
246	09/12/92	0.017	-	-	-	-
247	11/12/92	0.016	2 oocysts	-	-	-
249	16/12/92	0.008	1 oocyst	-	-	-
250	18/12/92	0.0065	0	-	-	-
251	21/12/92	0.0047	0	-	-	-
252	23/12/92	0.01	1 oocyst	-	-	-
253	27/12/92	0.005	-	-	-	-
255	03/01/93	0.01	-	-	-	-
258	11/01/93	0.01	-	-	-	-
259	13/01/93	0.026	-	-	-	1 oocyst
271	10/02/93	0.009	-	-	-	-

4.3.1.1.1. Viability of oocysts detected in raw water abstracted at Ross Priory for distribution.

Most of the oocysts detected in water samples taken from Ross Priory were non-viable. On 3/4/91 and 5/4/91 19 % of the oocysts recovered from the final pellet were considered to be viable. On 20/5/92 4 out of the 10 oocysts recovered from the final pellet were considered to be viable. In May and June 1992 bovine faecal samples analysed at SPDL were found to contain large numbers of viable oocysts. It was considered possible that the faeces was discharged onto land around the rivers Blane and Endrick.

4.3.1.2. Occurrence of oocysts in final water in distribution.

4.3.1.2.1. Strathclyde Regional Council.

Out of 188 samples of water of Loch Lomond origin taken from distribution in Strathclyde region, oocysts were detected in 7 (3.7 %). These data are shown in table 4.3.2 below. No viable oocysts were detected in any of these samples. There were no bacteriological failures reported on or around the times when oocysts were detected in water samples. There was no association with differences in other parameters (e.g. chlorine concentration) on or around the dates when oocysts were detected.

There was a significantly lower occurrence and concentration of oocysts in distribution water compared to sewage effluent. There was a significantly lower occurrence of oocysts detected in distribution water compared to oocysts detected in raw water, although there was no significant difference in the concentration detected. There was no significant difference in either the occurrence or concentration of oocysts detected in distribution water samples taken from the Blairlinnans line (SRC) compared to the Balmore line (LRC).

Compared to data collected between April 1989 and July 1990, there was a significantly greater occurrence of oocysts in the period 1989 to 1990 than in the present study (1991 to 1993). There was no statistically significant difference in the concentration of oocysts detected in either study.

Table 4.3.2. Occurrence of oocysts in samples from distribution, from SRC water department.

Sample	Date	Oocysts/l
Lenzie	2/12/91	0.021
Lenzie	9/11/92	0.0007
Lenzie	13/11/92	0.01
Lenzie	19/11/92	0.005
Port Glasgow	3/12/92	0.046
Port Glasgow	5/12/92	0.013
Port Glasgow	9/12/92	0.025

4.3.1.2.2. Lothian Regional Council.

Out of 111 samples taken, oocysts were detected in 7 (6.3 %). These data are shown in table 4.3.3 below. Oocyst viability ranged from 30 to 50 %. There were no bacteriological failures reported on or around the dates when oocysts were detected in distribution water.

There was a significantly greater occurrence and concentration of oocysts detected in sewage effluent compared to distribution water. There was no significant difference in the occurrence and concentration of oocysts detected in raw water compared to distribution water from the Balmore (LRC) distribution line. There was no significant difference in the occurrence and concentration of oocysts detected in LRC distribution water compared to SRC distribution water.

There was a significantly lower occurrence of oocysts in distribution water in the present study compared to the 1989/90 survey. The was no difference in the concentration of oocysts detected during either of the sampling periods.

Table 4.3.3. Occurrence of oocysts in samples from distribution, from LRC water department.

Sample	Date	Oocysts/l	Viability
Preston Tank	24/5/91	0.01	not tested
Preston Tank	2/3/92	0.032	1 oocyst
Humbie Tank	2/3/92	0.07	none viable
Gowanbank Tank	2/3/92	0.055	30 %
Humbie	18/6/92	0.013	50 %
Gowanbank	8/9/92	0.007	none viable
Gowanbank Tank	20/11/92	0.01	none viable

4.3.1.3. Occurrence of oocysts in sewage effluents.

Cryptosporidium oocysts were detected in 26 (38.24%) out of 68 samples of sewage effluent analysed for their presence. Oocysts were detected at a range of 0.024 - 2.3 oocysts/l (mean = 0.433 oocyst/l, standard deviation = 0.644 oocyst/l, median = 0.175 oocyst/l). The data are presented in table 4.3.4 and figure 4.5.

The occurrence and concentration of oocysts detected in sewage effluent samples was significantly greater than the occurrence and concentration of oocysts detected in samples of raw water from the abstraction point at Ross Priory and the two branches of the distribution network, sampled by SRC and LRC.

Table 4.3.4. Occurrence of oocysts detected in sewage effluent.

Date	Fintry	Balfron	Drymen	Croftamie	Strathblane	Killearn	Gartocharn
4/6/91	0	0	0	0	ns	ns	ns
5/6/91	ns	ns	ns	ns	0	0	ns
5/8/91	0	0	0.15	0	ns	ns	ns
6/8/91	ns	ns	ns	ns	0	0	0
7/8/91	ns	ns	ns	ns	ns	ns	ns
12/8/91	0.05	0	0.09	ns	ns	ns	0
13/8/91	ns	ns	ns	0	0.28	1.72	ns
20/8/91	0	0.76	0	0	ns	ns	0.043
21/8/91	ns	ns	ns	ns	0	0.35	ns
29/8/91	0	0.26	0.24	0	ns	ns	ns
17/12/91	ns	ns	ns	ns	0.59	0	ns
18/12/91	ns	ns	0.037	ns	ns	ns	ns
19/12/91	ns	ns	ns	0.77	ns	ns	0.12
20/12/91	0	0	ns	ns	ns	ns	ns
28/1/92	ns	0.1	ns	ns	ns	ns	ns
27/2/92	0.11	ns	ns	ns	0.024	0.03	ns
28/2/92	ns	ns	0.094	0	ns	ns	0.081
26/03/92	0	ns	ns	0	ns	ns	0
27/05/92	0	0	0	ns	ns	ns	0
28/05/92	ns	ns	ns	0	0	0	0
29/06/92	0	0	ns	ns	ns	ns	ns
24/07/92	ns	ns	0	0	ns	ns	ns
14/08/92	ns	ns	ns	ns	0	0	ns
18/09/92	ns	ns	0	ns	ns	ns	0
29/10/92	2.3	2.2	ns	ns	ns	ns	ns
29/11/92	ns	ns	0.2	0.3	ns	ns	ns
29/01/93	ns	ns	ns	ns	0.05	ns	ns
25/02/93	ns	ns	ns	ns	ns	0.32	ns

ns = not sampled

4.3.1.3.1. Viability of oocysts detected in sewage effluent.

The viability of oocysts detected in sewage effluent (table 4.3.4) ranged from 33 % to 100% (see table 4.3.5 below). Initially there was a problem in recovering oocysts in order to test viability. When recovered, the contaminating debris often obscured the oocysts so that dye inclusion/exclusion could not be viewed satisfactorily. The use of beads remedied this situation to some extent. The viable oocysts and percentage viability quoted in table 4.3.5 below are calculated from the number of oocysts on which viability was performed and not on the number which should have been present by back calculation from the concentration detected in the sample initially.

Table 4.3.5. Viability of oocysts detected in sewage effluent.

Sample	Date	DAPI +	DAPI -	PI +	Ghost	% viable
Fintry	27/2/92	1 oocyst	-	1 oocyst	1 oocyst	33 %
	29/10/92	1 oocyst	2 oocysts	1 oocyst	-	75 %
Balfron	29/8/91		1 oocyst		1 oocyst	50 %
	29/10/92	-	2 oocysts	-	1 oocyst	67 %
Drymen	29/11/92	2 oocysts	-	-	-	100 %
Croftamie	29/11/92	1 oocyst	-	-	2 oocysts	33 %
Strathblane	27/2/92	1 oocyst	-	-	-	100 %
Killearn	13/8/91	-	2 oocysts	1 oocyst	-	67 %
Gartocharn	28/2/92	1 oocyst	-	1 oocyst	-	50 %

4.3.2. Cryptosporidiosis in the community.

Throughout the duration of the study reports of cases of cryptosporidiosis were received from Dr D. Campbell of the Environmental Health (Scotland) Unit and from the relevant Health Board Laboratories. The occurrence of cryptosporidiosis showed the characteristic spring and autumn peaks. There were no reports of cases of cryptosporidiosis which were associated with the waterborne route. However an increase in cases was noted from the end of October (week 44) to mid December 1992 (week 49), primarily in Argyll and Clyde Health Board area, in the Inverclyde District, particularly Greenock and Port Glasgow. Up until November there had been an average of 4 cases per month. Twenty cases were recorded with onset dates spanning the period 13/11/92 to 3/12/92. Most cases were in school or pre-school children. There was no

obvious pattern in their geographical distribution. There did appear to be one family outbreak. Home and work or school water supplies were determined. Only 2 cases were definitely supplied with water of Loch Lomond origin, while 16 were supplied from other sources.

In Lanarkshire Health Board area there were 9 proven cases from the end of October to mid December. All appeared to be primary cases and most were over 18 years old (except for 2 children, one aged 6 years and one aged 14 months). Lomond water supplies about a third of the population of Lanarkshire (approximately 200,000 people). Seven out of the nine cases were found to receive Lomond water and 2 received water from South Lanarkshire.

In Greater Glasgow Health Board area there were 8 cases of cryptosporidiosis in November of which about half received Lomond water.

Lothian Health Board reported 22 cases of cryptosporidiosis in Lothian overall, of which 8 were in West Lothian, the area supplied with Lomond water. There were 3 family outbreaks included in these figures, with evidence of secondary spread. Only one case of cryptosporidiosis was recorded in someone not receiving Lomond water, but the remaining cases were not evenly distributed among the population zones. This seemed to mitigate against a common source outbreak originating from Loch Lomond water.

4.3.3. Cryptosporidiosis in animals.

There were no reports of cryptosporidiosis in the southern catchment area of Loch Lomond issued through the Communicable Diseases and Environmental Health in Scotland weekly reports. No reports of cryptosporidiosis were communicated to SPDL throughout the duration of the study. Only 2 positive cases in the southern catchment area of Lomond were detected. These were samples taken from calves with a history of scouring. The samples were collected from Veterinary practices in the area through Mr. W. Reilly (EH(S)U) and analysed at SPDL. Further details are given below.

4.3.4. Occurrence of oocysts in slurry and faecal samples.

Numerous samples of slurry and calf or cattle faeces were received from farms and veterinary practices around the southern catchment area of Loch Lomond, through Mr. W. Reilly (Environmental Health (Scotland) Unit). None of the samples of slurry was found to contain *Cryptosporidium* oocysts. Two samples of calf faeces contained oocysts, though little information was available on the farms of origin. The first was from a calf with a history of scouring, likely to be discharged onto land in the vicinity of the River Blane near Killearn.

Oocysts were detected at a concentration of approximately 10^6 oocysts/g faeces, with a viability of 86 % (27/5/92). The second was supplied by the veterinary practice in Balfron, with a viability of 80 % (29/6/92). No further information was supplied on this sample, although it is possible that the faeces was discharged onto land around the River Endrick.

Samples of bovine faeces were collected from the areas in the vicinity of the sewage effluent plants on most of the sampling occasions in table 4.3.4 above. No oocysts were detected in any of the samples from any site on any occasion.

Horse faeces was collected from fields on the banks of the River Blane, but no oocysts were detected.

4.3.5. Occurrence of oocysts in fish.

Fish were collected from the River Endrick, near to Balfron. No oocysts were detected.

4.4. Discussion

Oocysts were detected in raw water abstracted at Ross Priory before treatment and in final distribution water. This suggests that oocysts still penetrate water treatment. At least one viable oocyst was found in final water of Loch Lomond origin, although it cannot be concluded that it survived water treatment rather than originating from some source of post treatment contamination.

Seasonal occurrence showed that in the winter months, primarily December, the number of oocysts entering water treatment was greatest. This observation was statistically significant for the winter of 1992/3, while there was no statistical difference in occurrence of oocysts in winter 1991/2 compared to the other seasons. There was a significantly greater occurrence of oocysts in winter 1992/3 compared to winter 1991/2. There seems to be no reason for this pattern of oocyst detection. No farming practices at this time of year would lead to oocysts being discharged into water courses (Mr. W. Reilly, personal communication). It is possible that heavy rainfall might wash oocysts off the land into the rivers flowing into the loch, and into the loch itself. No correlation could be demonstrated between the occurrence and concentration of oocysts and rainfall. There did, however, appear to be high rainfall in the time prior to the detection of oocysts in the raw water.

Few viable oocysts were detected in raw water samples. The finding of viable oocysts in raw water in April 1991 and May 1992 coincided with finding cases of cryptosporidiosis in calves with a history of scouring. These cases occurred in May 1991 and June 1992 and originated from areas close to the rivers Blane and Endrick. Agricultural contamination of water supplies has been suggested as a major source of contamination of water with oocysts (Anon, 1990a; Rose, 1988) and has been implicated as a possible cause of at least one outbreak of cryptosporidiosis (Richardson *et al*, 1991). Agricultural contamination of water after treatment has also been suggested as the cause of another outbreak (Smith *et al*, 1989).

Oocysts were detected in sewage effluent being discharged into both the Endrick water, which flows into Loch Lomond, and into the Blane water, which flows into the Endrick and therefore ultimately to Loch Lomond also. The concentrations of oocysts detected in sewage effluent were low, with a range from 0.024 - 2.3 oocysts/l, although these levels were significantly higher than those found in raw or distribution water. There appeared to be a continuous detection of oocysts in sewage effluent, while this was not the case for the raw water at Ross Priory. Oocysts were detected with significantly greater frequency in sewage effluent than in raw water at the site of abstraction.

Cases of cryptosporidiosis were detected only sporadically in the Forth Valley Health Board area, which includes the Loch Lomond southern catchment area. On several occasions oocysts were detected in sewage effluent from sewage treatment works in the Forth Valley area, although only 6 cases of cryptosporidiosis were detected in the period from the beginning of September to the middle of November. Oocysts were detected in sewage effluent from Fintry and Balfron at the end of October 1992, although there were no reports of disease in the Forth Valley Health Board area at that time. At the end of November 1992 there was a case reported in the area and oocysts were detected in effluent from Drymen and Croftamie. It is possible that routine surveillance is not picking up continual low grade infections. Reports indicate that the minimum number of oocysts in human stool specimens that can be detected using the current coprodiagnostic techniques ranges from 5,000 to 50,000 oocysts/g of faeces depending on the state of the sample (watery or formed) and the concentration and detection method used (Weber *et al*, 1991).

It may be that the oocysts detected in abstracted water are of human and animal origin. Considering that the majority of oocysts detected at the abstraction point were non-viable it may be that oocysts are being shed into the environment and that they eventually reach the loch (and the abstraction point) at a much later stage than they first appeared in the environment. This may be a result of being shed or deposited on land close to tributaries of the loch, or the

loch itself, and being washed into the particular water course during or after a heavy period of rain. Oocysts may die whilst in slurry, depending on the storage time, leaving them non-viable when final discharged, or washed into water courses by runoff.

The occurrence of laboratory diagnosed cases of cryptosporidiosis in Scotland has remained similar over the last 3 years, with a baseline annual infection of around 900 to 1000 cases per year. It would appear that 1989 was an exceptional year for cryptosporidiosis, with the total number of cases in the region of 1200 cases for the year. In November 1992 there was an increase in cases of cryptosporidiosis observed in areas supplied with water of Loch Lomond origin. This increase was noted primarily in the Argyll and Clyde Health Board area (in the Inverclyde area, including Greenock, and Port Glasgow), which was supplied by Loch Lomond water via the Blairlinnans line after abstraction at Ross Priory. The initial cases (20 cases) were recorded with onset dates in the period 13/11/92 - 3/12/92.

The first positive water sample from Ross Priory around this time was detected on 23/11/92, the previous positive sample having been detected on 16/9/92. Oocysts were detected at low concentrations throughout December 1992 and early January 1993. In distribution, oocysts were detected earlier in samples from Lenzie (Dalmacoulter on Balmore distribution line), the first on 9/11/92 and subsequently on 13/11/92 and 19/11/92, than in the raw water (23/11/92). No cases of cryptosporidiosis were reported around the time of oocyst detection in distribution or raw water. This information is summarised in table 4.4.1 below.

On the Blairlinnans distribution line, the first positive water sample was detected on 3/12/92 in Port Glasgow, with 2 subsequent samples containing oocysts on 5/12/92 and 9/12/92. However, the onset of symptoms in the earlier cases of cryptosporidiosis began prior to the detection of oocysts in either raw water or Port Glasgow distribution water (although symptom onset ranged from 13/11/92 to 3/12/92 and the first positive in Ross Priory was 23/11/92), with ingestion likely to have occurred at least 3 to 4 days prior to the onset of symptoms (table 4.4.1). The occurrence of oocysts in raw water and in distribution water could not be correlated with the occurrence of disease in the community. Cases of cryptosporidiosis continued to occur in the area and oocysts continued to be found in the raw water. No further samples of distribution water were positive for oocysts.

Table 4.4.1. Occurrence of oocysts in raw and distribution water, date of onset of symptoms and approximate date of oocyst ingestion.

Origin of sample	Date of positive water sample	Symptom onset	Approximate ingestion date
Ross Priory	23/11/92	-	-
Lenzie	9/11/92	none	-
	13/11/92	none	-
	19/11/92	none	-
Port Glasgow	3/12/92	13/11/92	Approximately 3 to
	5/12/92	-3/12/92	4 days before onset
	9/12/92		of symptoms.

Most of the cases occurred in school or pre-school children. There was no obvious pattern in their geographical distribution. At least one family outbreak occurred. Home and work or school water supplies were determined and only 2 of the cases were definitely determined as having a water supply of Lomond origin.

Lanarkshire Health Board reported 9 proven cases from the end of October to the middle of December, all apparently primary cases, mostly over 18 years old, with two children aged 6 years and 14 months. Seven out of the nine cases received water from Loch Lomond. However, Lomond water supplies approximately a third of the population of Lanarkshire, around 200,000 people.

Eight cases of cryptosporidiosis were reported by Greater Glasgow Health Board in November. Approximately half of these received a water supply of Lomond origin, the rest from other sources.

Out of 22 cases reported by Lothian Health Board, 8 cases were in West Lothian. This was similar to the figure from the year before and included 3 family outbreaks, with evidence of secondary spread. In West Lothian approximately 104,000 people receive Loch Lomond water, while 60,000 receive water from other supplies. Only one case of cryptosporidiosis was recorded in somebody not receiving Lomond water, but the remaining cases were not evenly distributed among the population zones supplied by Lomond. This seemed to mitigate against a common source outbreak originating from Lomond water.

The relevant water authorities reported been no problems with, or changes in, water supplies in the recent past, prior to the incident described here, in any of the water department areas.

Central Regional Council Water and Drainage Department reported an increased use of Lomond water as a supply in the Kirkintilloch area. Sampling at Lenzie would detect oocysts present in this supply zone. Only sporadic cases of cryptosporidiosis were reported by Forth Valley Health Board, which covers this area.

The results suggest an increased incidence of cryptosporidiosis in Greenock. However, if it was water related it is unlikely to be associated with Lomond water. Other health board areas may have had a slight increase in the background number of cases, although again no association with Lomond water could be shown. There was no evidence of a common source for the increase in cases, water or other.

The occurrence of cryptosporidiosis in the population supplied with water from Loch Lomond remained close to background levels of infection. One increase in cases in an area supplied with Loch Lomond water was not attributed to a common water source.

There are several factors which may contribute to the fact that there was no identified connection with disease, despite the finding of oocysts in raw and final water and that cryptosporidiosis was reported in the communities supplied by Loch Lomond water. Individuals/communities may not have ingested oocysts, or a sufficient number of viable oocysts to establish infection. Few of the oocysts detected in water were viable, therefore if oocysts were ingested they may have been non-viable. High herd immunity in the community, possibly a result of long term exposure to low numbers of oocysts, may have resulted in reduced susceptibility, leading to a decrease in clinical signs of infection. In addition, the methods available for the detection of oocysts in water and faecal samples may not be sensitive enough to detect all the oocysts entering distribution or to detect infection in the community, especially where the infection was low grade.

Figure 4.1. Sewage treatment works on the Endrick Water.

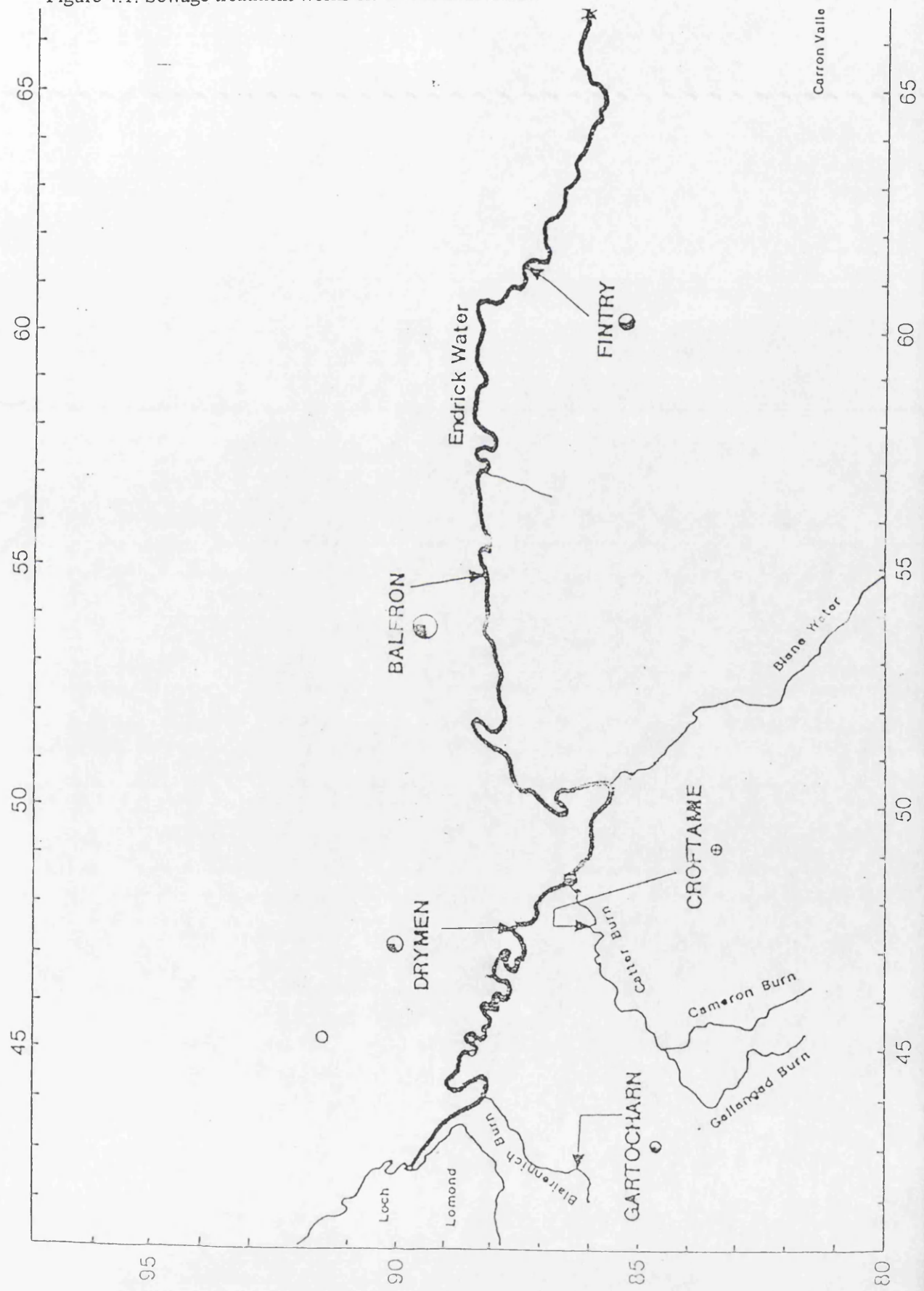


Figure 4.2. Sewage treatment works on the Blane Water.

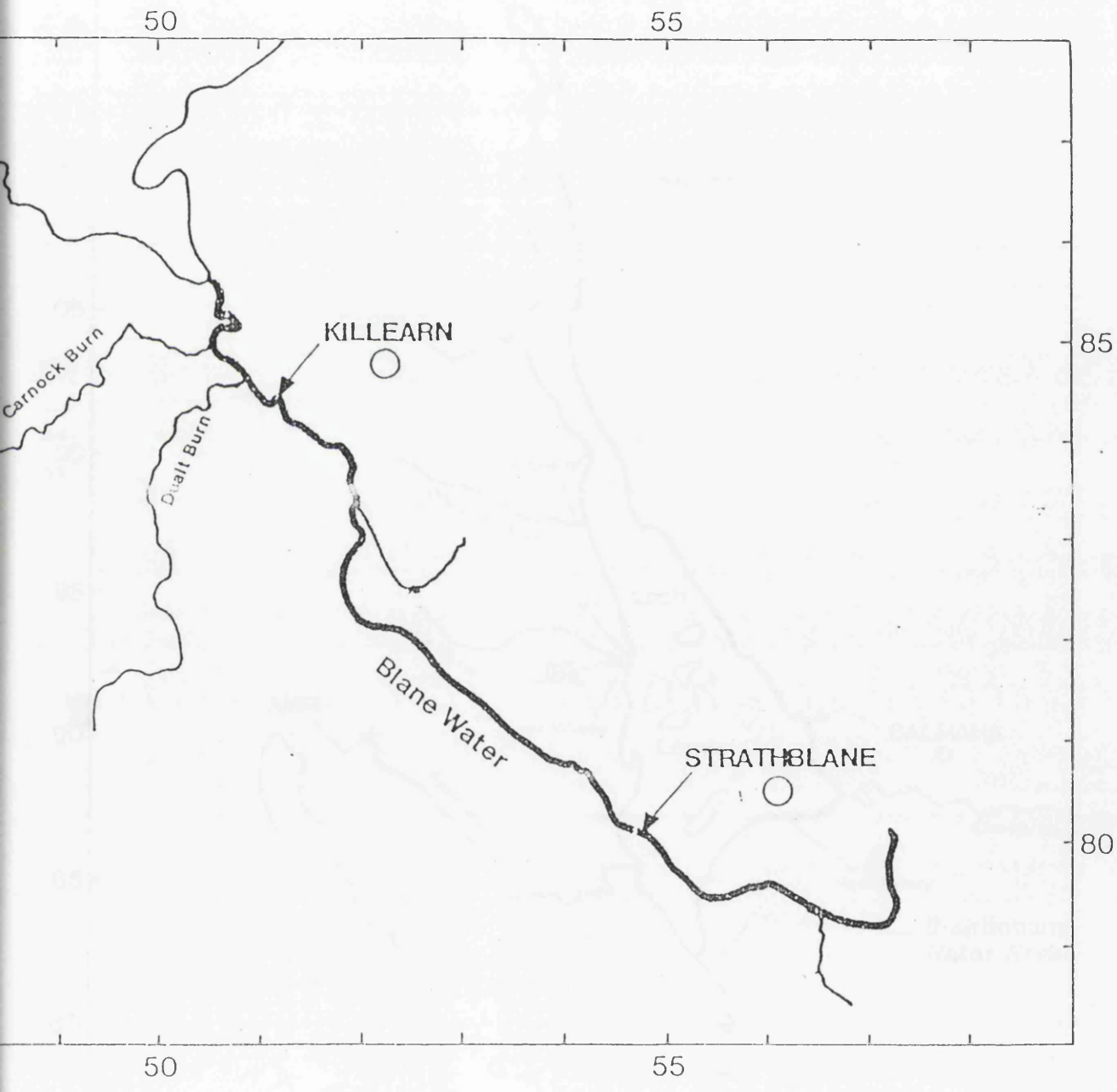
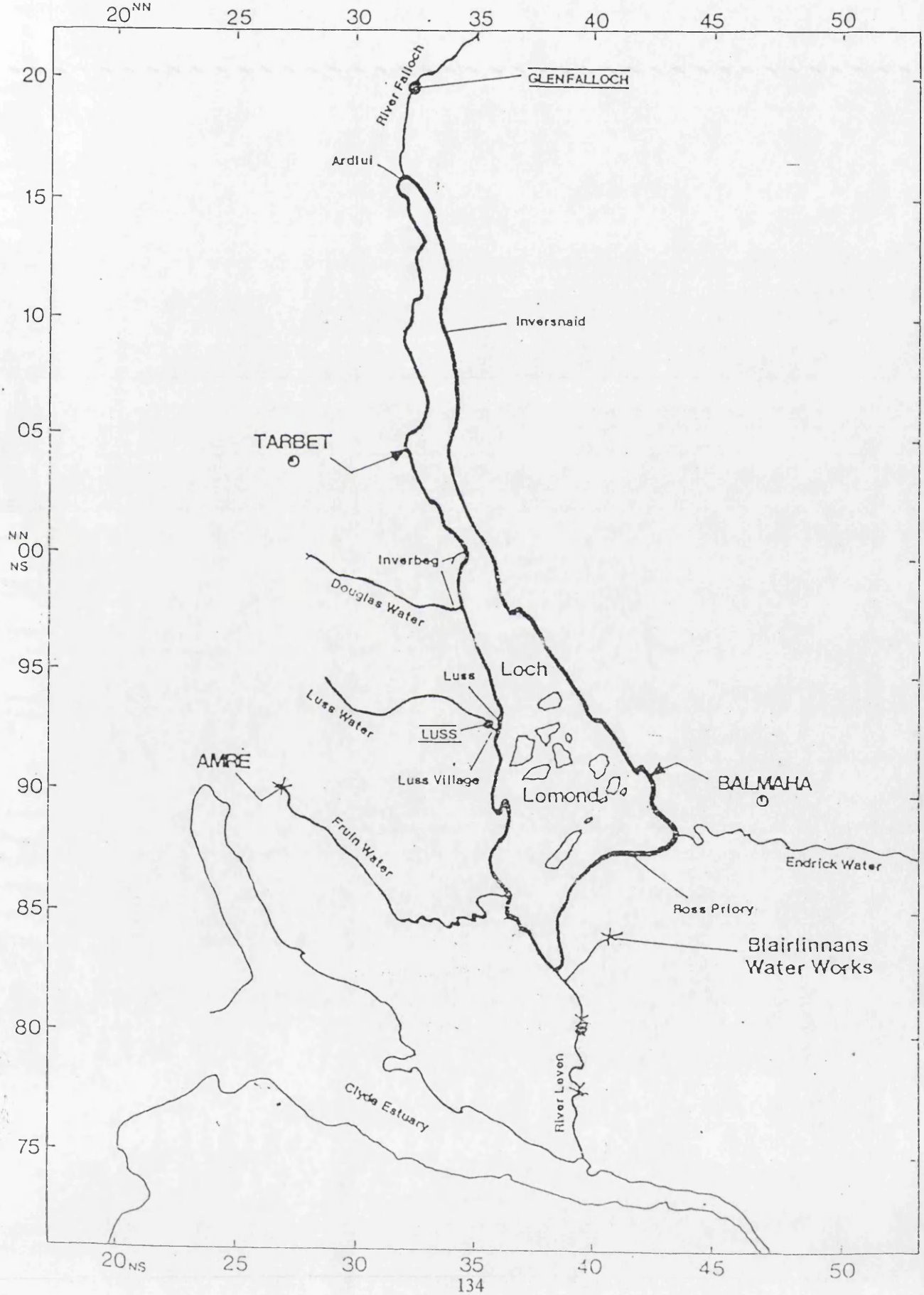
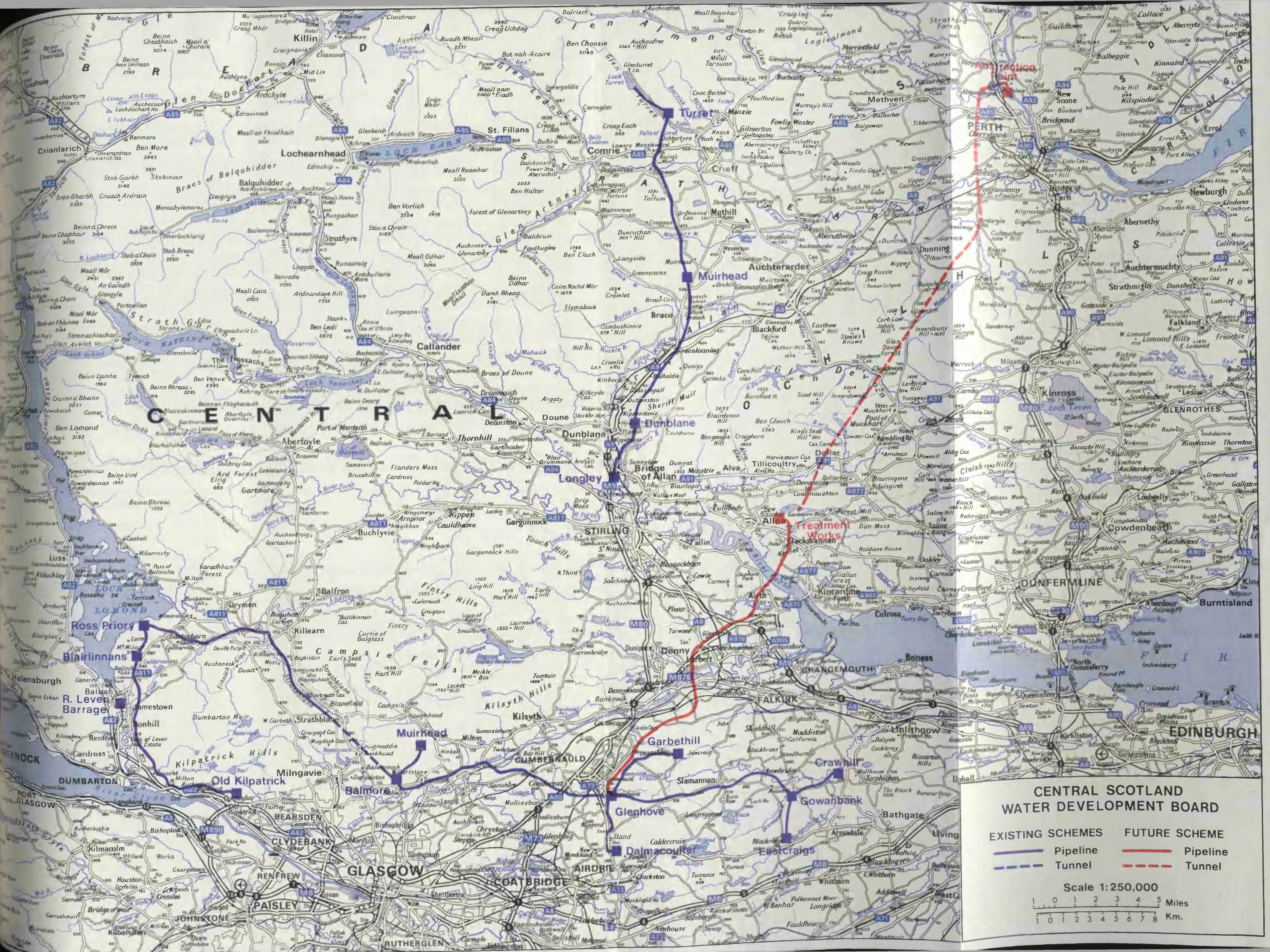


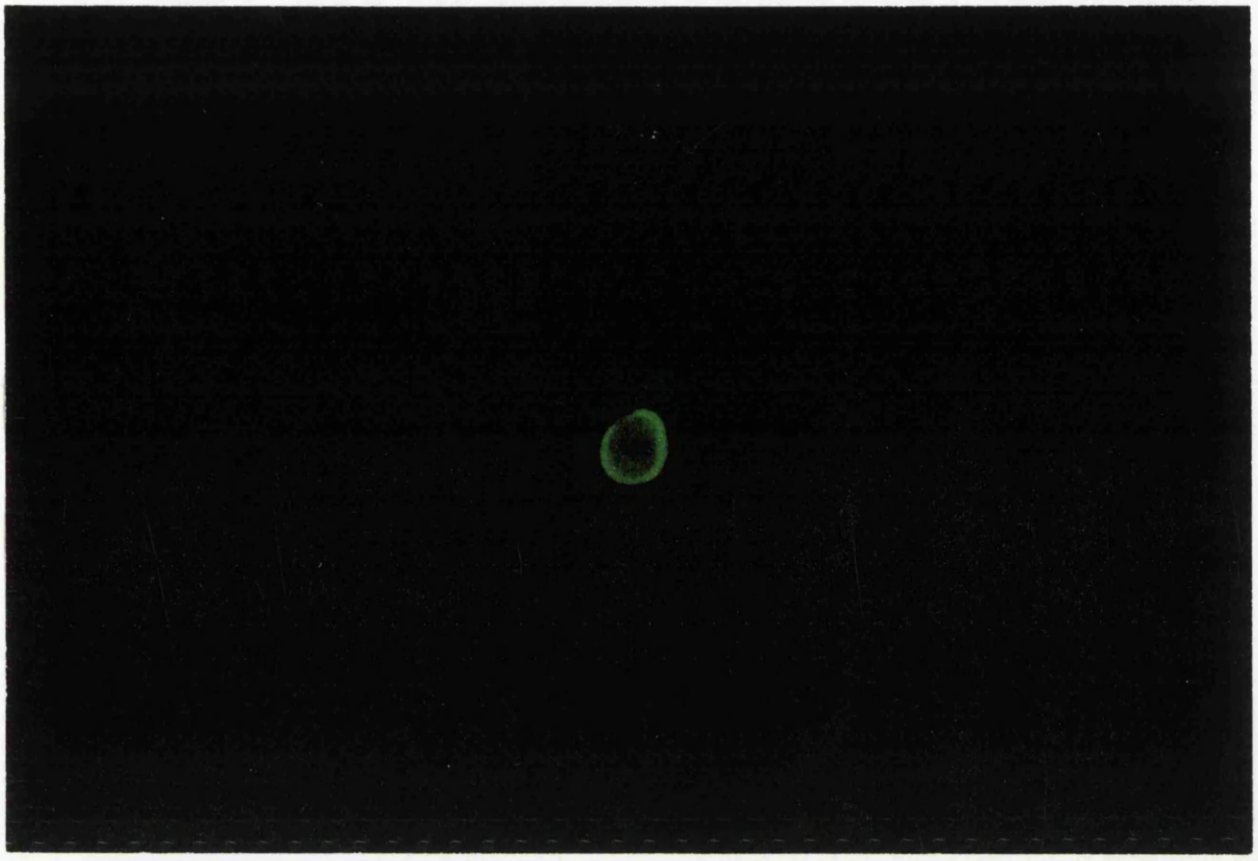
Figure 4.3. Diagram of Loch Lomond showing Ross Priory abstraction point and Endrick Water.




CENTRAL SCOTLAND
WATER DEVELOPMENT BOARD
EXISTING SCHEMES
Pipeline
Tunnel
FUTURE SCHEME
Pipeline
Tunnel
Scale 1:250,000
Miles
Km.
Abstraction Point
Treatment Works
Turret
Muirhead
Dunblane
Longley
Ross Priory
Blairlinnans
R. Leven Barrage
Old Kilpatrick
Balmore
Muirhead
Garbethill
Glenhove
Dalmacoulter
Gowanbank
Crawhill
Eastcraigs
CENTRAL
PERTH
STIRLING
GLASGOW
EDINBURGH
DUNFERMLINE
FALKIRK
CUMBERNAULD
PAISLEY
CLYDEBANK
COATBRIDGE
AIRDRIE

Photograph 4.1. A *Cryptosporidium* sp. oocyst detected in a water sample, labelled (A) with MAb and (B) with DAPI to aid identification. Bar = 5 μm.

A



B

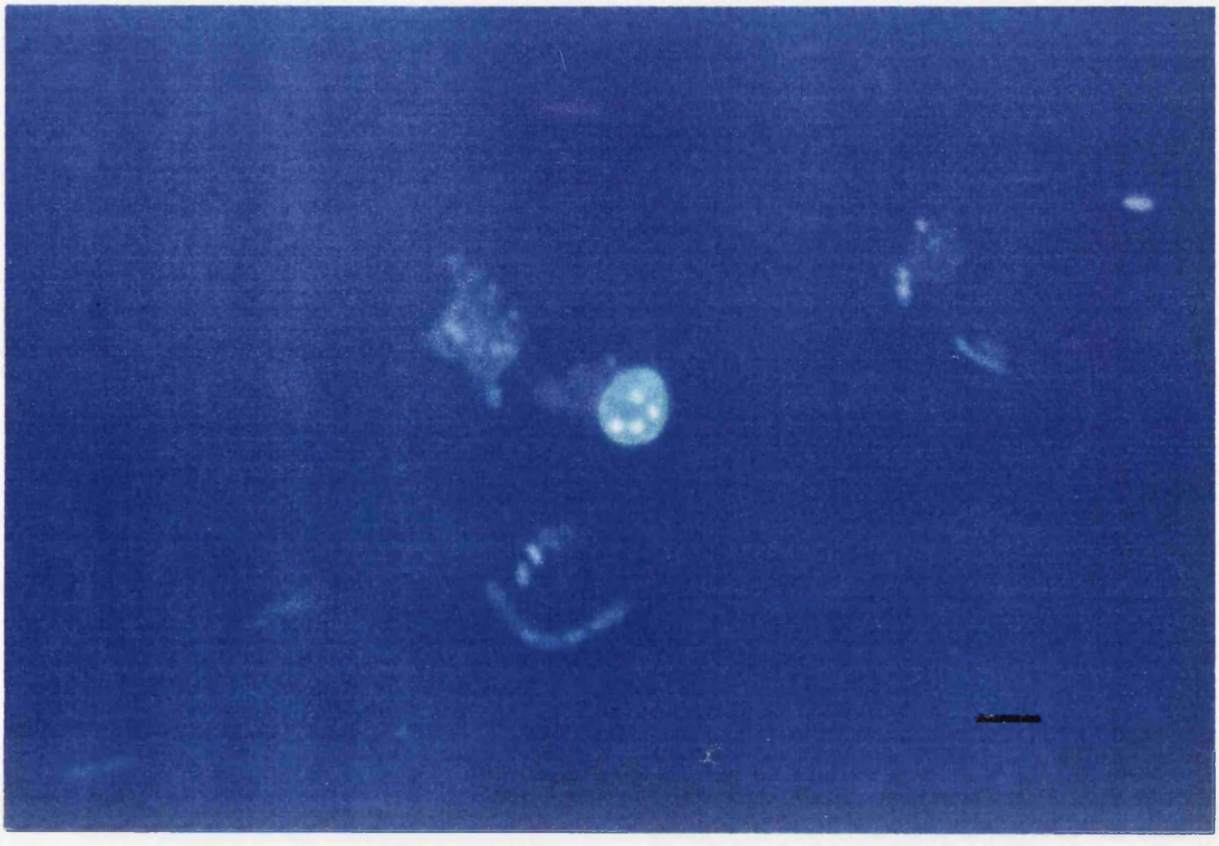


Figure 4.4. Concentration of oocysts detected in raw water samples from Ross Priory.

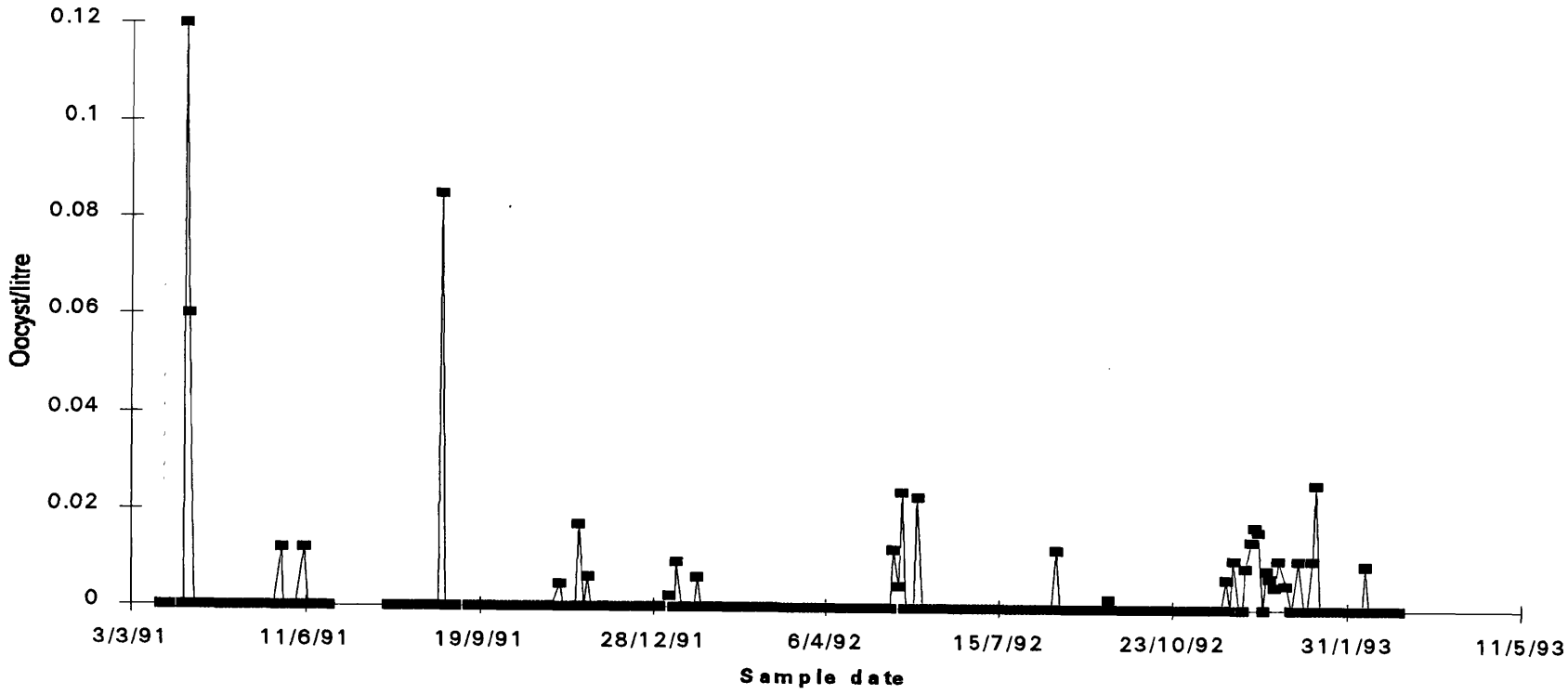
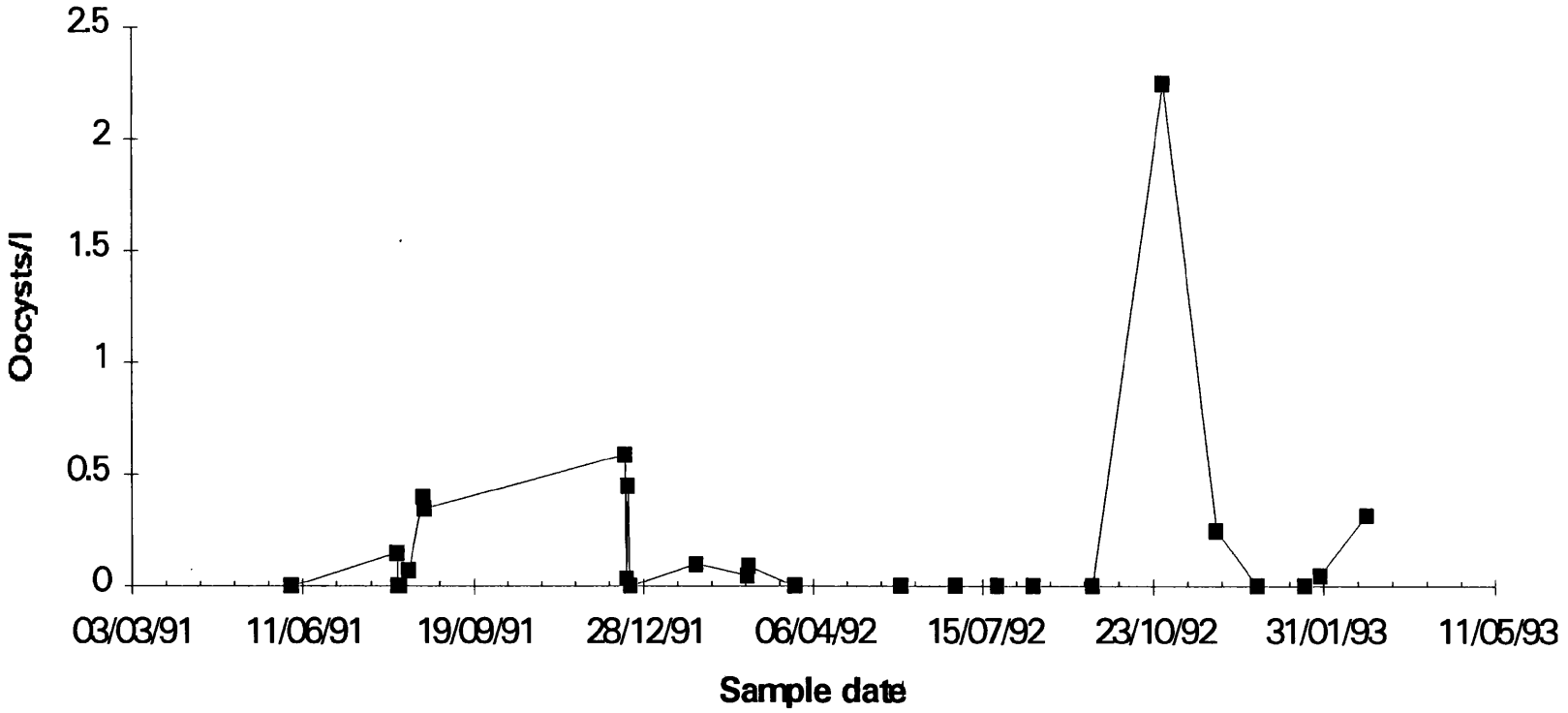


Figure 4.5. Mean concentrations of oocysts detected in sewage effluent.



CHAPTER 5. ALTERNATIVE CONCENTRATION TECHNIQUES: RECOVERY OF OOCYSTS BY IMMUNOMAGNETIC SEPARATION.

5.1 Introduction

The existing methods for the isolation, concentration and detection of *Cryptosporidium* oocysts and other protozoan parasites from water are time consuming, tedious and labour intensive (chapter 2, section 2.6. and chapter 3). A number of problems exist during the concentration phase (centrifugation and sucrose density flotation) and also in the detection of the organisms. These include contaminating debris, which cannot be wholly removed by current techniques, and the loss of oocysts during concentration of the sample. Oocysts may be trapped in contaminating debris and removed when such debris is discarded, rather than being recovered. In addition the presence of debris at the detection stage can obscure oocysts either so that they cannot be detected, or that if they are detected, positive identification and assessment of viability is difficult or impossible. Furthermore, if oocysts become damaged during isolation and concentration, this may lead to incorrect conclusions as to the viability of oocysts detected in water samples.

Various alternative methods have been or are being investigated (Smith *et al*, 1993). Vesey *et al*, (1992a) reported separating oocysts from contaminating debris using flow cytometry and fluorescence activated cell sorting (FACS). This method concentrates particles of defined shape and fluorescence intensity and sorts by light scatter and predefined fluorescence characteristics. The fluorescent sorted objects are automatically deposited onto slides and confirmation is made by conventional epifluorescent microscopy. This technique was found to give better recoveries and was faster and easier to perform than conventional routine water analysis. In addition, because this method was less laborious, a larger volume could be processed.

Centrifugation is currently used as one step in the concentration of oocysts (chapter 2, section 2.6.3.) and data (chapter 3, section 3.3.5. and table 3.9.) show recoveries of between 3 and 29 % after filter processing and large and small scale centrifugation. There are a variety of reasons for these losses. The centrifugal force applied may be inefficient at pelletting oocysts. Aspiration to remove the supernatant may therefore remove the oocysts. Increased centrifugal force should pellet the oocysts more efficiently, although Vesey *et al*, (1991) reported that centrifugation at a ***g*** value greater than 500 x ***g*** can damage oocysts, with a resultant reduction in detection. Stressed, damaged or old oocysts are more likely to be more susceptible to further damage, which may lead to oocyst disintegration, or render them unrecognisable with the result that they would not be identified or enumerated in a water sample.

A recent report (Whitmore and Carrington, 1992) showed that continuous flow centrifugation was unsatisfactory as an alternative concentration method. Vortex filtration and cross-flow (tangential) filtration were reported to give good recoveries (Whitmore and Carrington, 1992). Vortex flow filtration gave recoveries of between 30 and 40 %, but the length of time required to process large volumes of water was considered disadvantageous. Cross-flow filtration, where the sample flow was parallel to the filter surface and solids were concentrated in decreasing volume of recirculating retentate, gave recoveries of 40 - 80 %. The authors considered this method should be adopted for concentration, but it will be more useful if membranes with a pore diameter more than 0.2 μm become available. Hastie *et al* (1992) compared cross-flow filtration with centrifugation for the concentration of *Giardia* cysts and concluded that centrifugation was the method of choice.

Methods using coagulation or flocculation which entrap and sediment particles, including oocysts, may increase recoveries. Vesey *et al*, (1992b) reported a method of concentrating oocysts from water based on the precipitation of calcium carbonate. Calcium chloride and sodium bicarbonate solutions were added to water and the pH was raised to 10 by adding sodium hydroxide. The crystals of calcium carbonate which formed, enmeshed oocysts, were allowed to settle and the supernatant was discarded. The calcium carbonate precipitate was dissolved in sulphamic acid (NH_3O_3S). Further concentration could be achieved by centrifugation. This produced recoveries of 68 - 79 % from seeded deionised, tap and river water.

Any method using agents that coagulate, flocculate or precipitate particulate matter by non-specific physicochemical interactions must seek a balance between entrapping all the particles and retaining the ability to detect and identify oocysts in the concentrate. In addition, the effects of these treatments on oocyst viability may be deleterious (Robertson *et al*, 1992).

Methods based on the specific biological properties of oocyst surfaces are also being investigated and developed. These revolve around the immunoreactivity between exposed epitopes on the surface of the oocyst and commercial monoclonal antibodies. A combination of flow cytometry and fluorescence activated cell sorting (FACS), investigated by Vesey *et al*, (1992a, see above), has been shown to increase recoveries.

This chapter investigates the potential for the use of magnetic particles in the concentration and recovery of oocysts from water samples by immunomagnetic separation. Magnetic particles (Dynabeads) are superparamagnetic monodisperse polymer particles. They are coated in a thin polystyrene shell with an iron oxide (γFe_2O_3) core, allowing a consistent and reproducible reaction to a magnetic field.

Immunomagnetic particles have been used to separate and concentrate a variety of target cells from various biological fluids. A major advantage of this technique is that enrichment of a specific target cell requires no centrifugation step. One of the major uses for immunomagnetic separation is the separation of subpopulations of cells from a mixed population. Recent applications in microbiology have produced rapid selection, separation and concentration of bacteria, such as *Salmonella* (Tuley, 1992). The immunomagnetic particle system has also been used to separate viable *Salmonella enteritidis* from artificially contaminated egg yolk (Tuley, 1992).

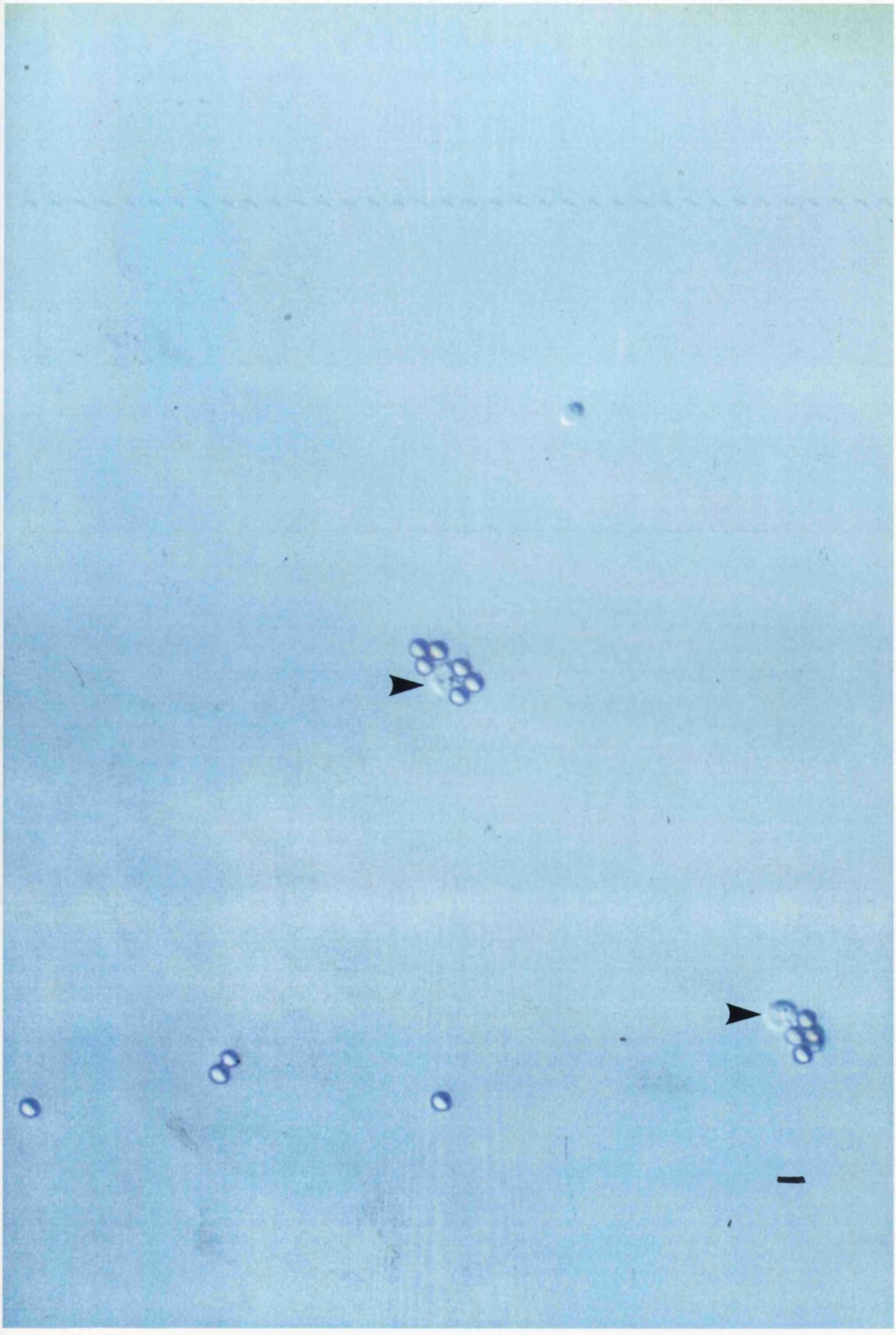
In the study reported here, different methods of bead coating and oocyst separation were investigated. The initial aim was to concentrate oocysts from water samples before sucrose density flotation, in an attempt to increase recovery efficiencies. The second method investigated the separation, purification and concentration of oocysts from the final pellets obtained at the end of filter processing (chapter 2, section 2.6.3.). This resulted from the need to recovery low numbers of oocysts from final pellets in order to assess oocyst viability. Initially M-450 Dynabeads with an anti *Cryptosporidium* MAb adsorbed onto the bead surface was used. Subsequently M-280 Streptavidin coated Dynabeads labelled with a biotinylated anti-FITC MAb were used to separate oocysts labelled with a commercial FITC conjugated MAb.

Beads were used to separate oocysts from seeded samples of RO water, PBS, raw water, sewage and diluted faeces. The recoveries were assessed and the number of beads required for most efficient separation was addressed.

5.2 Materials and Methods

The materials and methods are described in chapter 2, sections 2.8.

Photograph 5.1. Dynal beads M-280 attached to 2 *Cryptosporidium* sp. oocysts (arrowed) (x 400 magnification). Bar = 5μm.



5.3 Results

5.3.1. M-450 beads.

Dynabeads M-450 were coated with IgM anti *Cryptosporidium* MAb (supplied by PHLS, Collindale). When used to separate oocysts from seeded samples, good recoveries of up to 70 % were obtained. Detection of oocysts in these samples was, however, difficult due to the lack of FITC on the MAb.

5.3.2. Use of M-280 Streptavidin beads to concentrate large numbers of oocysts.

Three methods of coating beads and oocysts and subsequent separation were assessed (chapter 2, section 2.8.6.).

1) Beads plus anti-FITC MAb, washed, anti-*Cryptosporidium* MAb added , washed, added to oocyst suspension, followed by separation. This method gave recoveries of 10 to 35 %.

2) Beads, anti-FITC MAb, and anti-*Cryptosporidium* MAb mixed, washed, added to oocyst suspension, followed by separation. This method gave recoveries of 110 to 125 %.

3) Beads plus anti-FITC MAb, washed and added to oocysts labelled with anti-*Cryptosporidium* MAb, followed by separation. This method gave recoveries of 70 to 125 % and was the method chosen for further investigation.

5.3.3. Ratio of beads to target organism.

Methods 2 and 3 in 5.3.2. above were used to investigate the best ratio for optimum oocyst recovery. For complete depletion of a cell type, Dynal technical information suggested a ratio of between 4:1 and 40:1 beads to target organism.

Using a ratio of 40 beads to 1 oocyst in the system described for method 2 gave 100 % recoveries, while a ratio of 4 beads to 1 oocyst gave 30 % recovery of oocysts.

Using a ratio of 40 beads to 1 oocyst in the system described for method 3 gave 70 % recoveries, while a ratio of 4 beads to 1 oocyst gave 10 % recovery of oocysts.

5.3.4. Recoveries from filter backwash sludge.

Samples of oocyst free backwash sludge from cleaning water treatment filters were seeded with oocysts. From a seed of 1.2 x 10^5 oocysts/ml, 6 x 10^4 were recovered, yielding a recovery efficiency of 50 %, with a bead to oocyst ratio of 40:1. Tables 5.3.4.1 and 2 below show the recoveries from sludge seeded with a range of oocyst concentrations.

Table 5.3.4.1. Recovery of oocysts from seeded backwash sludge.

seed	1	10	100	1000	10^5	1.2 x 10^5
recovery	0	1	16	59	10^4	6 x 10^4
% recovery	0	10	16	5.9	10	50

Table 5.3.4.2. Recovery of oocysts from seeded backwash sludge.

seed	1	10	100	1000	10^4
recovery	2	1	1	58	266
% recovery	200	10	1	5.8	2.7
actual no.	2	-	106	800	7360
true % rec.	100	-	0.9	7.3	3.6

5.3.5. Recovery of varying (low) concentrations of oocysts from different water types.

Three types of water were seeded with theoretical concentrations of oocysts from 1 to 100 oocysts/ml and recovered using 10^4 or 10^6 beads, with oocysts labelled with two commercial FITC labelled MAbs. Preliminary experiments showed that a ratio of 40 beads to 1 target organism was insufficient to recover low numbers of organisms from final pellet samples. Consequently the use of 10^4 or 10^6 beads was investigated to assess the effect of bead numbers on oocyst recovery. The results are shown in tables 5.3.5.1 to 6 below. In tables 5.3.5.1, 3 and 5, the recoveries are shown and in tables 5.3.5.2, 4, and 6, the actual numbers present in the theoretical concentrations of oocysts are presented.

Although oocysts were recovered sporadically from the dilution groups 1, 5 and 10 oocysts/ml using both MAbs and 10^4 or 10^6 beads, the numbers were not sufficient to perform any statistical analyses on the data. However, sufficient numbers were recovered from the groups

which theoretically, by dilution, contained 50 and 100 oocysts/ml. In addition the number of oocysts actually present in each theoretical dilution group was ascertained so that comparison of recoveries could be made. Comparison of the effects of bead numbers, water type and MAb on oocyst recovery was performed.

5.3.5.1. Controls.

The dilutions of 1, 5, 10, 50 and 100 oocysts/ml were theoretical dilutions. To assess the actual number of oocysts present 1 ml samples were concentrated to 100 µl and 25 µl applied to each well of a 4 well slide and the number of oocysts counted. 100 % of the sample was analysed and this served as a control for the actual number of oocysts present in each sample. To assess the recovery by conventional techniques, 1 ml samples were concentrated to 100 µl and 10 µl applied to a slide. The number of oocysts was counted. 10 % of the sample was analysed and this served as a control for the number of oocysts recovered using the conventional method.

In dilutions of 1, 5 and 10 oocysts/ml, no oocysts were recovered. Table 5.3.5.1.1. below shows the number of oocysts recovered from 1 ml samples seeded with 50 and 100 oocysts/ml using the conventional method for the concentration and detection of oocysts in suspension. Table 5.3.5.1.2. shows the recoveries expressed as a percentage of the actual number of oocysts found to be present after analysis of the total volume of concentrate obtained from seeded 1 ml samples. All experiments were repeated 5 times.

Table 5.3.5.1.1. Mean number of oocysts ± standard deviation detected in control samples using conventional concentration techniques.

	PBS	Pond	Faecal
50 oocysts/ml	1 ± 1	1 ± 1	0.33 ± 0.58
100 oocysts/ml	0.67 ± 0.58	2.33 ± 0.58	3 ± 1.7

Table 5.3.5.1.2. Recovery efficiencies using conventional concentration techniques, expressed as percentages of the actual number of oocysts present.

	PBS	Pond water	Faecal water
50 oocysts /ml	3.3 %	4.9 %	1.03 %
100 oocysts/ml	0.94 %	8.2 %	4.7 %

The number of oocysts recovered using the conventional technique of centrifugation was low, even lower than the 10 % which might be expected from analysis of 10µl of 100µl concentrate

as described above, i.e. 5 oocysts from 50/ml and 10 from 100/ml. In terms of statistical analysis, little could be performed on the results given the numbers recovered. However, it can be seen from the results that recovery of oocysts using magnetisable particles was successful and generally exceeded the recoveries obtained using conventional techniques. Statistical differences were detected using concentrations of 10^4 and 10^6 beads to recover 100 oocysts/ml from the three water types, when compared to conventional techniques. Using 10^6 beads to recover oocysts from samples containing 50 oocysts/ml also demonstrated significantly better recoveries, while the higher recoveries seen using 10^4 beads were not significantly greater than those obtained using conventional techniques. This was largely a result of the range of recoveries obtained using the beads.

5.3.5.2. Comparison of oocyst recovery with actual numbers present.

a) For all three water types, using 10^6 and 10^4 beads and Cellab MAb to recover 50 oocysts/ml there were significantly more oocysts present in the sample than were recovered using the beads. The recoveries ranged from 2.7 - 24.3 %.

b) For all three water types, using 10^6 and 10^4 beads and Cellab MAb to recover 100 oocysts/ml in pond water there was no significant difference between the actual number in the sample and the number recovered using beads, indicating that recoveries were close to the actual number present. The recoveries from PBS and faecal water were less than the actual number present in the sample, giving a recovery range of 7.8 - 20.2 %.

Table 5.3.5.2.1. Mean number of *Cryptosporidium* oocysts recovered using beads from 1 ml final pellet volumes of water samples. Oocysts were labelled with Cellab MAb.

Sample	PBS	PBS	Pond	Pond	Faecal	Faecal
No. beads	10^6	10^4	10^6	10^4	10^6	10^4
1/ml	0.2 ± 0.4	0 ±	0 ±	0.2 ± 0.4	0±	0 ±
5/ml	0.2 ± 0.45	0.2 ± 0.45	0.4 ± 0.5	0 ±	0.6 ± 0.9	0.4 ± 0.5
10/ml	1.8 ± 1.9	0.4 ± 0.5	1.0 ± 0.7	0 ±	1.2 ± 0.8	0.8 ± 0.8
50/ml	2.0 ± 2.0	1.2 ± 1.3	7.2 ± 3.7	0.8 ± 0.8	5.4 ± 3.5	1.6 ± 1.5
100/ml	18.6 ± 8.9	7.2 ± 4.4	14.8 ± 10.1	2.4 ± 3.1	8.2 ± 1.9	8.0 ± 5.3

Table 5.3.5.2.2. Actual number (mean ± S.D.) of oocysts present in theoretical dilutions. Oocysts were labelled with Cellab MAb.

	PBS	Pond	Faecal
1 oocyst/ml	0	0	0.4 ± 0.5
5 oocysts/ml	0.4 ± 0.5	0.8 ± 1.3	1.4 ± 1.9
10 oocysts/ml	4.2 ± 3.9	1.8 ± 1.8	0.8 ± 1.8
50 oocysts/ml	33.4 ± 9.6	29.6 ± 7.2	34 ± 10.8
100 oocysts/ml	92.2 ± 8.6	25.4 ± 20.6	73.6 ± 11.6

Table 5.3.5.2.3. Percentage of *Cryptosporidium* oocysts recovered using beads from 1 ml final pellet volumes of water samples. Oocysts were labelled with Cellab MAb

Sample	PBS	PBS	Pond	Pond	Faecal	Faecal
No. beads	10^6	10^4	10^6	10^4	10^6	10^4
1/ml	0	0	0	0	0	0
5/ml	50	50	50	0	42.9	28.6
10/ml	42.9	9.5	55.6	0	150	100
50/ml	6	3.6	24.3	2.7	15.9	4.7
100/ml	20.2	7.8	58.3	9.4	11.1	10.9

c) Recoveries from all three water types, using 10^6 and 10^4 beads and NBI MAb to recover 50 oocysts/ml. In PBS, using 10^6 beads, there was no significant difference between the actual number in the sample and the number recovered using beads, indicating good oocyst recovery (92.5 %). However, using 10^4 beads in PBS, there was a significant difference, indicating fewer oocysts were recovered (19.5 %) than were actually present in the control. In pond water, there was no significant difference between the numbers recovered and the numbers actually present, irrespective of the number of beads used, indicating oocyst recoveries close to the number present in the original sample. In faecal water, there was a significant difference between the number of oocysts recovered and the actual number present using 10^6 beads (2.7 %), while no oocysts were recovered using 10^4 beads for the separation.

d) Recoveries from all three water types, using 10^6 and 10^4 beads and NBI MAb to recover 100 oocysts/ml. In PBS and pond water, using 10^6 or 10^4 beads for separation, no significant difference was detected between the actual number in the sample and the number recovered,

indicating that the number of oocysts recovered using the beads was close to the number of oocysts actually present in the 1 ml sample. However, in the faecal water there was a significant difference irrespective of bead numbers, indicating that fewer oocysts were recovered than were actually present in the sample before separation. Recoveries ranged from 6.3 to 9.2 %.

Table 5.3.5.2.4. Mean number *Cryptosporidium* oocysts recovered using beads from 1 ml final pellet volumes of water samples. Oocysts were labelled with Northumbria MAb.

Sample	PBS	PBS	Pond	Pond	Faecal	Faecal
No. beads	10^6	10^4	10^6	10^4	10^6	10^4
1/ml	0	0	0	0	0	0
5/ml	0.8 ± 0.8	0.6 ± 0.9	0.4 ± 0.5	0.2 ± 0.4	0.4 ± 0.5	0
10/ml	1.4 ± 1.1	1.0 ± 0.7	0.6 ± 0.9	0.8 ± 0.4	0.6 ± 0.9	0
50/ml	24.6 ± 13.5	5.2 ± 4.1	9.6 ± 5.7	3.2 ± 4.4	0.8 ± 0.8	0
100/ml	56.8 ± 17.6	37.6 ± 16.5	18.6 ± 6.4	2.0 ± 1.6	5.0 ± 2.0	3.4 ± 1.5

Table 5.3.5.2.5. Actual number of oocysts present in theoretical dilutions. Oocysts were labelled with Northumbria MAb.

	PBS	Pond	Faecal
1 oocyst/ml	0	0	0
5 oocysts/ml	1.6 ± 0.9	0.4 ± 0.9	0.8 ± 1.1
10 oocysts/ml	3.6 ± 2.2	0	2.4 ± 2.6
50 oocysts/ml	26.6 ± 12.1	11.6 ± 12.8	29.8 ± 6.3
100 oocysts/ml	53.0 ± 28.9	30.8 ± 32.3	54.4 ± 14.4

Table 5.3.5.2.6. Percentage of *Cryptosporidium* oocysts recovered using beads from 1 ml final pellet volumes of water samples. Oocysts were labelled with Northumbria MAb.

Sample	PBS	PBS	Pond	Pond	Faecal	Faecal
No. beads	10^6	10^4	10^6	10^4	10^6	10^4
1/ml	0	0	0	0	0	0
5/ml	50	37.5	100	50	50	0
10/ml	38.9	27.8	0	0	25	0
50/ml	92.5	19.5	82.8	27.6	2.7	0
100/ml	107.2	70.9	60.4	6.5	9.2	6.3

5.3.5.3. The effect of bead numbers, MAb used and water type on oocyst recoveries.

a) Comparison of number of beads (10^6 versus 10^4 beads) and MAb (Cellab versus NBI) to recover 50 oocysts/ml from PBS, pond and faecal water (table 5.3.5.3.1.).

(1) PBS.

Significantly more oocysts were recovered using 10^6 compared to 10^4 beads. The NBI MAb was significantly better when used for oocyst recovery compared with Cellab. In addition, there was interaction between the two variables, i.e. using NBI MAb with 10^6 beads increased the recovery of oocysts over and above the increase attributed to each variable alone.

(2) Pond water.

Significantly greater recoveries were achieved using 10^6 compared to 10^4 beads. There was no significant increase in recovery dependent on the MAb used and there was no interaction between variables.

(3) Faecal water.

Significantly more oocysts were recovered using 10^6 beads for separation compared to 10^4 beads, and using NBI MAb compared to Cellab. There was no interaction between the two variables.

b) Comparison of number of beads (10^6 versus 10^4 beads) and MAb (Cellab versus NBI) to recover 100 oocysts/ml from PBS, pond and faecal water (table 5.3.5.3.1.).

(1) PBS.

Significantly greater oocyst recovery was achieved using 10^6 beads for separation compared to 10^4 beads, and using NBI MAb compared to Cellab. There was no interaction between the two variables.

(2) Pond water.

Significantly more oocysts were recovered using 10^6 beads compared to 10^4 beads. No difference was detected between the recoveries attributed to the MAbs used. There was no interaction between the two variables.

(3) Faecal water.

No significant difference was detected between oocyst recoveries using 10^6 beads compared to 10^4 beads. A significant difference was detected when recoveries using NBI MAb compared to recoveries obtained using Cellab MAb. There was no interaction between the two variables.

c) Comparison of oocyst recoveries from different water types using 10^6 beads and both MAbs to recover 50 oocysts/ml (table 5.3.5.3.1.).

(1) PBS versus Pond water.

There was no significant difference detected in the number of oocysts recovered from PBS compared to the number recovered from pond water.

(2) PBS versus faecal water.

Significantly more oocysts were recovered from PBS compared to faecal water and NBI MAb gave better recoveries than Cellab. There was interaction between the two variables; significantly more oocysts were recovered from PBS using NBI MAb, than was achieved by each variable alone.

(3) Pond versus faecal water.

There was a significant difference in the number of oocysts recovered from pond water compared to faecal water. No difference was detected between the two MAbs and there was no interaction between the two variables.

d) Comparison of oocyst recoveries from different water types using 10^6 beads and both MAbs to recover 100 oocysts/ml (table 5.3.5.3.1.).

(1) PBS versus pond water.

Significantly more oocysts were recovered from PBS compared to pond water. Significantly more oocysts were recovered using NBI MAb compared to Cellab MAb. There was interaction detected between the two variables. Using NBI MAb in PBS increased oocyst recoveries over and above the recoveries which were attributed to each variable individually.

(2) PBS versus faecal water.

Significant differences were detected between the recoveries from the two water types and the two MAbs; greater recoveries were obtained from PBS and using NBI MAb. Interaction was detected between the two variables.

(3) Pond water versus faecal water.

Significantly more oocysts were detected from pond water compared to faecal water. There was no difference detected depending on MAb used and no interaction was detected between variables.

e) Comparison of oocyst recoveries from different water types using 10^4 beads and both MAbs to recover 50 oocysts/ml (table 5.3.5.3.1.).

(1) PBS versus pond water.

No significant difference was detected in the number of oocysts recovered from each water type, although using NBI MAb gave significantly better recoveries than using Cellab MAb. There was no interaction between the two variables.

(2) PBS versus faecal water.

There was a significant difference in the number of oocysts recovered from PBS compared to the number of oocysts recovered from faecal water. There was no significant difference detected between the recoveries obtained using the two different MAbs.

(3) Pond water versus faecal water.

There was no statistical significance between the number of oocysts recovered from pond water compared to faecal water. There was no significant difference in recoveries attributed to the MAb used.

f) Comparison of oocyst recoveries from different water types using 10^4 beads and both MAbs to recover 100 oocysts/ml (table 5.3.5.3.1.).

(1) PBS versus pond water.

Significantly more oocysts were recovered from PBS compared to pond water. Recoveries using NBI MAb were significantly greater compared to Cellab MAb. Interaction was detected between water type and choice of MAb.

(2) PBS versus faecal water.

Significantly greater recoveries were obtained from PBS compared to faecal water and using NBI MAb compared to Cellab MAb. There was interaction between the two variables.

(3) Pond water versus faecal water.

The recovery of oocysts from faecal water was significantly greater compared to pond water. There was no difference as a result of the MAb used and no interaction between variables was detected.

Table 5.3.5.3.1. Recovery (*percent recovery shown in brackets) of *Cryptosporidium* oocysts labelled with Cellab and Northumbria MAb from 1 ml final pellet volumes of water samples containing 50 and 100 oocysts/ml.

Sample MAb & oocysts/ml	PBS 10^6 beads	PBS 10^4 beads	Pond 10^6 beads	Pond 10^4 beads	Faecal 10^6 beads	Faecal 10^4 beads
Cellab, 50/ml	2 ± 2 (6 %)	1.2 ± 1.3 (3.6 %)	7.2 ± 3.7 (24.3 %)	0.8 ± 0.8 (2.7 %)	5.4 ± 3.5 (15.9 %)	1.6 ± 1.5 (4.7 %)
NBI, 50/ml	24.6 ± 13.5 (92.5 %)	5.2 ± 4.1 (19.5 %)	9.6 ± 5.7 (82.8 %)	3.2 ± 4.4 (27.6 %)	0.8 ± 0.8 (2.7 %)	0
Cellab, 100/ml	18.6 ± 8.9 (20.2 %)	7.2 ± 4.4 (7.8 %)	14.8 ± 10.1 (58.3 %)	2.4 ± 3.1 (9.4 %)	8.2 ± 1.9 (11.1 %)	8.0 ± 5.3 (10.9 %)
NBI, 100/ml	56.8 ± 17.6 (107.2 %)	37.6 ± 16.5 (70.9 %)	18.6 ± 6.4 (60.4 %)	2.0 ± 1.6 (6.5 %)	5.0 ± 2.0 (9.2 %)	3.4 ± 1.5 (6.3 %)

*Percentage recovery was calculated based on the mean recoveries.

Table 5.3.5.3.2. Actual number of oocysts present in theoretical dilutions of oocysts containing 50 and 100 oocysts/ml.

	PBS	Pond	Faecal
Cellab, 50/ml	33.4 ± 9.6	29.6 ± 7.2	34.0 ± 10.8
NBI, 50/ml	26.6 ± 12.1	11.6 ± 12.8	29.8 ± 6.3
Cellab, 100/ml	92.2 ± 8.6	25.4 ± 20.6	73.6 ± 11.6
NBI, 100/ml	53.0 ± 28.9	30.8 ± 32.3	54.4 ± 14.4

5.3.5.4. Recovery of oocysts from environmental samples.

Tables 5.3.5.4.1 and 2 below show the results for the recovery of oocysts from samples of raw water prior to abstraction for drinking water and for sewage effluent. The samples were first analysed according to routine water analysis procedures for the detection of oocysts in water (Anon, 1990b), as described in chapters 2 and 3. If a sample was found to contain oocysts, the number of oocysts in the remaining 80 - 90 % of the final pellet could be calculated. This calculated number is the predicted value quoted in tables 5.3.5.4.1 and 2 below.

Table 5.3.5.4.1. Recovery of oocysts from raw water samples, showing the number predicted by calculation from the number detected in 10 - 20 % of the sample.

predicted	6 oocysts	3 oocysts	6 oocysts	16 oocysts
recovered	0 oocysts	1 oocysts	1 oocysts	10 oocysts

Table 5.3.5.4.2. Recovery of oocysts from sewage effluent final pellets, showing the number predicted by calculation from the number detected in 10 - 20 % of the sample.

predicted	20 oocysts	23 oocysts
recovered	3 oocysts	4 oocysts

5.4. Discussion.

Initial conjugation of anti-*Cryptosporidium* MAb onto the 4.5μm diameter magnetisable particle (M-450 Dynabeads) followed by incubation of the coated bead with samples containing seeded oocysts gave good recoveries. It indicated the use of immunomagnetic separation as a potential alternative to large and small scale centrifugation and sucrose density flotation as a means of concentrating and clarifying oocysts from environmental samples. The number of experiments performed using these beads was small because of the scarcity of the anti-*Cryptosporidium* MAb which was obtained as ascites fluid and purified at SPDL. More importantly, it was rapidly discovered that although good recoveries could be obtained using the beads, detection of the oocysts once recovered was still painstaking and time consuming. The main reason for this was the absence of FITC on the purified anti-*Cryptosporidium* MAb, making detection difficult. The difficulty of detection was increased by the presence of the beads of similar size (4.5μm diameter), either bound to oocysts or not, present as a result of the separation, which served to contaminate the sample and obscure the oocysts.

As a result of, and in the light of these problems it was deemed necessary to use an anti-*Cryptosporidium* MAb with a fluorescein conjugate to aid the detection of oocysts. By attaching a biotinylated anti-FITC MAb to magnetisable particles coated in streptavidin, oocysts labelled with a commercial FITC conjugated MAb could be attached to beads via the anti-FITC MAb binding to the FITC group(s) on the anti-*Cryptosporidium* MAb and separation could be performed. These beads had a diameter of 2.8 μm (Dynabeads M-280 streptavidin).

Three methods of labelling beads and oocysts followed by separation were assessed. The first method, where beads were labelled with the biotinylated anti-FITC MAb, washed, labelled with the anti-*Cryptosporidium* MAb and washed again before being used proved to be the least successful. This was unfortunate as it was hoped that this method of labelling would allow concentration of oocysts from samples before the sucrose density flotation stage, thus eliminating the need for this stage. The second method, where beads, anti-FITC and anti-*Cryptosporidium* MAb were mixed together, washed using the magnetic particle concentrator (MPC) and then used to recover oocysts, gave high recoveries (over 100 %), although not consistently, there being occasions when this method did not work. Such high recoveries were possibly the result of the problems associated with preparation of accurate dilutions of oocysts and using the haemocytometer to count the low number of oocysts recovered. Labelling the beads with biotinylated anti-FITC and the oocysts with anti-*Cryptosporidium* FITC conjugated MAb, mixing the beads with the oocysts, followed by separation gave recoveries above 70 % on a constant basis. Therefore, methods two and three were considered for further evaluation.

For positive selection of a subset of cells from a mixed population a relatively low ratio of coated beads to target cell is required. A ratio of 3:1 is recommended for positive selection of T cell subsets (Lea *et al*, 1986; Dynal product application, 1989). For negative selection, the total elimination of a cell type, a ratio of 40 beads to 1 target cell is recommended, having been used to remove 99.98 % of T cells from human bone marrow (Vartdal *et al*, 1987; Dynal product application, 1989). When tested using both methods 2 and 3, recoveries of 70 to 100 % were obtained using a bead:target cell ratio of 40:1 compared to the 10 to 30 % recoveries obtained using a ratio of 4:1. When tested later on low numbers of oocysts, as would be expected in water samples (i.e. 1 to 100/ml) it was found that a ratio of 40 beads to 1 target organism was not sufficient to recover oocysts. As the results demonstrate, there was frequently a significant difference in the number of oocysts recovered from seeded samples using 10^6 beads compared to 10^4 beads.

The recovery of oocysts from sludge (obtained from the backwashing of water treatment filters) was not as successful as initial experiments had indicated, with recoveries dropping considerably to between 0.9 and 16 %. However, few oocysts if any would be recovered from sludge samples without considerable dilution and sample manipulation. In addition there was a considerable amount of magnetic contaminating debris which was attracted onto the MPC, making separation difficult. It is also likely that a large number of the oocysts were caught in the suspended matter and discarded with the supernatant. Analysis of the supernatant revealed some oocysts, but not enough to account for the losses. This method might be improved by mixing the beads and oocysts together for longer, more vigorous mixing, or both to enhance the collisions between beads and oocysts. Agitation during separation in the MPC to dislodge

contaminating debris which could interfere with the separation and attachment of beads onto the side of the tube in the MPC might further increase the number of oocysts recovered.

Recovery of low numbers of oocysts from different types of water produced some interesting results. Firstly, from the controls (section and table 5.3.5.1.) which were samples seeded with oocysts at the concentrations stated, recoveries of 0.33 to 1 oocyst/ml from 50 oocysts/ml (0.66 - 2 %) and 0.67 to 3 oocysts/ml from 100 oocysts/ml (0.67 - 3 %) were obtained. These were processed according to the normal procedures of centrifugation, aspiration to the lowest practical volume and resuspension in the lowest practical volume, usually in the region of 100 µl. Using beads, the lowest volume samples were resuspended in was 10 - 20 µl. If a sample contained 100 oocysts/ml and was reduced in volume to 100 µl, concentrating the oocysts into this volume, from which 10 µl was analysed, then one could only expect to recover 10 oocysts, or 10 %. Careful sample manipulation, reduction to less than 100 µl and analysis of more than 10 µl has provided better recoveries, although this procedure is time consuming.

As can be seen from the results, the recovery of oocysts from samples containing 1, 5 or 10 oocysts/ml was low. This prevented statistical analysis being performed on these results, but did demonstrate that oocysts can be recovered from samples containing very low numbers of oocysts, which conventional concentration procedures would be unlikely to recover. In addition the actual concentration of oocysts in the seeded sample was not always equal to the theoretical number calculated by dilution. The actual number of oocysts present in a sample was ascertained by reducing the sample to 100 µl, applying 25 µl to each well of a 4 well slide, allowing it to dry and analysing the entire sample for the presence of oocysts.

Comparison of the number of oocysts recovered by immunomagnetic separation compared to the actual number of oocysts present in a sample revealed that more oocysts were recovered from less turbid water. Using 10^6 beads compared to 10^4 beads provided significantly greater recoveries, while labelling oocysts with NBI MAb provided better recoveries compared with Cellab MAb. The greater the number of oocysts present in a sample, the more were recovered, although this was affected by the number of beads used and the type of water. There was a wide range of recoveries within each repeat of each separation experiment, perhaps reflecting the non-homogeneity or non-conformity even within samples of the same dilution. Certainly in some instances clumping of oocysts can be a problem and that could severely influence the number of oocysts present in a sample. Addition of Tween 80 (polysorbitan monoleate) and mixing may help combat clumping.

Comparison of the different parameters, within each water type, influencing the recoveries, revealed that using a higher number of beads achieved more successful separation, as did the

use of NBI MAb compared to Cellab MAb. There was interaction between the parameters such that the recoveries obtained using a combination of 10^6 beads with NBI MAb gave more significant recoveries than the recoveries attributable to each individual parameter alone.

A greater number of beads in the sample volume, where the number of target organisms is small, provides more opportunities for contact between beads and organisms to occur. The smaller size of the Dynabeads M-280, compared to the Dynabeads M-450 means that clusters made up of larger numbers of beads can form around the target organism, creating greater potential for magnetic attraction and therefore separation.

Little is known about the affinity of the paratope of either of the two commercial MAbs for its respective epitope, however, the higher the affinity, the better the binding. It is also possible that the recovery efficiency between the two MAbs varied because of the amount of FITC on each of the anti-*Cryptosporidium* MAbs. The use of anti-FITC coated beads meant that oocysts could be labelled with either of the FITC conjugated MAbs, which meant that detection of the oocysts was easier. One problem with the M-450 beads was that, although a much cleaner suspension of oocysts was obtained from environmental samples, the large number of beads present, and the beads binding the oocysts, obscured them, making detection and identification of the oocysts problematic. The beads were also of a similar size to oocysts. Using bright field or DIC microscopy it is difficult to detect small numbers of oocysts, so it was advantageous to develop a system whereby an FITC conjugated MAb could be used. When viewed using fluorescence microscopy the beads fluoresced weakly on a dark background and oocysts, whether covered by a clump of beads, or with only one or two beads attached, fluoresced brightly and clearly and could be easily identified.

When the recovery of oocysts from different water types was compared, using 10^6 beads for separation, recoveries from PBS were better than from pond water, both of which were better than the recoveries from faecal water. This was the case for oocyst concentrations of both 50 and 100 oocysts/ml. It would seem reasonable to conclude that the increased turbidity and contaminating matter in the pond and faecal water interfered to some extent with the binding of oocysts and beads, MAb and oocysts or with the magnetic separation of the bound bead/oocyst complex.

For the recovery of 100 oocysts/ml from the three types of water using 10^4 beads, the situation was the same as described above. However, when 50 oocysts were present per ml, recoveries from PBS were better than from faecal water, while recoveries from pond water were not. In this instance the reasons for the reduced recovery between water types are, as well as those

described above, compounded by the lower number of beads used for separation. Also, the more oocysts present in a sample, the greater the recoveries are likely to be.

The problem of recovering low numbers of oocysts from final pellets of environmental samples has made viability assessment of those detected a painstaking, time consuming and often unsuccessful task. Often, even if oocysts were detected after the viability assay had been performed, the contaminating debris covered or obscured the oocyst(s) to such an extent that not only was it impossible to decide whether dyes had been included or excluded, but it was also frequently difficult to positively identify an oocyst like body (olb) as an oocyst. The use of immunomagnetic separation has provided a method which can be used relatively simply to recover oocysts from a variety of environmental samples, leaving them in a clean final volume of 10 - 20 µl compared with 100 µl obtained from conventional methods. This allows the entire final pellet volume to be analysed in the same time that was previously required to analyse a tenth of the sample.

The success of this technique as an alternative concentration technique has been demonstrated. There remains room for improvement and further work on this and other applications of the technique are currently underway. Development of this technique such that it can be applied at an earlier stage of the concentration procedure, at least prior to sucrose flotation, would be of enormous advantage.

Having an anti-FITC MAb bound to the streptavidin coated beads provides the potential to separate any organism to which fluorescein groups can be attached, e.g. with FITC conjugated MAbs.

The length of time for which beads are incubated with oocysts is of importance, with an optimum incubation time around 90 min. Times below this give insufficient attachment, whereas times above 90 min may result in detachment (Arretxe, personal communication). Other methods of mixing and separation, including agitation during separation in the MPC are under investigation.

At the time when experimental work commenced on immunomagnetic separation as an alternative concentration technique, beads were only available from Dynal. It is now possible to obtain other similar products which can be used for the same purposes, including colloidal suspensions. Future work is aimed at investigating these other products with a view to comparing their efficacy in recovery of oocysts from environmental samples and investigate their wider applications.

Lectins, which are already used in cytochemistry and clinical microbiology are now being used to isolate and separate target organisms, particularly yeasts, in application to food microbiology (Tuley, 1992). It is possible that lectins may be incorporated into a method used for the detection and isolation of *Cryptosporidium* oocysts, perhaps even to species and viability status level, which is based on the use of magnetisable particles and lectins specific to sugars on the oocyst wall.

Immunomagnetic separation provided a successful method for separating oocysts from contaminating debris in environmental samples in order to assess their viability. Previously, assessing the viability of the low numbers of oocysts detected in water samples was time consuming, painstaking work and, because of the small numbers of oocysts in water samples, often no organisms were detected. Furthermore, contaminating debris could obscure the oocysts to the extent that it was impossible to determine the viability of the oocysts. The use of this magnetic separation technique reduced this problem considerably. It enabled the concentration of the sample to a smaller final volume and allowed the viability of the whole sample to be assessed within a reasonable period of time. Previously only 10 to 20 % of the sample could be analysed in the same time taken to analyse the whole sample, following IMS. Although there remains room for improvement in this technique, the assay enables an increased recovery of oocysts from water samples to be undertaken in a shorter period of time when compared with current technology. Further research into immunomagnetic separation is ongoing.

CHAPTER 6. THE EFFECTS OF OZONE, TEMPERATURE AND pH ON OOCYST VIABILITY.

6.1. Introduction.

Cryptosporidium spp. oocysts are widespread in the environment, being present in surface and ground water and in final water (Smith and Rose, 1990). There have been a number of outbreaks of cryptosporidiosis caused by water contaminated with oocysts which have survived water treatment, both filtration and disinfection, indicating that water treatment is not completely effective at removing viable oocysts.

Laboratory studies undertaken to investigate the resistance of oocysts to disinfection have focused on a number of compounds, including household disinfectants (Blewett, 1989) and forms of disinfection used in water treatment (Smith *et al*, 1989; Korich *et al*, 1990). These studies have shown that oocysts have great resistance to traditional forms of disinfection, with concentrations of chlorine as high as 80 mg/l to 16,000 mg/l over 2 h to 24 h required to kill all the oocysts. These studies all used fresh oocysts which were not environmentally stressed, as oocysts which may have been present in water for months, or passed through water treatment might be. Viability was assessed by *in vitro* excystation or by using animal models to assess infectivity, or both.

Interest in the water industry has turned to alternative disinfectants, including chlorine dioxide, monochloramine and ozone, of which ozone has shown the greatest promise compared to traditional disinfection with chlorine.

Ozone is a very powerful oxidising agent, possessing approximately twice the oxidising power of chlorine. It has been used in water treatment in parts of Europe, particularly France, since the early part of this century. It is employed primarily for the removal of organic and inorganic materials associated with colour and odour in the water. Although ozone is known to have excellent disinfecting powers, these have tended to be of secondary consideration since it is relatively insoluble and unstable in aqueous solution and thus the maintenance of a detectable residual is difficult (Glaze, 1987; Ransome *et al*, 1991).

Studies already conducted on the effects of ozone on oocyst viability have shown that oocyst viability can be reduced by relatively low concentrations of ozone. These studies also used *in vitro* excystation or animal infectivity to assess oocyst viability, and they correlated well with each other (Korich *et al*, 1990). Korich *et al*, (1990) also attempted to correlate these assays with uptake of the vital dyes fluorescein diacetate and propidium iodide, but although they had

been used successfully for determining *Giardia muris* viability, they were found to be unreliable for determining the viability of *C. parvum* oocysts. In studies using mouse or rat models to assess oocyst infectivity the animals were often immunosupressed and the number of oocysts required to cause infection was often high - 10^3 oocysts (Peeters *et al*, 1990), 10^4 oocysts (Perrine *et al*, 1990), although Korich *et al*, (1990) found the ID_{50} to be approximately 60 oocysts. These numbers are higher than the numbers encountered in water (chapter 3) and in addition, the number of oocysts required to cause infection (in lambs and mice) may be between 1 and 5 oocysts (Blewett *et al*, 1992; Kwa *et al*, 1992), indicating a need to assess the viability of individual organisms.

The use of disinfectants in water treatment is based on their effectiveness at inactivating microorganisms, with the concentration of disinfectant (C mg/l) and time of contact (T min) being major variables. Chick's Law (Chick 1908) characterised the inactivation of microorganisms with respect to time as a first order chemical reaction. The rate constant of Chick's Law has been shown to be responsive to disinfectant concentration, so that a given level of microorganism inactivation can be achieved by a combination of concentration and time.

This is usually written as: $C^nT = \text{constant}$

The power n relates to the biochemistry of the disinfectant and the microorganism. Both n and the constant are also dependent on the disinfectant chemistry and temperature. In most water works applications, the value of n is taken as unity, although this is based on experience with chlorine and coliform organisms. Variations in CT values have been encountered in work with other organisms (Singer 1990) and were attributed to the difficulties in performing quantitative work with ozone and microorganisms in water (ozone demand of water and organisms, inadequate analytical techniques for the measurement of residual ozone, and lack of uniformity of microorganisms among different laboratories).

A viability assay based on the incorporation of fluorogenic dyes developed at the Scottish Parasite Diagnostic Laboratory (SPDL), Glasgow (Campbell *et al*, 1992) has been used in studies to investigate the effect on oocyst viability of a number of treatments (Robertson *et al*, 1992a). The assay also correlated well with *in vitro* excystation ($r = 0.97$) and has the advantage of assessing the viability status of individual oocysts and would therefore give a good indication of the effects of ozone on oocyst viability. In preliminary studies conducted on a small scale to determine the concentration and time required to kill oocysts with ozone this assay was compared with *in vitro* excystation and good correlation was observed between the two methods (Joss, 1991).

This study aimed to investigate the effects on oocyst viability of three ozone concentrations over time at different temperatures and pH.

Initial work, performed in collaboration with N. Joss, aimed at finding a range of ozone concentrations and times which were required to kill oocysts. This work was performed at small scale (using universal bottles) in the laboratory, using different isolates of *Cryptosporidium* oocysts of bovine, cervine-ovine and human origin. Further laboratory work, on a larger scale, using Drechsel bottles was designed with the results from the previous work in mind, also taking into account some of the problems encountered in residual ozone measurement and variation in ozone concentration in the vessels used. These did not affect the results of the range finding study, but were considered important for the reproducibility of results in the larger scale study. The results obtained in the large scale system were used to set the parameters investigated in the pilot plant study, where oocysts were circulated in a water ozone contactor system. The same bovine isolate of oocysts was used in both the large scale laboratory study and the pilot plant as a result of the apparent differences in susceptibility to inactivation by ozone which was observed between the different isolates in the small scale study.

As a wide range of temperatures can be encountered in environmental water, from sub-zero to 30°C or more, dependent on origin or source and type of water, and climate, it was decided to conduct experiments at temperatures towards either end of this range. Theses were also readily attainable and maintainable in the laboratory.

6.2. Materials and Methods.

See chapter 2, section 2.9.

6.3. Results.

6.3.1. Small scale laboratory study.

A range finding study was carried out to find the required ozone concentration, temperature and contact time for the inactivation of oocysts.

Cryptosporidium spp. oocysts from bovine, cervine - ovine and human sources were exposed to varying concentrations of ozone over time at 20°C and 4°C. The viability of the oocysts was

assessed according to the inclusion or exclusion of fluorogenic vital dyes and by *in vitro* excystation.

Inactivation of oocysts was found to increase when exposed to increased ozone concentrations, a higher temperature or for prolonged contact time. Differences in susceptibility to ozone were also observed between the isolates used, including differences between two human isolates.

Complete (100%) inactivation of all isolates was achieved when oocysts were exposed to ozone concentrations of up to 7 mg/l at 20°C for a contact time of under 10 minutes, dependent on isolate, and at 4°C, 7.6 mg of ozone per litre for a contact time of 10 minutes reduced oocyst viability to 0%. At ozone concentrations between 2 and 3 mg/l the reduction in oocyst viability was less marked and was isolate dependent with a range in inactivation from 80 - 100 % at 20°C and 30 - 90 % at 4°C.

In vitro excystation and inclusion /exclusion of fluorogenic vital dyes for assessing viability correlated well throughout the experimental work.

6.3.2. Large scale laboratory study.

Comparison of ozone concentrations, temperature, pH and contact time required for the inactivation of *C. parvum* oocysts.

a) Comparison of concentrations

Results were considered to be statistically significant when $p \leq 0.05$.

1, 3 and 5 mg/l at pH 5 and 20°C

The results are presented in table 6.1 and figure 6.1.
Each concentration tested showed a significant reduction in oocyst viability compared to the controls. There was no statistically significant difference in the rate of oocyst death between the concentrations, viability being reduced to between 0 % and 1.3 % after 10 min exposure. In the first 2 min, at 1 mg/l, the effect of ozone was significantly less than at 3 and 5 mg/l.
The concentration x time ($C \times t'$) value under these conditions for 100 % inactivation was 30 mg/l.min. For 99 % inactivation a $C.t'$ value of 18 mg/l.min was required.

1, 3 and 5 mg/l at pH 5 and 5 ℃

The results are presented in table 6.2 and figure 6.2.
There was significantly more oocyst death after treatment with each concentration of ozone used compared to controls. There was also a significant difference between concentrations, with increased concentration causing a greater reduction in oocyst viability. A concentration of 5 mg/l caused a reduction in viability to 5.8 %, while 3 and 5 mg/l reduced viability by approximately 10 and 20 % respectively.

1, 3 and 5 mg/l at pH 7 and 20 ℃

The results are presented in table 6.3 and figure 6.3.
There were significant differences between controls and each concentration of ozone and significantly more oocysts were destroyed at ozone concentrations of 3 and 5 mg/l than at 1 mg/l. Between 3 and 5 mg/l there was no statistically significant difference in oocyst death. Viability was reduced to 10.7 % and 0.3 % after 2 min at 3 and 5 mg/l respectively.
The concentration x time ($C \times t'$) value under these conditions for 100 % inactivation was 20 mg/l min. A 2 log (99 %) inactivation was achieved under these conditions at a $C.t'$ value of 10 mg/l.min.

1, 3 and 5 mg/l at pH 7 and 5 ℃

The results are presented in table 6.4 and figure 6.4.
Oocyst viability was significantly reduced by exposure to each concentration when compared to controls. However, when comparisons were made between concentrations, no difference was demonstrated.

1, 3 and 5 mg/l at pH 9 and 20 ℃

The results are presented in table 6.5 and figure 6.5.
While a significant difference was detected between the controls and each concentration tested under these conditions, there was no difference demonstrated between the different concentrations, although oocyst destruction appeared to proceed more slowly at 1 mg/l than at the other concentrations. Oocyst viability was reduced to 0 % after 6 and 10 min exposure to 5 and 3 mg/l ozone respectively and to 3.9 % after 10 min exposure to 1 mg/l.
The concentration x time ($C \times t'$) value under these conditions for 100 % inactivation was 30 mg/l.min.

1, 3 and 5 mg/l at pH 9 and 5 ℃

The results are presented in table 6.6 and figure 6.6.
When compared to controls, oocysts exposed to a concentration of 1 mg/l ozone at pH 9 and 5° C showed no significant difference. There was, however, a significant reduction in oocyst viability when oocysts were exposed to 3 and 5 mg/l under the same conditions. There was no significant difference in oocyst killing between 1 and 3 mg/l, but between 1 and 5 mg/l and 3 and 5 mg/l, a statistically significant difference was detected, albeit not a large one.

b) comparison of the effects of temperature

When the two temperatures, 20°C and 5°C, were compared at each of the other parameters, eg. ozone concentration 1 mg/l at pH 5, there was significantly more oocyst killing at 20°C at all concentrations and pHs, except at a concentration of 5 mg/l at pH 5 and 7. Under these conditions oocysts were destroyed irrespective of temperature.The results are shown in tables 6.1 to 6.6 and figures 6.1 to 6.6.

c) comparison of the effects of pH

The results for the following conditions are presented in tables 6.1 to 6.6 and figures 6.1 to 6.6.

pH 5, 7 and 9 at 1 mg/l and 20 ℃

A significant difference was seen between pH 5 and 7 and pH 7 and 9, but not between pH 5 and 9. Oocysts were killed faster at pH 5 and 9 than at pH 7

pH 5, 7 and 9 at 3 mg/l and 20 ℃

There was no difference between any of the pH treatments at this concentration of ozone and temperature. Oocyst viability was reduced to 0, 0.3 and 0 % at pH 5, 7 and 9 respectively after 10 min, although even after 2 min the viability was drastically reduced to around 10 %.

pH 5, 7 and 9 at 5 mg/l and 20℃

There was no significant difference in oocyst destruction between groups of oocysts treated at 5 mg/l at 20°C at any of the 3 pH values. Oocyst viability was reduced to 0 % at each pH after 6 min.

pH 5, 7 and 9 at 1 mg/l and 5℃

There was a significant difference in oocyst viability between all three pH values, with greater reduction in viability associated with the lower pHs. Oocysts treated at pH 9 under these conditions of temperature and ozone concentration showed no difference from control oocysts.

pH 5, 7 and 9 at 3 mg/l and 5℃

No significant difference was observed between oocysts treated with 3 mg/l ozone at 5°C at pH 5 or pH 7, although under both sets of conditions significantly more oocysts were destroyed than in controls. Significantly more oocysts were killed at this concentration and temperature when the pH was 5 or 7 when compared to pH 9.

pH 5, 7 and 9 at 5 mg/l and 5℃

At the highest concentration of ozone tested, 5 mg/l, oocyst viability was more significantly reduced at pH 5 compared to 7 and 9. There was no significant difference between pH 7 and 9.

Table 6.1. Effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 5 and temperature 20°C.

	Exposure time in min				
Conc. O_3 (mg/l)	0	2	4	6	10
	Percentage of oocysts remaining viable				
1	97.9	75.8	20.4	6.4	1.3
3	97.9	8.7	7.9	0.9	0
5	97.9	3.9	1.8	0	0

Table 6.2. Effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 5 and temperature 5°C.

	Exposure time in min				
Conc. O_3 (mg/l)	0	2	4	6	10
	Percentage of oocysts remaining viable				
1	94.1	93.3	85.3	86.7	85.3
3	94.1	89.3	81.1	80.4	72.5
5	94.1	88.9	67.5	30	5.8

Table 6.3. Effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 7 and temperature 20°C.

	Exposure time in min				
Conc. O_3 (mg/l)	0	2	4	6	10
	Percentage of oocysts remaining viable				
1	95	60.7	52.2	39.5	29.3
3	95	10.7	5.2	0.6	0.3
5	95	0.3	0	0	0

Table 6.4. Effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 7 and temperature 5°C.

	Exposure time in min				
Conc. O_3 (mg/l)	0	2	4	6	10
	Percentage of oocysts remaining viable				
1	96.2	95.1	91.5	81.2	72.6
3	96.2	93.8	84.9	72.5	81.8
5	96.2	89.1	68	70.3	61.2

Table 6.5. Effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 9 and temperature 20°C.

	Exposure time in min				
Conc. O_3 (mg/l)	0	2	4	6	10
	Percentage of oocysts remaining viable				
1	93.6	95	76.8	46.5	3.9
3	93.6	13.2	4.6	1.9	0
5	93.6	12.2	4.2	0	0

Table 6.6. Effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 9 and temperature 5°C.

	Exposure time in min				
Conc. O_3 (mg/l)	0	2	4	6	10
	Percentage of oocysts remaining viable				
1	94	96.3	97.5	96.8	93.2
3	94	95.1	94.2	91.1	90
5	94	96.6	93.6	87.3	73.7

Figure 6.1. The effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 5, temperature 20°C.

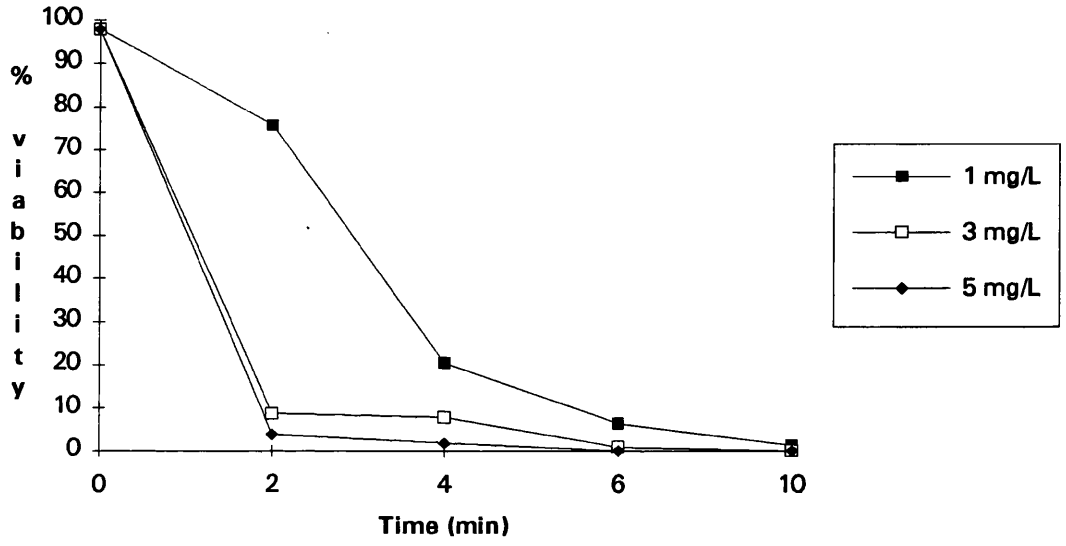


Figure 6.2. The effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 5, temperature 5°C.

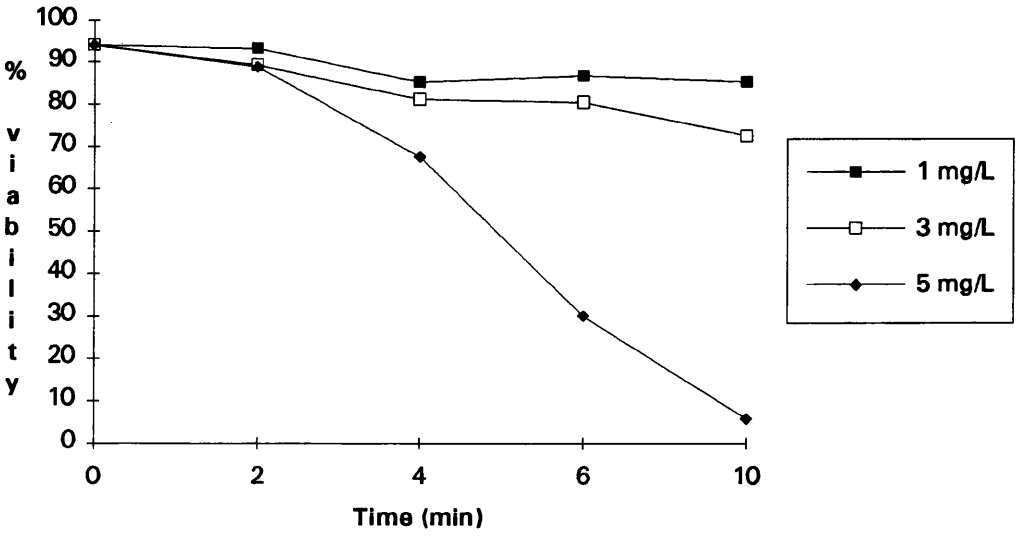


Figure 6.3. The effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 7, temperature 20°C.

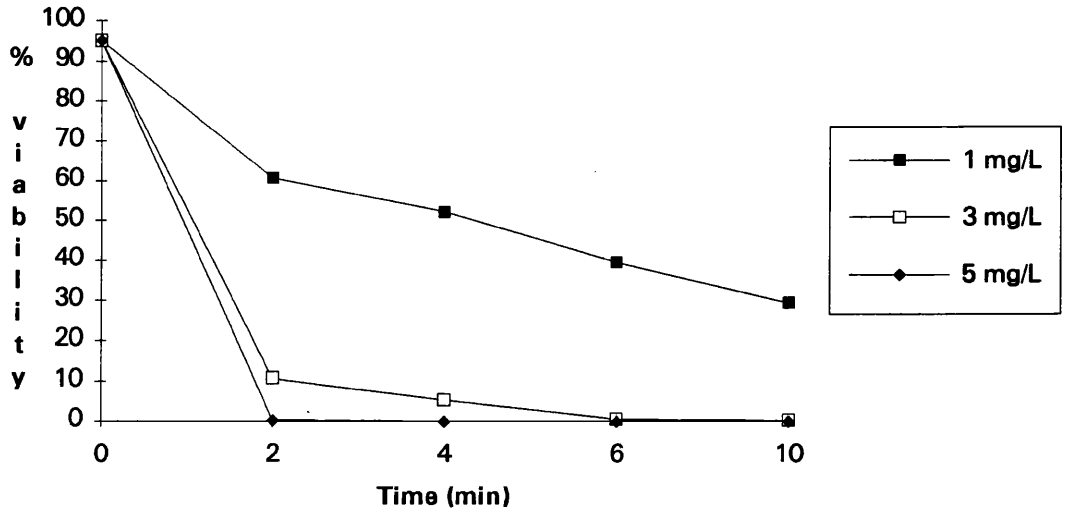


Figure 6.4. The effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 7, temperature 5°C.

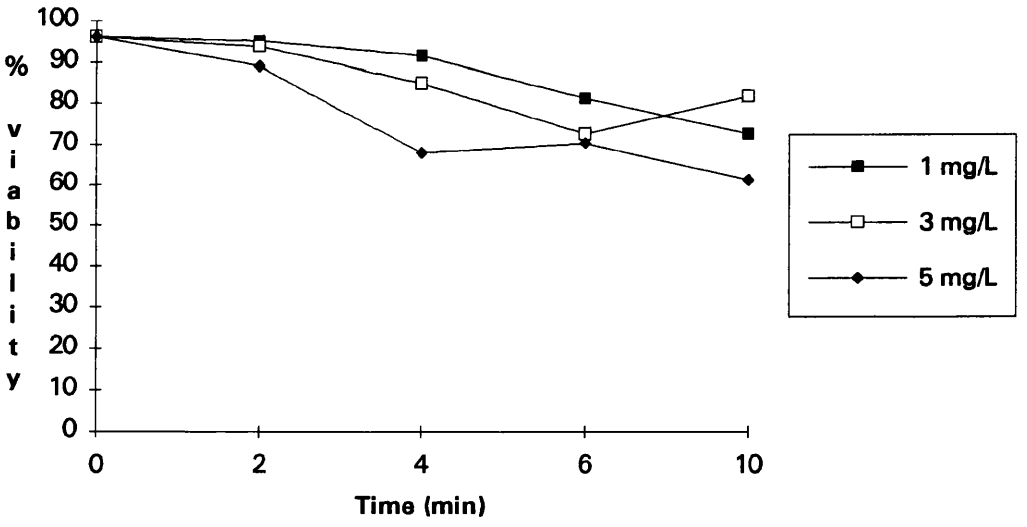


Figure 6.5. The effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 9, temperature 20°C.

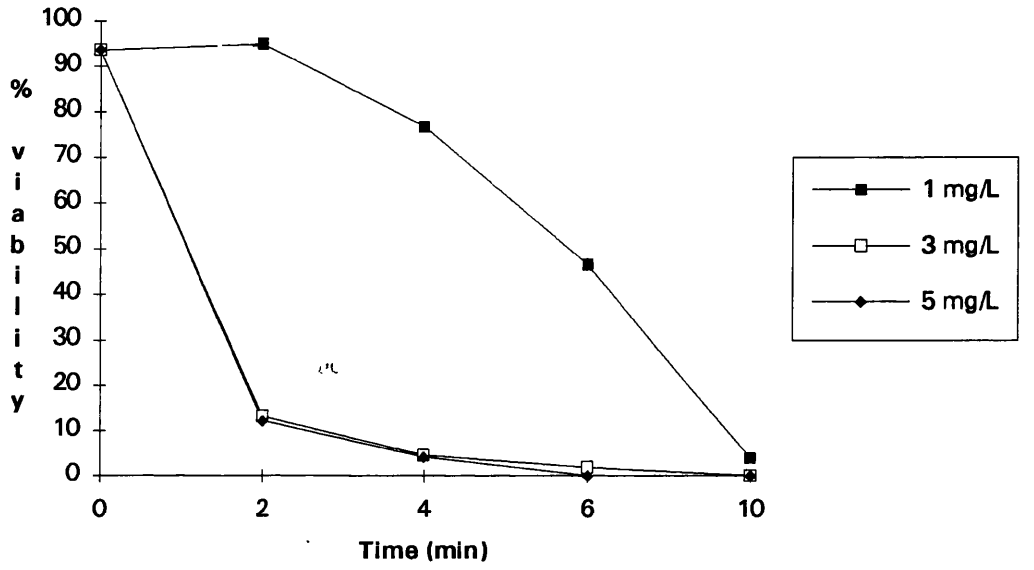
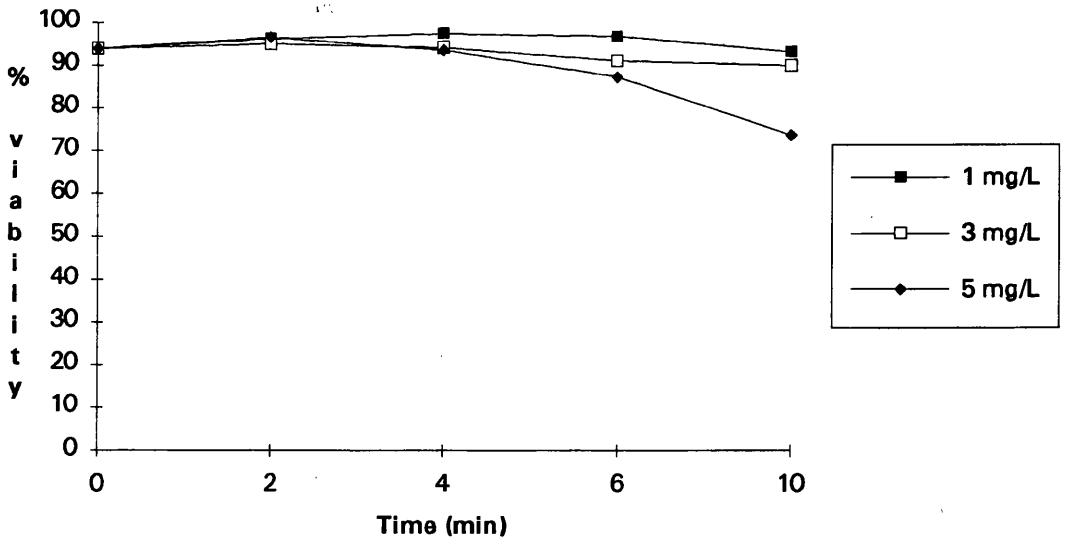
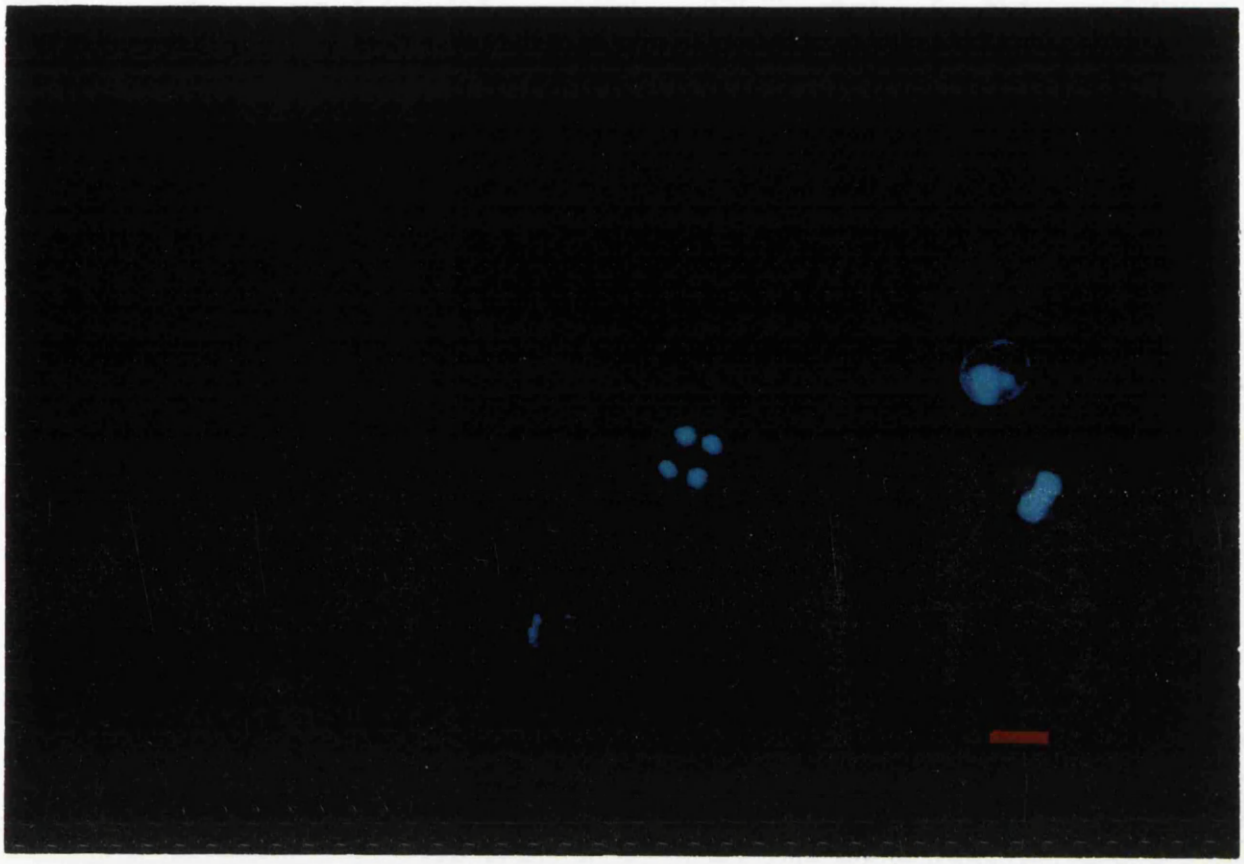


Figure 6.6. The effect of 1, 3 and 5 mg/l ozone on oocyst viability at pH 9, temperature 5°C.

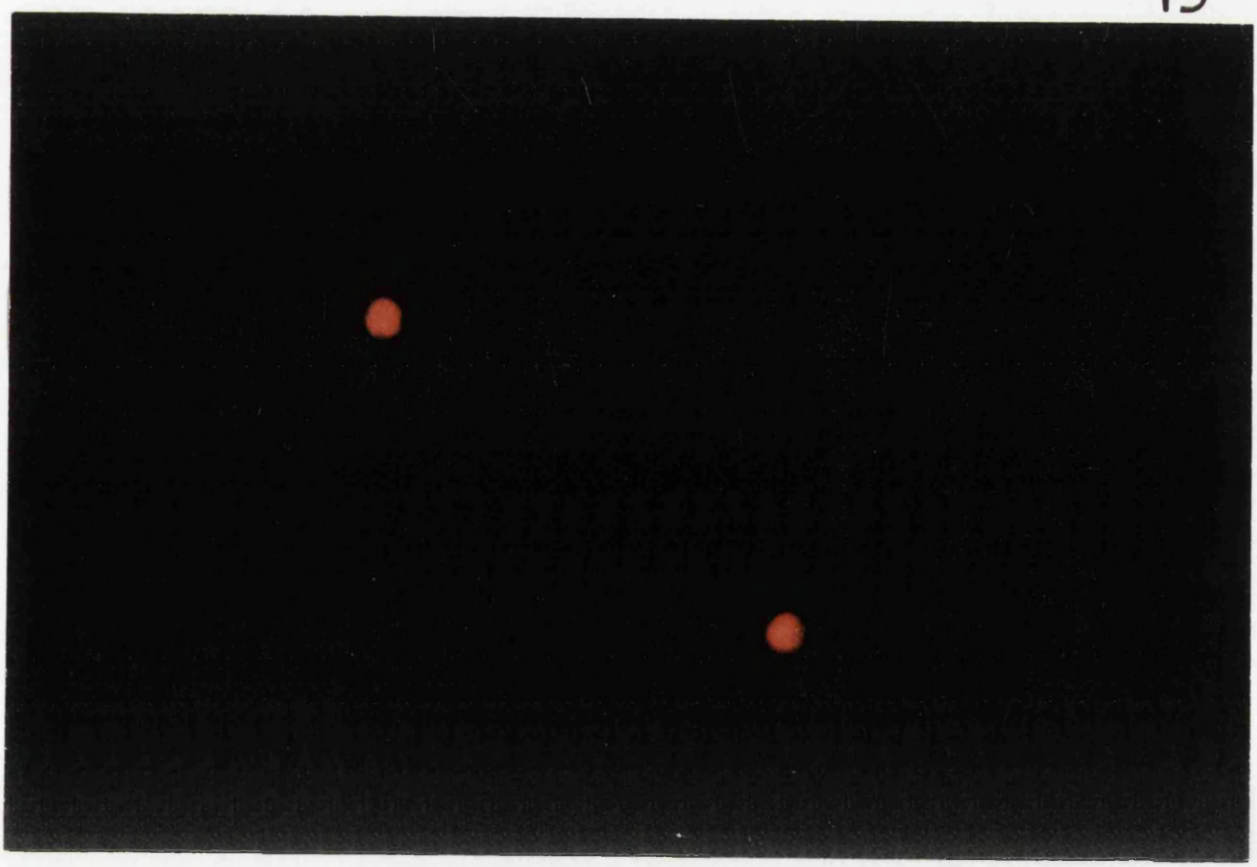


Photograph 6.1. of (A) a viable (DAPI+) oocyst, showing the four nuclei labelled with DAPI and (B) non viable (PI+) oocysts, having incorporated PI. Bar = 5μm.

A



B



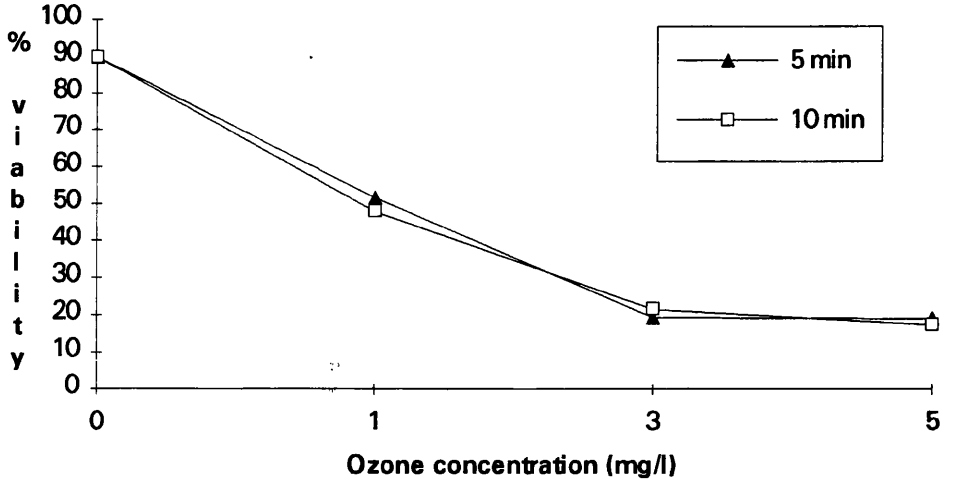
6.3.3. Results of exposure to ozone in contactor system (method 3)

Oocysts exposed to a dose of ozone (pH 7, temperature 5°C) in a contactor system, rather than to a residual concentration, showed a significant loss in viability. There was however, no significant difference in loss of viability between oocysts exposed to this treatment for 5 min or 10 min. Under these conditions of concentration, pH and temperature oocyst viability was reduced by approximately 70 % (table 6.7 and figure 6.7), initial viability of the control being 90 %.

Table 6.7. Viability of oocysts after exposure to ozone in contactor (method 3).

O_3 conc. dose	Viability after 5 min.	Viability after 10 min.
1 mg/L	51.6%	48%
3 mg/L	19.3%	21.6%
5 mg/L	19%	17.3%

Figure 6.7. Oocyst viability against ozone concentration.



6.3.4. Comparison of two methods of applying ozone for oocyst destruction, using residual concentration (method 2) and dosing in a contactor (method 3).

Under the conditions used to treat the oocysts (ozone concentrations 1, 3 and 5 mg/l, pH 7, temperature 5°C and time 10 min) the contactor method of ozone treatment achieved significantly greater oocyst inactivation ($p < 0.001$) at each concentration than the ozone residual treatment (table 6.8.).

Comparisons of the effects of different ozone concentrations within each treatment regime revealed significant differences between 1 and 3 mg/l and 1 and 5 mg/l ($0.01 < p < 0.05$) and between 3 and 5 mg/l ($p < 0.001$) for method 2 (residual concentration). Significant differences were seen between 1 and 3 mg/l and 1 and 5 mg/l ($p < 0.001$), but not between 3 and 5 mg/l, when viability was reduced to 21.6 % and 17.3 % respectively, in method 3 (contactor).

Table 6.8. Comparison of two methods for assessing the effects of varying concentrations of ozone on the viability of *C. parvum* oocysts.

	Method 2	Method 3	Method 2	Method 3
O_3 conc. Residual/dose	Viability after 5 min.	Viability after 5 min.	Viability after 10 min.	Viability after 10 min.
1 mg/L	87%	51.6%	72.6%	48%
3 mg/L	79%	19.3%	81.8%	21.6%
5 mg/L	75%	19%	61.2%	17.3%

6.4 Discussion.

The results presented show that ozone is capable of killing *C. parvum* oocysts. The most important factors influencing the destruction of oocysts by this method of disinfection appeared to be the residual concentration of ozone itself and the temperature at which the oocysts were exposed to the ozone. There was some effect of pH on the viability of the oocysts, a lower pH causing a greater reduction in viability than higher pH. This effect was seen mainly at the lower temperature tested (5°C), oocyst viability being reduced more effectively and faster at 20 °C. At 20°C, for ozone concentrations of 3 and 5 mg/l, the rate of decline in oocyst viability was fastest during the first 2 min, with 100 % inactivation occurring within 4 to 10 min. At 1 mg/l , although considerable reduction in oocyst viability was seen, the rate was somewhat

slower than at the higher concentrations. At pH 5 and 7 the graphs (figures 1 and 3) do indicate a slightly faster rate of reduction in oocyst viability over the first 2 to 4 min. However, at pH 9 (figure 5) this is not evident, although viability did eventually fall to < 4 %. Other workers noticed an increase in excystation at low concentrations of disinfectants (Reducker and Speer, 1985), before a reduction in viability was detected (Smith *et al*, 1989). Korich *et al*, (1990) detected a large apparent increase in excystation in oocysts exposed to high concentrations of disinfectant for a short time or to low concentrations for a long time. This apparently turned out to be an artifact of the counting procedure; they found that the contents of the oocysts had been destroyed and would be unable to excyst. Sporozoites were absent from the suspensions examined under the microscope.

As a method of disinfection for the water industry, when compared to other disinfection regimes tested (albeit, mainly under laboratory conditions), ozone is far more effective than most, especially chlorination. Smith *et al*, (1989) reported concentrations of free chlorine of 8,000 to 16,000 mg/l required to kill *C. parvum* oocysts (at a concentration of 10^3 oocysts/ml). Viability was assessed by excystation and by DIC microscopy. At pH 6 and 7, and a concentration of 8,000 mg/l, chlorine eliminated oocyst viability after 24 h at 5°C and 20 °C, while 16,000 mg/l reduced oocyst viability to 0% regardless of pH or temperature. Korich *et al*, (1990) conducted experiments at pH 7 and 25°C, using excystation and mouse infectivity to assess viability. After 120 min exposure to 80 mg/l free chlorine excystation was reduced to 0 % ($C.t' = 9600$). A 2 log inactivation in infectivity to mice was acieved in 90 min exposure to 80/mg/l ($C.t' = 7200$). Similar work on chloramination produced similar results, with $C.t' = 9600$ to stop excystsation, while mouse infectivity was reduced by only one log at $C.t' = 7200$ compared to the 2 log reduction seen with free chlorine.

Peeters *et al* (1989), using chlorine dioxide, achieved 1 - 2 log reductions in viability as assessed by infectivity to mice, at concentrations of 0.31 mg/l and 0.43 mg/l for times between 15 and 30 min. Korich *et al*, (1990) found a higher concentration (1.3 mg/l) for longer (1 h) was required to reduce excystation to near 0 %, while this gave only 1 log inactivation in infectivity to mice.

Peeters *et al* (1989) produced results which were encouraging for ozone as a potential practical disinfectant for water containing oocysts. Oocyst inactivation was assessed according to the ability of oocysts exposed to ozone to infect mice. They achieved 100 % infection of mice only after gavage with 10^3 oocysts and that the effect of ozone on oocysts depends on the number of oocysts used in the experiment. Ozone concentrations from 0.59 to 2.27 mg/l for times of 2 to 8 min were tested. No mice became infected when water containing 10^4 oocysts/ml was exposed to 1.11 mg/l ozone for 6 min, while for water containing oocysts at a concentration of

5 x 10^5 oocysts, 2.27 mg/l ozone was required for 8 min. These concentrations were the residual concentrations of ozone present at the start of the exposure time. Ozone concentration dropped during the course of the experiments (by approximately 33 %). The *C.t'* values given for this level of inactivation at the above concentrations were calculated as 6.7 and 18.2 respectively.

Korich *et al*, (1990) examined the effects on oocyst viability of ozone, chlorine dioxide, chlorine and monochloramine using excystation and mouse infectivity in order to assess viability. They found that ozone and chlorine dioxide were many times more effective than free chlorine and monochloramine in inactivating *C. parvum* oocysts. An ozone concentration of 1 mg/l applied and maintained over a 5 min period at 25°C reduced excystation from 84 % to 0 %. The rate of decrease was highest during the first minute. According to infectivity, 90 - 99 % inactivation was achieved after 5 min and 99 - 99.9 % inactivation was achieved after 10 min, giving an approximate *C.t'* value of between 5 and 10. The concentration of oocysts used in these experiments was 2.8 x 10^5/ml. *C. parvum* oocysts are, according to this work, many times more resistant than *G. lamblia* cysts to the disinfectants tested. The *C.t'* value of 5 to 10 quoted for ozone is 30 times that (0.18 mg/l.min) required for the same level of inactivation of *G. lamblia* cysts (Wickramanayake *et al*, 1985). In addition, Korich *et al*, (1990) found good correlation between excystation and infectivity as indicators of viability. This is interesting, and important because Campbell *et al*, (1992) demonstrated good correlation (r = 0.997) between vital dye uptake and excystation as a means of assessing oocyst viability. In a student project undertaken at the SPDL, excystation was used to verify the results of the vital dye viability assay, and good correlation was observed in that instance also (N. Joss, 1991). Although others (Korich *et al*, 1990) state that the vital dyes were totally unreliable, the information obtained through these experiments is that vital dyes gave a good indication of oocyst viability. It is possible that oocyst viability is overestimated to some extent when using the fluorogenic vital dyes as a method of assessing viability, as has been shown for *Giardia* (Labatiuk *et al*, 1991), especially as it indicates the viability status of individual organisms. It remains to be seen just how well the vital dye viability assay correlates with infectivity as a means of assessing viability. Infectivity in neonatal mice, used by Peeters *et al*, (1989) required at least 10^3 oocysts to establish infection and consequently they used a minimum infection level of 10^4 oocysts/ml during the trials. This level is a factor of at least 1,000 times higher than the levels detected in surface waters (Madore *et al*, 1987; Smith and Rose, 1990; Anon, 1990a; Barer and Wright, 1991). Korich *et al*, (1990) used neonatal mice to determine the effects of disinfectants, and they determined the ID_{50} to be approximately 60 oocysts. Inability to produce infection in mice with an incremental $\log_{10}$ increase in dose of the ID_{50} implied a level of oocyst inactivation of 1 log. Perrine *et al*, (1990) used infectivity in immunosupressed rats to assess viability and found that a minimum infection level of 10^4 oocysts per rat was

necessary to induce a 100 % rate of infection. All three of these methods use larger numbers of oocyst to assess viability or infectivity than the vital dye assay, but the fact that both animal infectivity and vital dye viability assessment correlate with *in vitro* excystation suggests that they are useful techniques with which to assess the viability of *C. parvum* oocysts.

From the work performed in this study other important factors influencing the inactivation of oocysts by ozone have been demonstrated. One of these is the temperature at which the experiments were carried out. The concentration of oocysts used in the experiments was shown to influence the inactivation of *C. parvum* oocysts; a higher concentration of the parasite required a higher concentration of ozone for a longer period of time (a greater *C.t'* value). Also of importance is whether oocysts were 1) added to water with a residual concentration of ozone which was not maintained, 2) added to water with an established concentration of ozone which was maintained for the duration of the experiment or 3) added to water which was then dosed with and maintained at a particular concentration of ozone. The results of the comparison of ozone treatments presented in section 6.3.5. show that there was a statistically significant difference in inactivation of oocysts; greater inactivation observed when a particular dose was applied and maintained for the contact time (method 3) as opposed to a particular dose being applied to achieve a particular residual concentration and not being maintained (method 2). Peeters *et al*, (1989) do not state the temperature at which ozonation and incubation of the oocysts was carried out. Their ozonization procedure was similar to method 2 used here, where a concentration of ozone was produced and measured at time zero when the oocysts were added, but the concentration was not maintained, allowing the ozone to decay. Korich *et al*, (1990) performed experiments at 25°C and dosed water with ozone such that the residual concentration was 1 ppm (mg/l) and maintained it at that concentration for the exposure time. Perrine *et al*, (1990) conducted experiments using an initial concentration of ozone which was not maintained (as in method 2) in water at 20°C.

Comparison of the results obtained by different groups on the inactivation of oocysts is difficult because of the different effects of conditions such as temperature, ozonation technique, assay for the assessment of viability and the concentration of oocysts used. The results from Peeters *et al*, (1989) give *C.t'* values of 6.7 and 18.2 for 10^4 and 5 x 10^5 oocysts/ml respectively. Korich *et al*, (1990) found that a *C.t'* value of between 5 and 10 was sufficient to give 99 % inactivation of 2.8 x 10^5 oocysts/ml. Perrine *et al* (1990) found that 0.44 mg/l (mean residual concentration) ozone for a contact time of 6 min, giving a *C.t'* value of 2.64, was sufficient to give a 2 log (99 %) inactivation of oocysts. From the results presented in this study a *C.t'* value of 30 (based on the measured residual concentration of ozone at time zero) was required to give 100 % reduction in oocyst viability, with an oocyst concentration of 1.25 x 10^5 oocysts/ml. A 2 log (99 %) inactivation occurred at *C.t'* values between 10 and 18

mg/l.min for the same initial concentration of oocysts in water. *C.t'* ratios must be treated with a certain degree of caution because some of the tests have not shown the interchangeability of *C* and *t* to provide a required *C.t'* value (Anon 1990a). Only in this study, in fact, were *C* and *t* values interchangeable, eg 3 mg/l for 10 min, or 5 mg/l for 6 min gave 100 % reduction in viability at pH 5 at 20°C.

Several water companies use ozone as a means of disinfecting water. A residual ozone concentration of 0.4 mg/l for 6 min is achieved and maintained by injecting 1.5 to 4 mg ozone per litre of water (Peeters *et al*, 1989; Perrine *et al*, 1990). This is the level Perrine *et al*, (1990) found to be effective in inactivating 99 % (2 log) of oocysts. Considering that it is rare to find a contamination level of surface water higher than 10^2 oocysts/l, they suggested this would be "an effective prophylactic mean for defeating the transmission of waterborne cryptosporidiosis". Similarly Peeters *et al*, (1989) felt that ozone constituted a means of disinfecting drinking water which was contaminated with *Cryptosporidium* oocysts. Korich *et al*, (1990) concluded that disinfectants alone, with the possible exception of ozone, could not be expected to inactivate *C. parvum* oocysts. The *C.t'* values obtained from this study (ranging from 10 to 30) are high and one must speculate as to whether ozone treatment would be economically viable. A recent report by the Drinking Water Inspectorate put the indicated *C.t'* values for 99 % inactivation of oocysts at between 5 and 15 mg/l.min. These would be achievable on full scale treatment works, although the upper values lie outwith current design practices and may make installing ozone equipment in an existing plant difficult.

An important consideration in determining the effectiveness of ozone as a disinfectant is the mechanism by which inactivation of the microorganisms occurs. The National Research Council (NRC) in America (NRC 1987; NRC 1980) reported that the biocidal action of ozone is a result of its reaction with double bonds of fatty acids in bacterial cell walls and cell membranes and the protein capsid of viruses. In the case of bacteria the oxidation results in a change in cell permeability and a leakage of cell contents into solution. In the case of viruses alteration of the protein capsid prevents the virus being taken up by the susceptible cells. In the case of *Giardia* cysts, the cyst wall is attacked by ozone, permitting attack on the plasma membrane and ultrastructural components of the organism (Sproul, 1986; Singer, 1990). The mechanism by which ozone inactivates *Cryptosporidium* oocysts is not known, although the mechanisms outlined above would provide some areas for investigation.

A further important point, related to the mode of action, is the reactivity of ozone and its stability in water. Ozone decomposes rapidly in water above pH 7 and readily reacts with contaminants (such as natural organic matter), so its persistence in such water is short lived. At elevated pH values it is difficult to maintain any measurable dissolved ozone residual. If

molecular ozone is the main oxygen species responsible for microorganism inactivation, then disinfection by ozone will only be effective in water at lower pH (pH < 7) in which molecular ozone is more stable. The relatively poorer effects of ozone on oocyst viability seen at pH 9 compared to pH 5 and 7 suggests that molecular ozone contributes to, but is not solely, responsible for the inactivation of oocysts. This would require further investigation. However, if any of the radicals produced by the decomposition of ozone show strong disinfecting properties, then the effectiveness of ozone is not limited to waters in which molecular ozone is stable (Singer, 1990). That some oocyst inactivation was observed at pH 9, suggests that perhaps a combination of radicals and molecular ozone, or both individually may have a destructive effect on oocysts. Adjusting the conditions at which experiments are carried out to favour either molecular ozone, or radical production would give more insight into which is more successful at oocyst inactivation, and perhaps indicate a mode of action by which oocysts are destroyed.

Where oocysts have been present in the environment for some time or have been damaged during some stage of water treatment, and are less resistant to disinfection, then lower $C.t'$ values may prove more successful at inactivating oocysts. This is a matter for further research, some of which has commenced and is presented in a later section (chapter 7).

CHAPTER 7. THE DESTRUCTION OF OOCYSTS OF CRYPTOSPORIDIUM PARVUM BY SAND AND CHLORINE.

7.1. Introduction.

Surveys of the occurrence of *Cryptosporidium* spp. oocysts in water in the USA and the UK have demonstrated that they can occur commonly in both raw and treated water (Smith and Rose, 1990), and outbreaks of waterborne cryptosporidiosis have been the result of both pre- and post-treatment contamination of water supplies (Anon, 1990a). In addition, samples of backwash water (which is a result of cleaning the filters used in the treatment of drinking water by forcing water through the filters in the reverse direction) from sand filters can contain large numbers of oocysts (Rose, 1988; Richardson *et al*, 1991). At some water treatment plants, following settlement in lagoons, clarified backwash water is returned to the head of the water treatment works, whilst the sludge is disposed of on a designated waste disposal site or on agricultural land. It has been recommended that when contamination of sludge with oocysts is suspected special arrangements should be made for disposal to a designated waste disposal site and not to agricultural land (Anon, 1990a). Reusing oocyst-contaminated clarified backwash water may increase the oocyst concentration as a result of recycling, providing increased opportunity for them to enter and pass through water treatment processes. At present there is no cost-effective method for treating backwash water which would guarantee either the inactivation or removal of oocysts.

Recently, a fluorogenic assay for assessing the viability of individual *C. parvum* oocysts has been developed in this laboratory (Campbell *et al*, 1992). This provides a means for investigating the effect of environmental pressures and laboratory mock-ups of water treatment processes on oocyst viability (Robertson *et al*, 1992a). Such an assay can highlight regimes which are injurious to oocysts and may aid in the development of strategies for the inactivation of waterborne oocysts. This chapter addresses (a) the effect of shaking *C. parvum* oocysts with sand, and (b) the effect of chlorination on sand-damaged *C. parvum* oocysts, at laboratory scale, using the fluorogenic viability assay.

7.2. Materials and Methods.

Materials and methods are described in full in chapter 2, section 2.10.

7.2.1. Shaking of oocysts with sand.

Oocyst-free sand (average particle size 0.33 mm, uniformity coefficient 1.56) from a slow sand filter was washed several times in reverse osmosis (RO) water and aliquots (9.5 g dry weight) were placed in multiple sterile 50 ml centrifuge tubes. To each sand containing tube, 2 ml of RO water and 100µl (3.4×10^5 oocysts) of the oocyst stock suspension were added, resulting in a total volume of approximately 7.5 ml. Tubes were placed on a horizontal shaker (Luckham R100 Rotatest; Denley Luckham Ltd., Billinghurst, U.K.) and rotated at 128 rpm, for 5, 30, 60, 90 min, 2 h and 24 h. Three types of controls were used: 1. oocysts sampled directly from the stock suspension; 2. oocysts left in unshaken sand; and 3. oocysts shaken in RO water, and sampled at the above time points.

At sampling times a single tube was removed from the shaker, the sand allowed to settle, and 2 ml of the fluid pipetted into a 2 ml microfuge tube (Sarstedt (U.K.), Leicester). This was centrifuged at 12,500 x ***g*** for 15 sec and the pellet washed in 2 ml of (isotonic) Hanks' Balanced Salt Solution (HBSS) without phenol red (Gibco Ltd., UK., cat. no. 041-04025M). Oocyst viability was then assessed as described in chapter 2, section 2.4.1.

7.2.2. Chlorine disinfection of oocysts shaken with sand.

Oocysts were treated with chlorine after shaking with sand for 5 min. After allowing the sand to settle, 2 ml of fluid were removed, added to a 50 ml centrifuge tube containing 25 ml of 1 mg/l free chlorine and allowed to stand for 5 min at 20°C. Sodium thiosulphate was then added to neutralise the chlorine and the tube was centrifuged at 900 x ***g***. The supernatant was aspirated, leaving approximately 1 ml and the pellet resuspended, decanted into a 2 ml microfuge tube, and washed in HBSS. Viability was assessed as described in chapter 2, section 2.4.1.

7.3. Results.

The percentage of non-viable oocysts increased from 17.6 ± 3.2% to 50 ± 2% after 5 min shaking with sand, with a further increase to 86.7 ± 4.9% after 30 min (table 7.1, figure 7.1). At 60 and 90 min, 99.3 ± 0.6% and 99.7 ± 0.5% of oocysts, respectively, were non-viable (table 7.1, figure 7.1). Statistical analysis demonstrated a significant increase in the percentage of non-viable oocysts after 5 min shaking with sand, compared to controls ($p < 0.001$). No live oocysts were found after shaking with sand for periods from 2 to 24 hours. Oocysts were devoid of contents (ghosts), appeared damaged, and the majority became fragmented over the

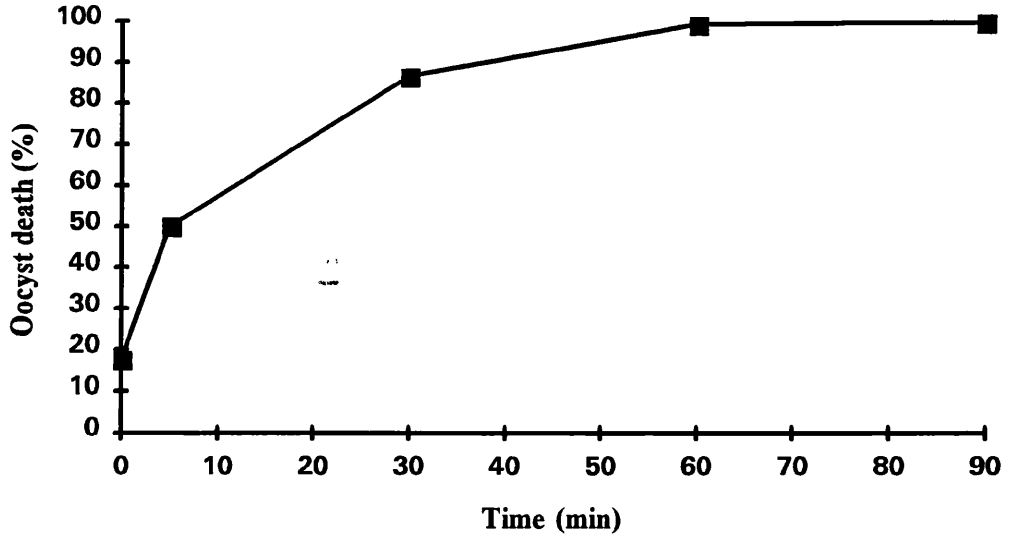
24 hour period. Viability of control oocysts was the same at the start and end of the incubation with sand.

When dead oocysts were examined for the presence or absence of internal contents, the proportion in which internal contents were absent increased with time. Whilst 93.3% of non-viable control oocysts contained internal contents, as demonstrated by PI inclusion, the number of oocysts which contained internal contents following shaking with sand decreased to 41.3% at 5 min and to 0.3% at 90 min (table 7.1, figure 7.2).

Table 7.1. The effects of sand on oocyst viability.

		% viable oocysts	% non-viable oocysts	% non-viable oocysts	
Time	n	mean ± s.d.	mean ± s.d.	with contents	without contents
control	5	82.4 ± 3.2	17.6 ± 3.2	93.3	6.7
5 min	3	50 ± 2	50 ± 2	41.3	58.7
30 min	3	13.3 ± 4.9	86.7 ± 4.9	8.9	91.1
60 min	3	0.7 ± 0.6	99.3 ± 0.6	1.9	98.1
90 min	3	0.3 ± 0.5	99.7 ± 0.5	0.3	99.7

Figure 7.1. Percentage of oocysts rendered non-viable by shaking with sand, over time.



When oocysts were shaken with sand for 5 min and then exposed to 1 mg/l free chlorine for a further 5 min the number of non-viable oocysts increased significantly compared to controls (untreated oocysts), and the number of non-viable oocysts increased compared to those treated with sand only (from 53.8% to 68.02%) (table 7.2). Oocysts which were chlorinated after shaking with sand were examined for the presence of internal contents (table 7.2). Although the percentage of non-viable oocysts increased after chlorination, treatment with chlorine did not affect the proportion of oocysts which contained internal contents (table 7.2).

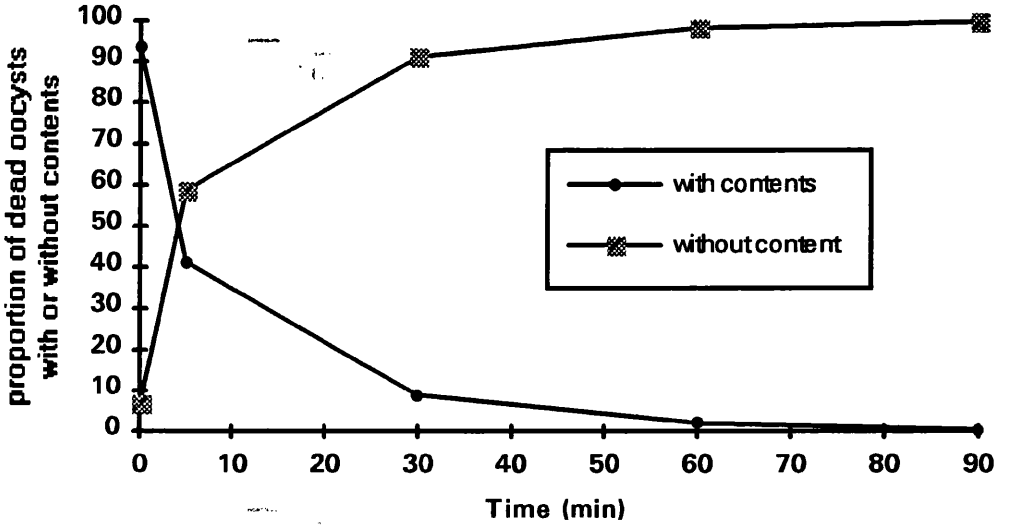
Table 7.2. The effects of sand and chlorine on oocyst viability[1].

		% viable oocysts	% non-viable oocysts	% non-viable oocysts	
Experiment	n	mean ± s.d.	mean ± s.d.	with contents	without contents
control	3	89.6 ± 0.013	10.4 ± 0.013	81.2	18.8
sand	3	46.2 ± 1.197	53.8 ±1.197	29.3	70.7
sand + Cl	3	31.98 ± 1.392	68.02 ± 1.392	30.4	69.6

n = number of experiments, s.d. = standard deviation, sand = oocysts shaken with sand for 5 min, Cl = chlorination (1 mg/l for 5 min).

[1] means and standard deviations calculated after arc-sine transformation of percentages

Figure 7.2. Percentage of dead oocysts with or without internal contents over time.



7.4. Discussion.

The results indicate that shaking oocysts with sand, for as little as 5 min is sufficient to kill a significant number of *C. parvum* oocysts, while shaking for up to 90 min kills 99.7% of oocysts. Shaking with sand for periods from 2 to 24 h was observed to kill all oocysts, causing the majority to fragment. In addition, if oocysts are treated with a concentration of chlorine suitable for the disinfection of water, after shaking for 5 min with sand, more oocysts are killed.

LeChevallier *et al*, (1991b) noted that out of 23 oocysts detected in samples of potable water, only 2 were considered viable, in that they contained sporozoites, whilst one third of the oocysts detected in raw water contained sporozoites. These workers implied that water treatment processes inactivated oocysts, and suggested that it was the disinfection practices of the plants studied (prechlorination and postchloramination) which were effective in oocyst inactivation.

Whilst the shaking of oocysts with sand is undoubtedly a more severe treatment than the passage of oocysts through sand filters, it does indicate that collision between oocysts and sand grains is detrimental to oocyst viability. In addition, the abrasive effect of a 5 min exposure of oocysts to sand may be sufficient to render them susceptible to concentrations of free chlorine which have been shown to be ineffective on oocysts isolated from human faeces (Smith *et al*, 1989). Although the concentration of chlorine used in these experiments did not significantly affect oocyst viability when compared with oocysts shaken with sand, further replicates of these experiments, a higher concentration (*e.g.* 3 mg/l), a longer exposure time (*e.g.* 10 min), or a combination of these might give more significant results. Further work could address the percentage oocyst loss over time, through disintegration, during their incubation with sand.

Abrasion of oocysts may occur during their passage through rapid sand filters, and subsequent disinfection could have a greater effect on oocyst viability than has been demonstrated previously in laboratory-based studies (Korich *et al*, 1990; Ransome *et al*, 1991). Further studies should examine the effects of prechlorination regimes and the use of disinfectants such as ozone or chloramine on oocyst viability following oocyst passage through sand filters.

Where large numbers of oocysts can be detected in backwash water from filters, the data indicate that it may be possible to accomplish both the large-scale destruction of oocysts and the reuse of clarified backwash water following agitation with sand. Systems which may be compatible with this concept include collapse-pulsing during rapid filter backwashing particularly with mixed air and water, and the external washing processes employed for skimmed, dirty sand from slow sand filters. Examination of other mechanical systems for

agitation, or ultrasonication may be productive in improving the efficiency of sand in killing oocysts.

CHAPTER 8. ANTIOXIDANT PROTECTION MECHANISMS IN *CRYPTOSPORIDIUM PARVUM.*

8.1. Introduction.

Intracellular parasites are exposed to oxidative killing from various sources, including normal cellular processes and the host immune response, which produce a number of oxidants, such as superoxide ions (O_2^-), hydroxyl radicals (HO·), oxygen singlets ($^1O_2$), hydrogen peroxide (H_2O_2) and nitric oxide (NO). Cells and parasites are protected from the damaging effects of these oxidants by specific enzymes. Among these enzymes is superoxide dismutase (SOD) which catalyses the dismutation of superoxide anions to hydrogen peroxide (McCord and Fridovich, 1969).

SOD was first recognised and described by McCord and Fridovich (1968, 1969). It exists in three different forms, characterised by the metal cofactors present in the active site of the enzyme, and by their sensitivities to different inhibitors. Copper/Zinc (Cu/Zn) containing SOD is sensitive to cyanide (KCN) and H_2O_2 and has been found in the cytosol of a wide range of eukaryotic cells, including animals, plants, yeasts and helminths (Callahan *et al*, 1988; Henkle *et al*, 1991;Callahan *et al*, 1993). Manganese (Mn) containing SOD is insensitive to cyanide (KCN) and H_2O_2 and has been isolated from eukaryotic mitochondria (Fridovich, 1975; Weisiger and Fridovich, 1973) and from bacteria (Keele *et al*, 1970). Iron (Fe) containing SOD is insensitive to cyanide but sensitive to H_2O_2, and has been demonstrated in many prokaryotes (Yost and Fridovich, 1973; Lumsden and Hall, 1974), some plants (Lumsden and Hall, 1974) and protozoa (Meshninick and Eaton, 1981; Le Trant *et al*, 1983; Kitchener *et al*, 1984; Sibley *et al*, 1986; Barra *et al*, 1990; Tannich *et al*, 1991; Becuwe *et al*, 1992).

It is mainly iron containing SOD which has been detected in parasitic protozoa, although endogenous manganese containing SOD has been detected in *Plasmodium falciparum* (Fairfield *et al*, 1988; Ranz and Meshnick, 1989) and *Eimeria tenella*, which contains the three forms of SOD during different stages of its development (Michalski and Prowse, 1991).

Table 8.1. Anti-oxidant enzymes of protozoa and helminths.*

PROTOZOA	Range of SOD (U/mg protein)
Trypanosoma brucei	4.83
Trypanosoma cruzi	0.054 - 0.56
Leishmania donovani	4.1 - 12.8
Leishmania tropica	6.4 - 23.0
Crithidia fasciculata	9.16 - 14.2
Toxoplasma gondii	6.1 - 14.6
Plasmodium berghei	host derived
Plasmodium falciparum	6.3 - 25.1
Babesia hylomysci	2.92
Babesia divergens	2.50
Eimeria tenella	4.8 - 380.4
Entameoba histolytica	18.7
HELMINTHS	
Trichinella spiralis	6.8 - 30.8
Nippostrongylus brasiliensis	15.34
Nematospiroides dubius	31.63
Onchocerca volvulus	8.1
Onchocerca cervicalis	27.6 - 29.5
Dirofilaria immitis	35.2 - 37.0
Turbatrix aceti	27.1 - 27.8
Caenorhabditis elegans	3.8 - 17.1
Taenia taeniaeformis	34.29
Hymenolepis diminuta	7.9
Moniezia expansa	9.5

*Adapted and modified from Callahan *et al*, (1988).

C. parvum is ultimately inactivated by ozone (O_3), but requires greater concentrations than many other organisms (Peeters *et al*, 1989; Korich *et al*, 1990; chapter 6). It is likely that the oocyst wall protects the sporozoites contained within and acts as a physical barrier. However, it is also likely that the sporozoites themselves have a further protection mechanism against oxidant damage. This may be necessary for protection against oxidants produced by the host immune response when sporozoites excyst following ingestion and establish infection in the

epithelial cells of the small intestine and for later developmental stages in the life cycle of the parasite. The protection against oxidant mediated damage is likely to come from enzymes such as SOD.

Decomposition of ozone produces a number of oxygen species which are damaging to microorganisms, including O_2^-, OH·, $^1O_2$ and H_2O_2. Other antioxidants, such as the enzymes catalase and glutathione peroxidase and the sugar mannitol, scavenge some of these products (Callahan *et al*, 1988) and the presence of mannitol, which scavenges HO· radicals has been demonstrated in *C. parvum* oocysts (Schmatz, 1989).

As a consequence of the resistance shown by oocysts to inactivation by ozone, this chapter investigates the possibility that the enzyme superoxide dismutase is present in sporulated oocysts of *C. parvum*.

8.2. Materials and Methods.

The materials and methods are described in chapter 2, sections 2.11. Four methods were used to assay for superoxide dismutase, two of which provided methods of assessing the activity of the enzyme, if present.

8.2.1. Spectrophotometrical measurement of superoxide dismutase activity.

Superoxide anions can be produced in solution (using xanthine-xanthine oxidase to generate O_2^-) and the rate of superoxide production can be followed and measured spectrophotometrically (see section 2. 11). The addition of sporulated oocysts, if they contain SOD would inhibit the colour change observed as a result superoxide production and the presence of SOD can be established and quantified by the percentage inhibition measured.

8.2.1.1. 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium reduction assay.

This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase activity is then measured spectrophotometrically (550 nm) by the degree of inhibition of this reaction.

8.2.1.2. Ferricytochrome *c* reduction assay.

The reduction rate of cytochrome *c* by superoxide radicals can be monitored spectrophotometrically (550 nm) using xanthine and xanthine oxidase as the source of superoxide ions. SOD competes for superoxide and decreases the reduction rate of cytochrome *c*. This is similar to the system described above.

8.2.2. Electrophoretic detection of superoxide dismutase.

8.2.2.1. Horizontal agarose gel electrophoresis.

Methyl thiazolyl tetrazolium (MTT) is reduced by electron donors to form a dark blue-purple insoluble formazan. The reaction proceeds rapidly in the presence of phenazine methosulphate (PMS) which acts as an intermediary catalyst. After exposure to light, the enzyme can be seen as pale zones on the dark background formed by the formazan.

8.2.2.2. Polyacrylamide gel electrophoresis.

Nitro blue tetrazolium (NBT) is reduced by transfer of electrons from photo-reduced riboflavin, resulting in the formation on the gel of a blue colour. Where SOD was present an achromatic zone was visible in contrast to the blue colour.

8.3. Results

8.3.1. Spectrophotometrical measurement of superoxide dismutase activity.

8.3.1.1. 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium reduction assay.

Superoxide dismutase activity was examined in freeze thawed fractions of three different isolates of *C. parvum* oocysts, *Eimeria tenella* oocysts and whole blood. The majority of *E. tenella* oocysts were sporulated. Low levels of activity of superoxide dismutase were detected in all of the fractions tested, as shown in table 8.3.1. Oocysts of *C. parvum* isolated from sheep (C1/93), humans and lambs showed mean superoxide dismutase activities of 6.19 ± 1.17 U/mg protein, 5.44 ± 2.4 U/mg protein, 9.27 ± 1.35 U/mg protein respectively. The mean superoxide dismutase activity detected in freeze thawed fractions of *E. tenella* was 8.39 ± 0.81 U/mg protein and in whole blood was 2.4 ± 0.14 U/mg protein. The concentration of SOD per ml and the number of organisms and concentration of blood per ml are shown in tables 8.3.2 and 8.3.3.

The percentage inhibition, calculated from the change in optical density per minute, was plotted against the $\log_{10}$ concentration of a series of superoxide dismutase standards (of known concentrations). The concentration of superoxide dismutase in samples and controls was obtained by extrapolating the percentage inhibition to concentration on the graph (data not shown). The concentration and percentage inhibition of the superoxide dismutase standards are shown in table 8.3.4. One unit of SOD activity is defined as the amount of enzyme required to inhibit the reaction by 50 %.

Table 8.3.1. Concentration of superoxide dismutase detected in *C. parvum* oocysts, *E. tenella* oocysts and whole blood.

Sample/isolate analysed	Units of SOD per mg soluble protein
C. parvum (C1/93)	5.36 - 7.02
C. parvum (Human)	3.74 - 7.14
C. parvum (Lamb)	8.32 - 10.22
E. tenella	7.82 - 8.96
Whole blood	2.3 - 2.5

Table 8.3.2. Concentration of superoxide dismutase detected in assay.

Sample/isolate	U SOD/ml	U SOD/mg	U SOD/ml	U SOD/mg
C.parvum (C1/93)	0.263	5.36	0.603	7.02
C. parvum (Human)	0.692	3.74	0.28	7.14
C. parvum (Lamb)	0.24	8.316	0.295	10.22
E. tenella	0.977	7.82	2.239	8.956
Whole blood	276	2.3/mg haem.	295	2.5/mg haem.

haem. = haemoglobin. There were 118 mg haemoglobin/ml sample of whole blood.

Table 8.3.3. Concentration of organisms used for each assay.

Sample/isolate analysed	Concentration of organisms/ml
C.parvum (C1/93)	3.4×10^8
C. parvum (Human)	5.8×10^7
C. parvum (Lamb)	5.6×10^7
E. tenella	1.14×10^7
Whole blood	118 mg/ml

Table 8.3.4. Concentration and percentage inhibition of superoxide dismutase standards/controls.

concentration SOD U/ml	% inhibition
0	0
0.156	4.17
0.469	29.16
0.938	41.67
1.875	79.17
3.75	91.67

8.3.1.2. Ferricytochrome *c* reduction assay.

Low superoxide dismutase activity was detected in oocysts by measuring the reduction rate of cytochrome *c* using xanthine-xanthine oxidase as a source of superoxide anions. The activity detected was in the range of 0.39 U/ml to 0.64 U/ml, although activity was also detected in the lysis buffer at a concentration of 0.2 U/ml and in the negative control at a concentration of 0.36 U/ml. This was not reproduced in further repeats of the assay, but did indicate that SOD might be present and that further investigations should proceed.

8.3.2. Electrophoretic detection of superoxide dismutase.

8.3.2.1. Horizontal agarose gel electrophoresis.

No superoxide dismutase was detected in *C. parvum* oocysts using horizontal agarose gel electrophoresis and staining with methyl thiazolyl tetrazolium (MTT) and phenazine methosulphate (PMS). However, the enzyme was detected in lysed fractions of red blood cells.

8.3.2.2. Polyacrylamide gel electrophoresis.

No superoxide dismutase was detected in *C. parvum* oocysts using native polyacrylamide gel electrophoresis and staining with nitro blue tetrazolium. However, the enzyme was detected in one control dilution of a known concentration of superoxide dismutase.

8.4. Discussion

The results presented indicate that the antioxidant enzyme superoxide dismutase (SOD) is present in oocysts of *C. parvum* at a range of 3.74 - 10.22 U/mg protein. The enzyme is most likely to be present in the sporozoites present in the oocysts, rather than in the oocyst wall, although its location in the sporozoite, i.e. whether it is membrane or cytoplasm associated has not yet been ascertained. However, performing the same experiments described here on purified samples of sporozoites and empty oocysts (i.e. oocyst walls) would indicate whether SOD is present in the oocyst wall or in the sporozoite. SOD has already been shown to be present in a number of other parasites, as described in section 8.1. (and Callahan *et al*, 1988), including *E. tenella* (Michalski and Prowse, 1991), where it is present at levels ranging from 380.4 - 9.8 U/mg protein in sporulating oocysts from 0 h to 48 h of sporulation respectively, and in sporozoites from 4.8 - 12.9 U/mg protein. The concentration of SOD detected in *C. parvum* falls within this range and the results are similar to the levels detected in various other parasites (Callahan *et al*, 1988).

Superoxide dismutases are found in three forms in various kinds of organisms, the Mn-containing form of the enzyme found in the mitochondrial matrix and Cu/Zn and Fe containing forms of the enzyme found in the cytosol (Asada *et al*, 1980; Michalski and Prowse, 1991). The metal content (as well as the amino acid sequences) distinguishes the different forms of enzyme from one another. Determination of the metal content is usually performed by differential inhibition. Cu/Zn SOD is cyanide and H_2O_2 sensitive, whereas Fe and Mn SOD are affected by H_2O_2 and sodium dodecyl sulphate (SDS) respectively. Most intracellular parasites have Fe and Mn SOD. Cu/Zn SOD is found in various parasitic helminths (Callahan *et al*, 1988), *T. cruzi* (Giulivi *et al*, 1988) and also in erythrocytic stages of malarial parasites which derive and concentrate Cu/Zn SOD of host origin (Fairfield *et al*, 1983). However, late stages of *P. falciparum* contain cyanide insensitive SOD in addition to other forms, which may represent an endogenous enzyme (Fairfield *et al*, 1988). Michalski and Prowse (1991) detected several forms of SOD in *E. tenella*, four being identified as Fe SODs and one Mn SOD, while the major SOD isoenzymes in unsporulated oocysts appeared to be Cu/Zn containing; these observations being the result of differential inhibition experiments, using cyanide (KCN), H_2O_2 and SDS. SOD activity diminished during sporulation, with sporulated oocysts containing one sporozoite associated Mn form of the enzyme.

The role of SOD enzymes (and other antioxidant enzymes) to parasites (and any other organisms) is to protect against oxygen mediated damage. Oxidants are produced during normal cellular metabolic processes and as a consequence of the host immune response. Unsporulated oocysts of *E. tenella* require oxygen for the sporulation process to proceed and it

is possible that the Cu/Zn containing SOD may protect the parasite from oxygen radicals during this process.

The lack of the major Cu/Zn SOD in *E. tenella* sporozoites, an invasive form of the parasite, would theoretically reduce its chances of survival in the face of the host response. It is possible that *E. tenella* sporozoites can acquire host SOD during the intracellular stage of development, or as reported for helminths (Callahan *et al*, 1988), the sporozoites may produce an extracellular SOD which would protect it against oxidative damage and help it evade the host immune response. It is also possible that *E. tenella* uses other antioxidant systems, such as glutathione peroxidase, glutathione transferase or mannitol for protection against the host immune response. *E. tenella* contains large amounts of mannitol and enzymes of the mannitol pathway have been detected in a range of coccidian oocysts, including other *Eimeria* species and *C. parvum* (Schmatz, 1989).

The viability of *E. tenella* sporozoites is dramatically affected by exposure to superoxide ions in aqueous solution (Michalski and Prowse, 1991). Catalase only partially protected sporozoites, indicating that H_2O_2 (a product of dismutation of superoxide ions) is not directly responsible for parasite killing. Similarly, the lack of protection given by histidine excludes singlet oxygen ($^1O_2$) from this role. However, SOD and mannitol (a scavenger of hydroxyl radicals) had maximum effect on parasite viability, which strongly suggested the involvement of superoxide and hydroxyl radicals in parasite killing, since it has been demostrated that $\cdot OH$ is a product of an interaction between H_2O_2 and $O_2^{\cdot-}$.

Oocysts of *C. parvum*, although inactivated by ozone, require considerably higher concentrations than many other organisms, suggesting some form of protection against ozone and its oxidative killing mechanisms, such as superoxide ions and hydroxyl radicals. A degree of this protection may well be due to the oocysts wall. However, the presence of SOD in freeze thawed fractions of oocysts (containing sporozoites) suggests that this enzyme adds to the resistance of *C. parvum* oocysts to oxidative killing mechanisms.

The presence of these antioxidants may give insight into the mechanisms involved in the inactivation of oocysts by ozone and which oxygen species (or combination of oxygen species) is more likely to cause the inactivation of the oocysts. The difference in inactivation of oocysts by ozone under different physico-chemical conditions, which affect the oxygen species most prevalent (e.g. oxygen radicals, superoxide ions, hydroxyl radicals and molecular ozone) may be a result of antioxidant enzymes such as SOD, catalase and glutathione peroxidase, or other antioxidant mechanisms (e.g. mannitol) which scavenge, dismutate or uncouple free radicals, nascent oxygen and other oxidants. Killing of oocysts in ozonated water at pH 9, conditions

favouring the production of radicals produced during the decomposition of ozone, was siginificantly less than at other pH values. It is possible that the presence of enzymes which protect against these oxidants (by dismutation, scavenging or uncoupling) is responsible for the reduced susceptibility of the parasite to inactivation by ozone.

The location of SOD in sporozoites is not known. It may be membrane associated, or cytoplasm/cytosol associated. Its presence may be for protection of the parasite against oxidative killing mechanisms associated with its lifecycle in the host and for defense against the host immune response. Sporozoites may require SOD for protection during cell invasion. Examination of other life cycle stages of *C. parvum* might reveal the presence of SOD, which may indicate the stage where antioxidant protection is most required. Protection of sporozoites within the environmentally robust oocyst is probably largely due to the oocyst wall, however, analysis of empty oocysts (i.e. oocyst walls), following excystation may reveal the presence of SOD, indicating a possible protective role in the transmissive stage.

The presence of SOD in oocysts, most likely the sporozoites, is important for its survival within the host, where numerous oxidants are produced, both in normal cellular metabolic processes and in the host immune response. In *E. tenella*, unsporulated oocysts require oxygen for the sporulation process to proceed, and SOD may provide protection for the parasite from oxygen radicals produced during this process. If sporulation of *C. parvum*, which unlike *E. tenella* occurs within the host, is susceptible to oxidant damage, then the presence of SOD may provide protection for the parasite, as in *E. tenella*. However, it is not yet known whether SOD is present in other life cycle stages of *C. parvum*, so at which stage its protective qualities are required is unknown.

The difference in susceptibility of various isolates of oocysts to ozone (Parker *et al*, in preparation; see also chapter 6) may be explained by different amounts of SOD present in particular isolates. Greater concentrations of ozone were required to inactivate oocysts of cervine-ovine origin compared to two human isolates and an isolate of bovine origin. The two human isolates required different concentrations for inactivation of oocysts, and oocysts of bovine origin were more susceptible to inactivation than any of the other isolates. This may be a result of the amount of SOD produced in response to the amount of oxidants produced by the host from which the isolates originated. The amount of SOD detected in the isolates tested all fell within a fairly narrow range (3.74 - 10.22 U/mg protein) and whether this is sufficient to be responsible for the differences in isolate susceptibility remains to be elucidated. It is possible that attempts to establish the infectious dose for *Cryptosporidium* (Smith *et al*, 1993) may be affected by using an isolate which has low SOD content to infect a host with higher production of oxidants.

The fact that SOD was not detected using the other assays described was almost certainly due to the use of insufficient numbers of oocysts. The amount of SOD present in lower numbers of oocysts would have been below the detection limits of the assays. Further, confirmatory work should begin with using these assays to detect SOD and to determine the metal content using inhibition studies. Thereafter it would be of interest to localise the enzyme to assess whether it is membrane or cytosol associated. In addition to the presence of SOD in *Cryptosporidium* it would be of interest to analyse the parasite for other antioxidant enzymes. Finally, as a means of assessing which of the products of ozone decomposition is responsible, or predominantly resposible for the inactivation of oocysts, it may be possible to assess the effect of individual oxygen species on oocyst viability by selective production of each metabolite, or by addition of selected agents to protect oocysts against for example H_2O_2, $HO\cdot$, $^1O_2$ and O_2^-, such as histidine, mannitol and catalase.

CHAPTER 9. DISCUSSION AND CONCLUSIONS.

9.1. Discussion.

Cryptosporidium spp. oocysts and *Giardia* spp. cysts were shown to be widespread in the environment. They were detected in a whole range of water and water related samples, including river water, loch and reservoir water, swimming pool water, raw sewage influent, sewage effluent and treated drinking water. The presence of oocysts and cysts in drinking water, prior to and following water treatment indicated the potential for waterborne transmission of both cryptosporidiosis and giardiasis. The occurrence of both parasites was, on occasions, within the levels detected during the course of documented outbreaks of disease (Hayes *et al*, 1989; Smith *et al*, 1989; Anon, 1990a; Richardson *et al*, 1991). The techniques used for the recovery, isolation, concentration and detection of oocysts and cysts from water samples are inefficient, which means that the actual occurrence and concentration of the parasites may be considerably more frequent and higher than the occurrence and concentrations quoted. In surface water, the frequency of occurrence of both parasites was similar, although *Cryptosporidium* oocysts were detected at higher concentrations than *Giardia* cysts. In treated water *Cryptosporidium* oocysts were detected more frequently and at higher concentrations than *Giardia* cysts.

Following the survey performed to ascertain the occurrence of *Cryptosporidium* and *Giardia* in the aquatic environment, attempts were made to assess the efficiency of different forms of water treatment in the removal of the environmentally robust oocyst and cyst stages. Problems were encountered initially in locating suitable treatment works with sufficient numbers of the organisms in the raw water. When oocysts or cysts were detected they were usually found in low numbers. Considering the poor recovery efficiency of the sampling and sample processing method, working with low numbers at the limits of detection makes calculation of filtration removal efficiencies difficult. Many of the problems encountered in this work could be overcome by using pilot scale plants, in which the number of organisms being introduced to the treatment plant and their viability at the outset could be determined and controlled.

In water undergoing pressure filtration and water undergoing conventional (coagulation, flocculation and rapid sand filtration) treatment, oocysts and cysts were detected in the water prior to treatment, while only oocysts were detected post-treatment. There were insufficient data to calculate removal efficiencies in these treatment works, although the presence of oocysts in the final water indicates that *Cryptosporidium* oocysts do pass through these water treatment works. Oocysts were detected in water entering a slow sand filtration water treatment works. Sampling of both raw and final water indicated that oocysts were also detected in the

final water and that oocysts were still detected in the final water after no further oocysts were detected in the raw water. The removal efficiency calculated on the data available, indicated a removal efficiency of approximately 92 % of the number of organisms detected prior to treatment. Unfortunately, at the time, there was no way of ascertaining the viability of the low numbers of oocysts detected.

Attempts should be made to determine at which depths oocysts occur most commonly in slow sand filters and what effect, if any, the 'schmutzdecke' has on the viability of oocysts. The rate of progress of oocysts through sand filters and in particular the biologically active 'schmutzdecke', could be assessed and laboratory scale experiments could be devised to assess the impact of these conditions on oocysts over the time taken for oocyst passage through the filter.

Cryptosporidium oocysts and *Giardia* cysts were also detected in water prior to its abstraction and treatment by microstraining. Approximately 62 % of the oocysts were removed, although no significant difference was determined in the occurrence or concentration of oocysts detected before and after treatment. Cyst removal was approximately 81 % and cysts were detected significantly less frequently and at lower concentrations after treatment, compared to before treatment.

In the light of the number of recent outbreaks of waterborne cryptosporidiosis and giardiasis (D'Antonio *et al*, 1985; Craun, 1988; Hayes *et al*, 1989; Smith *et al*, 1989; Anon, 1990a; Richardson *et al*, 1991), and the presence of both *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in raw water and treated water in distribution (Madore *et al*, 1987; Smith and Rose, 1990; LeChevallier *et al*, 1991a; LeChevallier *et al*, 1991b), one must question the success of water treatment processes in the removal of these pathogens and the integrity of the distribution network conveying the water to its destination. This is of concern in relation to public health and has obvious consequences for all users of water. In outbreaks when a point of contamination has been identified, both pre- and post-contamination has been indicated (Smith *et al*, 1989; Anon, 1990a; Richardson *et al*, 1991). Assessing the possible source of oocysts which contaminate potable water and enter water treatment, whether they survive water treatment, whether they are viable in the distribution network and retain the ability to cause infection is important in assessing the risk posed to human health from oocysts present in water.

The initial survey work performed on water samples from various origins gave a great deal of information on the occurrence and distribution of both *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in the aquatic environment. Continuous analysis of Loch Lomond water

sampled from the abstraction point at Ross Priory gave an indication of the occurrence of oocysts entering water treatment. Ross Priory is the sole abstraction point for water distributed to the central belt of Scotland. A possible outbreak of cryptosporidiosis in 1989 had been linked to water (in which oocysts had been detected) of Loch Lomond origin, although this has not been proven (Anon, 1990a).

An attempt was made to ascertain the origin and fate of oocysts in potable water by sampling the water abstracted from Loch Lomond and water in distribution, and by monitoring the occurrence of cryptosporidiosis in humans and animals in the southern catchment area of Loch Lomond (where individuals with the disease may contribute to contamination of the water) and in the areas supplied with Lomond water. In addition, sewage effluents discharging into tributaries of the loch were sampled to establish the input of oocysts from this possible source of origin. Agricultural runoff has been implicated as a source of contamination of water with oocysts (Madore *et al*, 1987; Ongerth *et al*, 1988; Rose *et al*, 1988) and this was assessed by monitoring disease in animals, analysis of animal slurry and faeces from areas around the loch and water courses leading to it. The viability of any oocysts detected was assessed.

Oocysts were detected in both raw and final water samples at low concentrations and with generally low occurrence, however, there was a peak in winter. There was little evidence of any increase in cases of cryptosporidiosis above background levels. An increase in cases seen in one area supplied with Lomond water was not associated with the water supply, or any other common source. Most of the oocysts detected were non-viable, although on two occasions viable oocysts were detected in Ross Priory water. These occasions coincided with the detection of large numbers of viable oocysts being excreted by scouring calves on farms bordering two rivers leading to the loch. It may be that oocysts are being excreted, or spread onto land surrounding the loch and its tributaries (possibly during the spring or autumn peaks of disease detected in animals) and that these are eventually washed into the water supply. If the interval between excretion of oocysts and their occurrence and detection in the water supply is lengthy (perhaps a matter of six months) this may explain the winter peak in oocyst detection, the lack of viable oocysts and the fact that no disease was associated with Lomond water, even on occasions when oocysts were detected in the water. The familial spread of cryptosporidiosis in cases which were investigated consolidates the concept that the major spread of cryptosporidiosis is likely to be person to person.

There are several factors which may contribute to a lack of association with disease, despite the finding of oocysts in raw and final water and that cryptosporidiosis was reported in the communities supplied by Loch Lomond water. Individuals/communities may not have ingested oocysts, or a sufficient number of viable oocysts to establish infection. Few of the oocysts

detected in water were viable, and therefore if oocysts were ingested they may have been non-viable. High herd immunity in the community, possibly a result of long term exposure to low numbers of oocysts, may have resulted in reduced susceptibility, leading to a decrease in the clinical signs and symptoms of infection. In addition, the methods available for the detection of oocysts in water and faecal samples may not be sensitive enough to detect all the oocysts entering distribution or to detect infection in the community, especially where the infection is low grade.

Oocysts were detected in sewage effluents on numerous occasions and this is undoubtedly another source of contamination of water supplies in general. While the bacterial faecal indicator organisms used to indicate faecal contamination were commonly detected in raw water at abstraction, there were no bacteriological failures in treated water, either in water of Lomond origin, or other water samples, which proves that these standard indicators are not sufficient to indicate protozoan contamination of water. This may be a consequence of the survival of oocysts when exposed to disinfectants used in water treatment which inactivate other organisms in water.

The majority of oocysts detected in Loch Lomond water, sampled at Ross Priory, were non-viable. The existence of a viability assay (Campbell *et al*, 1992) which could be performed on low numbers of oocysts made the assessment of the viability of waterborne oocysts possible. In previous work, oocyst viability could only be assessed by the appearance of the oocyst(s), whether intact sporozoites were present, using Nomarski differential contrast microscopy (DIC) on a wet preparation of the sample (LeChevallier *et al*, 1991a; LeChevallier *et al*, 1991b; chapter 3). Furthermore, the number of organisms present in these samples was too few to perform an excystation assay. However, the problem still remained of how to recover sufficient oocysts from the final pellet, which in raw water and sewage effluent was usually fairly turbid, in order to perform a viability assay on them. In addition, when viability assessment was performed and oocysts were detected, the debris present in the sample often obscured the oocysts to the extent that positive assessment of viability could not be made because the inclusion or exclusion of the dyes could not be confirmed.

In order to overcome this problem, an adaptation of immunomagnetic separation (IMS) was employed, using magnetisable beads coated with streptavidin as a solid phase to which a biotinylated monoclonal antibody (MAb) raised against fluorescein isothiocyanate (FITC) could be bound. These were mixed with the sample containing oocysts which had been incubated with the relevant dyes and an anti-*Cryptosporidium* MAb. Separation was performed by placing the tube containing the sample in a magnetic particle concentrator and withdrawing the sample fluid. The sample was resuspended in a small volume of clean water and was then

analysed. This provided a successful method for separating oocysts from environmental samples and assessing their viability. Previously, assessing the viability of the low numbers of oocysts detected in water samples was time consuming, painstaking work and often no organisms were detected. On occasions, contaminating debris obscured the oocysts to the extent that the viability could not be stated. The use of this magnetic separation technique reduced this problem considerably. This enabled concentration of the sample to a smaller final volume and allowed viability of the whole sample to be assessed within a reasonable period of time. Previously 10 to 20 % of the sample was analysed in the same time taken to analyse the whole sample following IMS.

However, further work remains to be undertaken in order to optimise this technique. The data indicated that a large number of beads were required to concentrate small numbers of oocysts, one monoclonal was better than the other tested and that recovery was affected by the turbidity of the sample. Recently it has been observed that particles present in sewage samples have magnetic properties and are attracted to the magnet in the MPC, thus concentrating these particles as well as the beads. This reduces the efficiency of the technique for concentrating samples to a smaller volume and removing contaminating debris.

One of the initial considerations for the use of magnetisable particles was to use it as a concentration technique before the final pellet was obtained. For instance, beads coated with streptavidin, biotinylated anti-FITC and the FITC labelled anti-*Cryptosporidium* MAb could be added to the water sample prior to sucrose density flotation (Anon, 1990b)), thus eliminating the necessity for flotation and the subsequent concentration of the sample by centrifugation. However, the initial attempts at these labelling techniques did not provide particularly promising recovery efficiencies. Further work is required in order to titrate the optimum concentration of reagents and establish the best times and methods of mixing and separation.

Recently new products have been marketed for immunomagnetic separation which are colloidal suspensions of iron based particles. These are used on the same principle as the larger Dynal beads used in this study but have certain advantages over them. Being a colloidal suspension alleviates the problem of continuously resuspending the sample because the particles remain in suspension. However, they are expensive and the apparatus required to perform immunomagnetic separation (e.g. the MPC) is also expensive. Cost may negate the use of such techniques unless they can be shown to improve the recovery efficiency of oocysts from samples considerably, or speed up the isolation and concentration method.

Oocysts have been shown to survive the disinfection regimes used in the water industry and concentrations well in excess of acceptable limits (80 - 16,000 mg/l) were required to inactivate

them (Smith *et al*, 1989; Korich *et al*, 1990). As a result a great deal of effort has been directed at testing numerous alternative disinfectants for their oocysticidal properties. Of the many tested, few have shown promise, although agents such as chlorine dioxide (ClO_2) and especially ozone have been demonstrated to have good activity against other organisms which show resistance to disinfection, such as *Giardia* cysts and *Cryptosporidium* oocysts (Wickramanayake *et al*, 1984; Peeters *et al*, 1989; Korich *et al*, 1990; Perrine *et al*, 1990; Labatiuk *et al*, 1991). Work performed at the laboratory scale has indicated that concentrations of ozone (1 - 5 mg/l) within both a physically and an economically attainable limit of ozone production, for standard, to upper limit, contact times (5 - 10 min) were sufficient to render oocysts non-infective to mice (Peeters *et al*, 1989; Korich *et al*, 1990) or rats (Perrine *et al*, 1990) and also to prevent *in vitro* excystation of oocysts. However, this work was performed (by various workers) using oocysts isolated from different hosts, e.g. calves, horses, humans and using different concentrations of oocysts, different methods for the exposure of oocysts to ozone, different methods for assessing the viability of oocysts and under different conditions of pH and temperature, which are two important factors in the treatment of water. These differences in experimental conditions prevent full comparisons being drawn between the work of different groups.

The concentrations of ozone and the contact time required for 2 log inactivation of oocysts found in this study (giving a $C.t'$ range of 10 - 18 mg/l.min) were similar to the range $C.t'$ values (5 - 18 mg/l.min) quoted by other investigators (Peeters *et al*, 1989; Korich *et al*, 1990), although these are higher than the $C.t'$ value quoted by Perrine *et al*, (1990) of 2.6 mg/l.min for the same level of inactivation. However, when the temperature of the water in which the oocysts were exposed to ozone was altered, oocyst survival was affected. At lower temperatures (5°C) oocysts show greater resistance to ozone than at higher temperatures (20° C). In addition, at higher pH (pH 9) oocyst killing was less marked, especially at 5°C where there was very little oocyst inactivation, than at lower pHs (pH 5 and 7). This difference in inactivation of oocysts may be a result of the different oxidants produced during the decay of ozone under different pH conditions, pHs above 7 favouring the production of radicals such as superoxide and hydroxyl radicals and pHs of 7 and below favouring stability of molecular ozone.

Elevated temperatures may have the effect of making the oocyst wall and perhaps the sporozoite membrane more permeable to ozone, or the free radicals produced during its decomposition, thus allowing the nuclear material to be damaged. Temperature plays an important part in the excystation of oocysts and for the uptake of particular vital dyes, both of which are used for the assessment of viability. Water in which oocysts are present and which is likely to be ozonated either for the inactivation of oocysts, or as a routine method of

disinfection, is more likely to be at temperatures around 5°C, especially at the times when oocysts are present, in the winter months. At low temperatures, oocyst inactivation by ozone is less than at 20°C (chapter 6, Perrine *et al*, 1990), suggesting that the efficacy of ozone as a suitable agent for the inactivation of oocysts is less practicable in terms of water treatment. However, ozone would still appear to be the most useful disinfectant for inactivation of oocysts in water.

There are numerous additional factors to consider in the treatment of water with ozone for inactivation of microorganisms. Amongst these is the fact that ozone is unstable, highly reactive and decays rapidly. Numerous by products may be produced by reactions with natural organic and inorganic substances (Glaze, 1987; Singer 1990), some of which promote biological growth in the distribution system and others, such as bromate, which may be hazardous to public health. Water has an ozone demand, which must be satisfied before saturation of the water with ozone, which will have disinfectant or oxidising properties, can occur. The earlier in the water treatment process and the more contaminated the water is, the more ozone will be required to be produced and applied to the water in order to satisfy the ozone demand of the water and to attain a particular residual concentration, because it will have a high ozone demand, than would be necessary to attain the same residual concentration in partially treated water with less contamination and a lower ozone demand.

The method of application of ozone to oocysts was shown to be important at laboratory scale (chapter 6), in terms of continuous production and maintenance of a dose of ozone when compared to production of a residual dose, which then gradually decreases over time. There was significantly greater reduction in the viability of oocysts which were exposed to ozone in a contactor system, where oocysts were in contact with a stream of bubbles of ozone in water which was being constantly circulated and maintained at a particular ozone concentration, compared to the addition of oocysts to a container in which a residual concentration of ozone had been produced and which was allowed to decay over the time course of the experiment. Another point to consider is the point of application of ozone. Water may be ozonated for different reasons, either before or after filtration stages, depending on the major function for which it is required, i.e. removal of colour, odour, pesticides, as an aid to coagultion, or as a final disinfection step. In some situations water may be ozonated early in treatment and late in treatment. Such a regime may damage oocysts during the first ozonation step to a sufficient extent for them to be inactivated at the second ozonation step. Passage of oocysts through sand filters may have abrasive effects, or damaging effects that would make oocysts more susceptible to inactivation by, not only ozone, but also to other disinfectants which have shown reasonable efficacy against oocysts. These include disinfectants such as chlorine dioxide and monochloramine, and even chlorine which has so far been ruled out in its oocysticidal

properties. Further work on a large scale basis, i.e. pilot plants, is required to test these parameters, where point and number of ozone applications can be varied. The effects of the passage of oocysts through both slow and rapid sand filters is important to assess in connection with the effects of disinfectants on oocyst viability.

In order to investigate the possible effects of sand from (slow) sand filters on oocysts, a series of experiments was performed in which oocysts were incubated with sand for varying periods of time, with agitation. Following incubation and agitation with sand, oocysts were exposed to a low concentration of chlorine to assess the effects of disinfection subsequent to contact with sand. Gentle agitation of oocysts mixed with sand had marked effects on oocyst viability within a short period of time (5 min to 90 min) and following this with disinfection with chlorine for 5 min further enhanced the destruction and inactivation of oocysts.

Whilst the shaking of oocysts with sand is probably a more severe treatment than the passage of oocysts through sand filters, it does indicate that collision between oocysts and sand grains is detrimental to oocyst viability. Perhaps passage of oocysts through rapid sand filters or pressure filters would be more likely to cause the abrasive, detrimental effects observed. Subsequent disinfection regimes, such as chlorine, chlorine dioxide, chloramine or ozone, may have much greater effects on oocyst viability than previous disinfection studies have indicated.

Large numbers of oocysts have been detected in backwash water from filters and filters themselves can trap and concentrate oocysts (Rose, 1988; Richardson *et al*, 1991). Cleaning of slow sand filters is accomplished by shaking the sand with water, basically washing it with agitation. Washing rapid sand and pressure filters is by forcing water through the filters in the reverse direction to normal water flow (backwashing). At some water treatment plants, following settlement in lagoons, clarified backwash water is returned to the head of the treatment works. The sludge is normally disposed of on a designated waste disposal site, or on agricultural land. However, when contamination (with oocysts) is suspected, special arrangements must be made for disposal of sludge to a designated waste disposal site (Anon, 1990a). Reusing oocyst-contaminated, clarified backwash water may increase the oocyst concentration on the filter as a result of recycling, providing increased opportunity for oocysts to enter and pass through water treatment processes. The data accrued in this study indicate that it may be possible to accomplish both the large-scale destruction of oocysts and the reuse of clarified backwash water following agitation with sand. This would also ease the problems of disposal of the sludge obtained following filter backwashing.

Although oocysts were shown to be susceptible to inactivation by ozone, the concentration required was considerably higher than that required for most other organisms encountered in

water treatment which require disinfection, for example faecal indicator organisms (Wickramanayake *et al*, 1984; chapter 6). Ozone has much greater oxidising power (Glaze, 1987; Ransome *et al*, 1991) than other disinfectants, such as chlorine to which oocysts are remarkably resistant. Much of the resistance must be a result of the robust oocyst wall which protects the sporozoites within. However, it is highly likely that further resistance is conferred on the organism by superoxide dismutase (SOD), an enzyme which catalyses the dismutation of superoxide ions. SOD has been detected in a wide range of organisms and in all the parasitic protozoa and helminths tested so far (Callahan *et al*, 1988; chapter 8) and in *Cryptosporidium* in this study at levels of 5.44 - 9.27 U SOD/mg protein. Its presence in *Cryptosporidium* is important for its survival in the host (assuming that it is present in the developmental stages of its life cycle which take place within the host) and for the survival of the sporozoites during invasion of the epithelial cells of the host's small intestine The presence of SOD in the environmentally robust oocysts may add to its anti-oxidant defences, increasing its survival in the environment. This is an unfortunate additional problem for the water industry.

Further work on SOD is necessary, to determine its levels in other species of *Cryptosporidium*, isolates of different animal origin and other life cycle stages of the parasite. It may be that SOD is present at considerably higher levels in intracellular stages and may be important in protecting the parasite from oxidants produced as a consequence of the host immune response. Most of the SOD enzymes detected in the parasitic protozoa tested are iron (Fe) containing, although recently copper/zinc (Cu/Zn) and manganese (Mn) containing forms of the enzyme have been detected in some protozoa (Callahan *et al*, 1988). Assessing which form(s) of the enzyme is/are present in the parasite may indicate the origin of the enzyme, whether it is host derived or endogenous. Experimentally, determination of the metal content of the enzyme can be performed by inhibition reactions, using cyanide, H_2O_2 or SDS.

In addition to SOD there are other anti-oxidant enzymes and mechanisms. Determination of catalase and glutathione peroxidase (GSH-px), which scavenge H_2O_2, may lead to further information on the resistance of the parasite to a range of disinfecting and oxidising agents. The enzymes mannitol-1-phosphate dehydrogenase (M1PDH) and mannitol-1-phosphatase (M1Pase) have been detected in the oocysts of a number of *Eimeria* species and in *Cryptosporidium parvum*. These enzymes are part of the mannitol cycle and it is thought that the presence of this pathway may be important for the production and utilisation of energy reserves for the parasites. Mannitol may also act as an osmoregulator, keeping the oocyst rigid until the wall hardens during sporulation, and it may be a component of the sporocyst and/or oocyst wall (Schmatz, 1989).

Mannitol is also a scavenger of oxygen associated free radicals, including hydroxyl (·OH) radicals and its presence in oocysts may confer further protection against oxidant-mediated damage or killing.

9.2. Conclusions.

This study has demonstrated that *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts are widespread in the aquatic environment and shown that water treatment removes some but not all of the organisms. Some of the sources of oocysts have been established and some of the oocysts detected in water and water-related samples were viable. It appears that low numbers of oocysts can be detected in the water supply with no apparent effects on the health of the communities supplied.

A new method using immunomagnetic separation has been developed for the concentration and detection of oocysts in water samples, which shows promise. Low numbers of oocysts can be recovered from water samples and their viability assessed. Other methods of oocyst destruction have been investigated, such as inactivation by ozone and shaking oocysts with sand, followed by chlorination. Ozone shows particular promise as a method for disinfecting water contaminated with oocysts, although a few drawbacks have been detected in the conditions under which it is applied. The method of application of ozone to oocyst contaminated water was shown to be important. Shaking oocyst with sand was shown to destroy oocysts rapidly and subsequent disinfection with low concentration chlorine was shown to increase this destruction and inactivation further.

Finally the presence of the antioxidant enzyme superoxide dismutase (SOD) was demonstrated. This enzyme protects the parasite against oxidant killing by superoxide, an oxygen radical which is also produced during the decay of ozone. Its presence may be important for its survival not only in the environment, but also, importantly, in the host.

9.3. Further work.

Continuous sampling of Lomond water for abstraction should be continued. This would provide an early warning to water authorities in the event of oocysts (possibly at high concentrations) being detected. Further and more intense work should be undertaken on ascertaining the disease status of the animal population and on the farming practices in the southern catchment area, with cooperation from farmers and veterinarians, to form a more comprehensive picture of areas of potential contamination. The unique monitoring situation set up and used for the reporting of cryptosporidiosis in this project should be continued. This

would enable greater use to be made of data obtained from continued sampling. The level of interaction already achieved with the Environmental Health (Scotland) Unit and the Communicable Diseases Surveillance Unit throughout this project in conjunction with the proximity of the departments and personnel should be used to full benefit in continuing surveillance of cryptosporidiosis in the Lomond southern catchment area and in areas supplied with Loch Lomond water. This type of study could be beneficially implicated in other suitable areas.

The immunomagnetic separation technique used to recover oocysts for viability assessment could be improved, and adapted for use earlier in the recovery and isolation of oocysts from water samples. Other particles for immunomagnetic separation are now available, such as Ferrofluids (Scotlab, UK) and Magnetic Controlled Pore Glass (Hoefer, UK) and these should be assessed for their recovery of oocysts from water samples.

Further work is required to assess the effects of ozone on oocyst viability in pilot scale water treatment plants, where the effects of water treatment combined with ozonation can be assessed for the removal and destruction or inactivation of oocysts. Establishing the mode of oocyst inactivation by ozone and which oxygen species has the greatest oocysticidal effect would be of benefit for further work on the inactivation of oocysts in water by ozone.

Media other than sand could be assessed for their destructive effects on oocysts, following the work described in chapter 7. Higher concentrations of chlorine and longer contact times could be evaluated for inactivation of sand damaged oocysts. Disinfectants which have hitherto proved unsuccessful for inactivation of oocysts in water could be assessed for their ability to inactivate oocysts following slow or rapid sand filtration.

Having established ther presence of superoxide dismutase in freeze-thawed fractions of *C. parvum* oocysts, further work should be performed to characterise the protein and discover its metal cofactor. This may be of help in identifying whether the enzyme is of host or parasite origin. Assaying for SOD in pure suspensions of sporozoites would indicate whether the enzyme is present in the sporozoites, or if there is any association with the oocyst wall.

Further work should be performed to analyse *C. parvum* for other antioxidant enzymes, such as catalase and glutathione peroxidase. The importance of the mannitol cycle to *Cryptosporidium* oocysts should be elucidated.

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