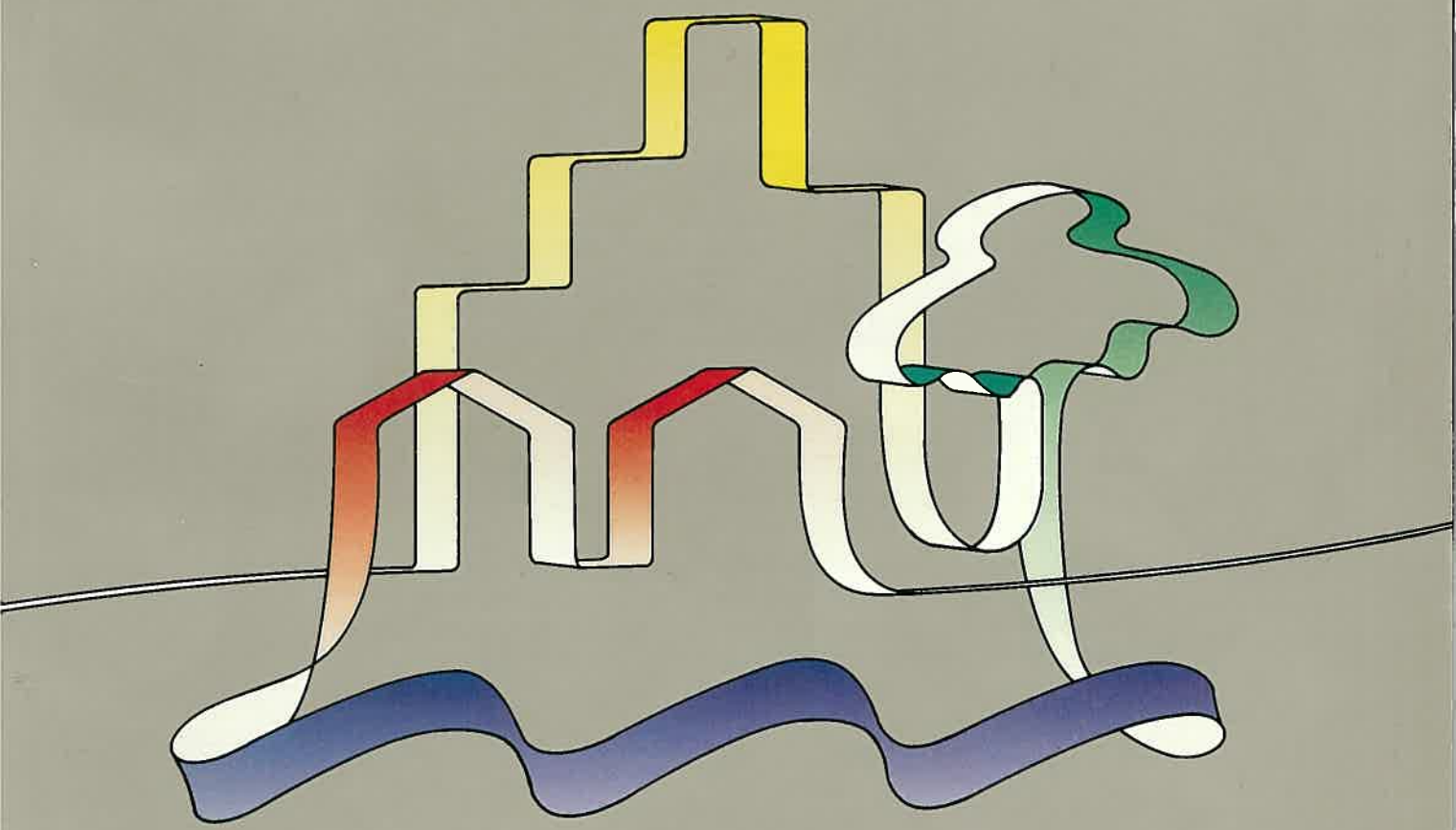
Urban Water Research Association of Australia

Methods for Detection of *Giardia* and *Cryptosporidium* in Water: A Preliminary Assessment



Research Report No. 25

URBAN WATER RESEARCH ASSOCIATION OF AUSTRALIA

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Methods for Detection of *Giardia* and *Cryptosporidium* in Water : A Preliminary Assessment

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Australian Centre for Water Treatment
and Water Quality Research

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FOREWORD

This report is based on UWRAA Research Project No WS-13: 'Development of methods for detecting *Giarda* and *Cryptosporidium* in water' which was undertaken during the period August 1988 - February 1991. Organisational responsibility for the project was as follows:

Sponsoring Authority	:	Engineering and Water Supply Department, South Australia
Project Officer	:	Mr B S Robinson Engineering and Water Supply Department
Research Agency	:	Australian Centre for Water Treatment and Water Quality Research, Adelaide, South Australia
Principal Researcher	:	Ms C A Bee, Australian Centre for Water Treatment and Water Quality Research

The project was funded by the Urban Water Research Association of Australia, the Australian Centre for Water Treatment and Water Quality Research, and by the Engineering and Water Supply Department.

SYNOPSIS

Giardia and *Cryptosporidium* are significant causes of human gastroenteritis that can be transmitted by several routes. Water-related outbreaks of giardiasis and cryptosporidiosis are known from North America and Britain, and have involved from fewer than ten to several thousand infections.

Recognition of infections by *Giardia* and *Cryptosporidium* is increasing throughout Australia. While there is little evidence yet of transmission by water, there are strong perceptions among the public and the medical and laboratory community that water is a source of infection, particularly by *Giardia*. Methods for detecting these organisms in water would permit more specific investigation of disease outbreaks as well as prospective studies.

Detecting *Giardia* and *Cryptosporidium* in water is essentially a three-stage process, involving **primary concentration** to reduce a large sample to a volume that can be handled easily in the laboratory, **secondary concentration and separation**, and **microscopic examination**, usually facilitated by a stain. Current methods for detecting *Giardia* and *Cryptosporidium* in water are time-consuming and give poor recovery or are poorly reproducible. There appears to be scope for simplifying the initial concentration step, particularly in the most widely-used method for concentrating large volumes in the field (the "Reference Method").

This study concentrated initially on a thorough examination of the secondary concentration and microscopic steps. The **secondary concentration and separation** step has been simplified significantly. Centrifugation in a sucrose density gradient permitted recoveries of *Giardia* or *Cryptosporidium* exceeding 90%, while segregating mineral material and much of the organic material into separate fractions. Recovery of these organisms in practice will depend on the number and sizes of other organisms present in a sample. Use of monoclonal antibodies and fluorescent microscopy for **microscopic examination**, in conjunction with n-propyl gallate to stabilise the fluorochrome fluorescein isothiocyanate (FITC), improves the recognition of *Giardia* and *Cryptosporidium* significantly.

The secondary concentration and microscopic steps were used to make a preliminary assessment of crossflow microfiltration as an alternative method for **primary concentration**. The important variables affecting recovery of *Giardia* and *Cryptosporidium* have been identified, but further trials are needed to establish the optimum conditions for this process.

Further study will concentrate on the reproducibility of primary concentration, which is essential to quantitative analysis, and on field investigations in areas with potential sources of contamination by *Giardia* and *Cryptosporidium*.

CONTENTS

INTRODUCTION

Giardia and *Cryptosporidium* are enteric protozoan parasites which can cause moderate to severe gastroenteritis in humans: the diseases called giardiasis and cryptosporidiosis respectively. The organisms can be transmitted by various mechanisms that maintain a faecal-oral cycle. Waterborne outbreaks of giardiasis were first reported in North America around 1970, while transmission of *Cryptosporidium* by water has only been recognised in the 1980s. The incidence of both infections appears to be increasing in many parts of Australia. At present, no Australian laboratory conducts analyses of water for *Giardia* or *Cryptosporidium*, except on a very *ad hoc* basis.

If the role of water in the transmission of *Giardia* and *Cryptosporidium* in Australia is to be understood, it is important that laboratory services be available to investigate contamination of water directly, in epidemiological or prospective studies.

This report presents research conducted at the South Australian State Water Laboratory, on methods for detecting *Giardia* and *Cryptosporidium* in water. The project has been funded partly by the Urban Water Research Association of Australia, partly by Australian Centre for Water Treatment and Water Quality Research and partly by the Engineering and Water Supply Department of South Australia.

OBJECTIVES

The objectives of this study were to review the published methods for detecting *Giardia* and *Cryptosporidium* in water and to develop and calibrate methods using materials and equipment available in Australia. One important aim was to develop a common method for *Giardia* and *Cryptosporidium*, or methods with as many common steps as possible. This would increase the value of field investigations, particularly prospective studies when either organism might be present.

The intended outcome of the project is a method handbook suitable for use by water-microbiology or clinical laboratories. To this end, a survey of potential users of analyses for *Giardia* and *Cryptosporidium* was conducted, to assist in the design of the study and handbook.

Part of the method development (assessment of primary concentration) is incomplete and will continue at the State Water Laboratory, along with application of the methods to specific water quality problems.

REVIEW

Giardia, *Cryptosporidium* and Human Infection

Giardia has been known as a human parasite for 200 years, but has been regarded seriously as an agent of disease only since the 1960s (Craun, 1979). Until recently, *Cryptosporidium* was best known as a parasite of pasture animals, but is now regarded as an important human pathogen, particularly in immuno-compromised patients (Current, 1987).

The active stage of *Giardia* is a flagellate which attaches to the intestinal wall by an adhesive disc, where it may inhibit absorption of nutrients and cause gut peristalsis that results in diarrhoea. On the other hand, many human carriers of *Giardia* lack any symptoms of gastroenteritis. The infectious stage (a cyst) is shed in faeces, intermittently but in large numbers. *Cryptosporidium* has a more complex life cycle with intracellular development in the gut wall and sexual and asexual reproduction. Thick-walled oocysts, passed in faeces, are responsible for transmission.

In principle, both giardiasis and cryptosporidiosis can be spread by any mechanism that maintains a faecal-oral cycle: direct person-to-person contact, contaminated food, contaminated drinking water, or zoonosis (transmission from an infected animal). In practice, the most common identified mechanisms of infection are direct contact, particularly in communal groups of various kinds (Harley, 1988), and drinking water (see following section). Food has only occasionally been identified as a source of infection by *Giardia* (Harley, 1988; Peterson et al., 1988).

One important aspect of the biology of these parasites that influences transmission is **host range**. As well as infecting humans, *Giardia* is common in domestic cats and dogs (Lewis, 1988). In North America, it has been detected in many animals that may occupy protected water catchments, including aquatic mammals such as beavers and muskrats (Wallis et al., 1986). *Cryptosporidium* may infect domestic pets as well as pasture animals (Current, 1987). It is usually assumed that animals infected by either parasite are a potential source of direct or indirect infection for humans.

There is strong evidence of diversity within *Giardia*, with morphological differences among parasites from different hosts. Recent genetic evidence suggests that *Giardia* isolates from human infections belong to several species (Andrews et al., 1989). Despite this diversity, cross-infectivity has been demonstrated for some *Giardia* isolates from humans and a range of animal hosts (Erlandsen et al., 1988).

A large number of *Cryptosporidium* species have been described in the past, based largely on assumptions about host specificity. The present view is that the *Cryptosporidium* infecting mammals belong to only two species (Current, 1987). Transmission of *Cryptosporidium* to humans from animals has been demonstrated or inferred (usually calves but possibly domestic pets: Reif et al., 1989; Soave and Johnson, 1988).

The size of the infective, resistant stages of *Giardia* and *Cryptosporidium* has implications for the effectiveness of treatment processes in removing them from contaminated water. The maximum dimension of *Giardia* cysts is about 8 to 18 μm and of *Cryptosporidium* oocysts, about 4 to 6 μm.

Significance in Water

Waterborne outbreaks of giardiasis are known from the USA (reviewed by St Louis, 1988), Canada (Wallis et al., 1986) and Great Britain (Jephcott et al., 1986). In North America, the early outbreaks were recognised by epidemiological methods: statistical association of cases with particular water sources and correlation with the quantity of water consumed (Witherell and Herbert, 1976).

The first successful attempts to detect *Giardia* in water were made during investigation of an outbreak of giardiasis in Rome, New York in 1975 which affected approximately 5000 people. The Centres for Disease Control (CDC) concentrated samples totalling more than 10^6 litres (discussed in **PUBLISHED METHODS**, below). Part of the concentrate was fed to *Giardia*-free puppies, which became infected; part was examined directly. One *Giardia* cyst was recognised during microscopic examination (Shaw et al., 1977).

In the United States, more than 90 outbreaks of giardiasis linked to drinking water had been recognised by 1984 (Craun, 1988), involving from fewer than 10 to hundreds of infections. The outbreak in Rome, New York, remains the largest. With improved laboratory methods, *Giardia* has been detected in water sources, reticulated water or wastewater in a number of studies, often in the absence of faecal indicators (Kirner et al. 1978; Lippy, 1978). Water supplies implicated in outbreaks have often used apparently "pristine" sources, with no treatment other than disinfection, or have experienced "treatment deficiencies", such as poor filtration, undetected in bacteriological monitoring. Giardiasis outbreaks have also been attributed to contaminated swimming pools and a water-slide (St Louis, 1988).

The search for sources of contamination has concentrated on point-sources of human waste and on possible animal reservoirs of *Giardia*. A strong theme in the North American literature is the role of aquatic mammals, such as beavers and muskrats, in the contamination of streams and water storages (Dykes et al. 1980; Navin et al., 1985). Control strategies have included removing infected beavers. However, a minority of authors have argued that the presence of infected animals in a catchment may be coincidental (since identity of parasites collected from animals with those from humans or from water cannot be demonstrated), or may reflect exposure to common sources of *Giardia* (Bemrick and Erlandsen, 1988). Certainly, animal sources cannot be assumed for all *Giardia* detected in water.

The persistence and viability of *Giardia* cysts in water will be important, particularly in assessing the significance of intermittent contamination. In a careful study of cysts suspended in lake and river water *in situ*, DeRegnier et al. (1989) showed that survival was determined predominantly by water temperature. A high percentage of cysts survived for 30 days or longer at or below 5°C, but lost viability more rapidly at higher

temperatures. Other studies report that a proportion of *Giardia* cysts can survive in water for months (Meyer and Jarroll, 1980).

The first water-related outbreak of cryptosporidiosis was recognised in the USA in 1984, and involved distribution of water from a contaminated well (bore) in Texas (D'Antonio, 1985). Methods for detecting *Cryptosporidium* in water had not been developed and the source of infection was inferred from a formal epidemiological study. In a second outbreak studied in the same way, swimming in and drinking untreated surface water were implicated (Rose, 1988). In later outbreaks in the USA and Britain involving public water supplies, *Cryptosporidium* was detected in water samples, often in the absence of faecal coliforms (Hayes et al., 1989; Smith et al., 1989). The largest outbreak in the USA involved an estimated 13000 cases; the largest in Britain, about 550 cases. An outbreak in Britain (over 70 cases) was attributed to a contaminated swimming pool (Galbraith, 1989). Owing to its small size, *Cryptosporidium* is more likely than *Giardia* to penetrate filter systems of various kinds.

Several estimates of the density of *Giardia* and *Cryptosporidium* in the environment have been published. Sykora et al. (1988) reported *Giardia* densities up to 400 L^{-1} in sewage and up to 13 L^{-1} in wastewater effluents. Ongerth (1989) detected *Giardia* cysts in river water at densities between 0.1 and 5.2 L^{-1}. Madore et al. (1987) reported *Cryptosporidium* densities as high as 1.4×10^4 L^{-1} in raw sewage and 2.6×10^3 L^{-1} in effluents from activated sludge treatment. Densities in surface water varied from below 1.0 L^{-1} to 5.8×10^3 L^{-1}. Ongerth and Stibbs (1987) detected *Cryptosporidium* in water from relatively pristine rivers, at densities between 2 and 110 L^{-1}. Among the variables that influence recovery of *Giardia* and *Cryptosporidium* are particulates, including clay and algae, collected during concentration of the sample (Rose et al., 1989).

In North America, much of the current research on *Giardia* and *Cryptosporidium* is being directed toward assessing the efficiency of water treatment and disinfection processes (e.g. Ongerth et al., 1989).

Significance in Australia

Giardia has been recognised as a common enteric parasite in Australian children, at least since the late 1950s (Court and Stanton, 1959). Increasing recognition of the importance of *Giardia* as a pathogen culminated in a conference-workshop in Canberra in 1989, the proceedings of which summarise the present knowledge of its significance in Australia (Wade and Yapp, 1989).

The overall incidence of *Giardia* infections in Australia is hard to judge, since the disease is not universally notifiable. Most Australian studies have examined selected subjects (either a particular age or community group, or symptomatic patients). In one study which examined households at random (Boreham & Phillips, 1986), the average carriage rate was 5%, with one to five-year-olds the most frequently infected age group (12%). The literature as a whole suggests that *Giardia* infections may be more prevalent in rural than in urban communities, and particularly common among Aboriginal groups.

During the period that giardiasis has been notifiable in South Australia (1980-88), the number of notifications has increased steadily, reflecting either its incidence or recognition. Cases that occur as outbreaks most often involve close communal groups in which hygiene is difficult to supervise, such as day-care centres (Cameron, 1989). As many as 20% of children in such environments may carry *Giardia* without symptoms (Grimmond et al., 1988). There is also a significant level of infection endemic among children and adults in the wider community, for which sources of infection are difficult to identify.

Two epidemiological studies have included a statistical analysis aimed at testing hypotheses about the role of water in transmission of *Giardia* in Australia. A study in Mt Isa, Queensland, showed no correlation of infection with the source of drinking water, whether or not water was treated in the home, or with recreational contact with water perceived as a source of infection (Boreham and Phillips, 1986). Cameron (1989) analysed the incidence of giardiasis in Adelaide, in separate areas defined by the source of domestic water. In this large sample, there were no significant differences among five areas receiving filtered or unfiltered water, from local catchments or from the River Murray. A study in New South Wales also revealed no link with drinking water, despite perceptions by the community and general practitioners (Walker et al., 1986).

Analysis of *Giardia* infections in Logan, southern Queensland, showed a significantly higher incidence in children from a community reliant on septic tanks for waste disposal than in children from a neighbouring sewered community (Boreham et al., 1981). Seepage and overflow were common problems, suggesting that contact with septic tank waste or contaminated soil contributed to infection.

There is less data on the incidence of *Cryptosporidium* infections in Australia. Cruikshank et al. (1988) reported the prevalence of *Cryptosporidium* among clinical specimens examined by a single laboratory over 12 months. This organism was the third most common enteric pathogen, after Rotavirus and *Giardia*. Twelve cases were associated with an outbreak in a day-care nursery in Townsville, Queensland, presumably spread person-to-person. The remaining patients were individual cases, with no readily identified source of infection.

PUBLISHED METHODS

Detecting *Giardia* and *Cryptosporidium* in water is essentially a three-stage process, involving **primary concentration** to reduce a large sample to a volume that can be handled easily in the laboratory, **secondary concentration and separation**, and **microscopic examination**, usually facilitated by a stain.

Published methods have been based on techniques for detecting *Giardia* and *Cryptosporidium* in clinical specimens. The primary concentrate is treated essentially as faecal material, using sedimentation and flotation steps to reduce the volume further and to remove material that may interfere with recognition of the organisms.

Improvements in clinical techniques are therefore readily included in methods for water.

The first successful attempt to detect *Giardia* in water (Rome, New York) used a bulky, sand-filled swimming pool filter for primary concentration (Shaw et al., 1977). In subsequent investigations, this was replaced by a more portable sampling process developed by the Health Effects Research Laboratory (USEPA). This process, widely known as the "Reference Method", uses yarn-wound filters with non-fraying orlon or polypropylene yarn (Jakubowski, 1985). Further variations to methods for water have mostly involved this primary concentration step. Filtration processes tested have included cartridges containing a pleated, acrylic copolymer filter (Hausler et al., 1984), flat polycarbonate membranes (Wallis and Buchanan-Mappin, 1985; Ongerth, 1989) and a "cassette" system that distributed the sample to a parallel stack of membranes by tangential flow (Isaac-Renton et al., 1986).

Recovery rates for *Giardia* have been reported as ranges, means or medians, varying from below 1% to 15% for yarn-wound filters; 31% for tangential-flow filtration, and 22% to 53% for flat polycarbonate membranes. Direct comparisons suggest that recovery of cysts from yarn-wound filters is the most difficult and least efficient (Isaac-Renton et al., 1986; Ongerth, 1989).

The first detections of *Cryptosporidium* in water or wastewater were reported in 1986 (Rose et al., 1986). Spun polypropylene yarn filters (Madore et al., 1987; Musial et al., 1987; Rose et al., 1989) or flat polycarbonate membranes (Ongerth and Stibbs, 1987) have been used for the primary concentration step. Recovery rates between 5% and 20% have been reported.

The secondary concentration/separation steps have also been varied by some authors, with flotation techniques using zinc sulphate, formalin-ethyl acetate or potassium citrate. The length of time that cysts or oocysts have been in the water influences their recognition by fluorescent microscopy (Rose et al., 1989). It is not clear how this is related to their viability.

The shortcomings of the methods available for *Giardia* were summarised by Jakubowski (1985). They included the long time involved in processing each sample, the experience required for identifying cysts, the lack of information about the viability of *Giardia* detected and the unreliability of any conclusions about density of the organisms. Most of these difficulties still exist and apply equally to *Cryptosporidium*. However, monoclonal antibodies have now replaced less specific staining materials, making the microscopic examination less reliant on specific experience with identification of parasites (Sauch, 1985). Some recently developed monoclonal antibodies are reported to distinguish between *Giardia* and *Cryptosporidium* from man and those from some animals (Rose et al., 1989). Application of fluorescein diacetate (Schupp and Erlandsen, 1987) promises to provide sound estimates of viability.

APPROACH TO THE PRESENT STUDY

The three stages in analysis of a water sample (see preceding section) were examined separately. A "back-to-front" approach was used, as it was necessary to optimise the **microscopic examination** in order to test **secondary concentration and separation**, and it was important to know the recovery rates of the secondary stage to assess the **primary concentration** stage. A prime consideration in the method development is to try to simplify the primary concentration step, which seems very cumbersome in the published methods. A preliminary examination was made of crossflow microfiltration, which promises to be less time-consuming. Continuous centrifugation is another option for smaller samples which has not yet been assessed. This part of the project is incomplete, and comparison with the published methods is continuing.

MATERIALS AND METHODS

Sources of *Giardia* and *Cryptosporidium*

For testing the laboratory methods, *Giardia* cysts from patients with gastroenteritis were obtained from Dr G. Mayrhofer (University of Adelaide, Department of Microbiology). *Cryptosporidium* oocysts from calves were obtained from Mr R. Lumm (Institute of Medical and Veterinary Science, Adelaide).

Solutions

Phosphate Buffered Saline (PBS), 0.01M, pH 7.5
(sachets from Meridian Diagnostics, made up to 500mL).

Stabilising solution for the fluorochrome fluorescein isothiocyanate (FITC):
n-propyl gallate, 0.1% in glycerol.

Sucrose for density gradients:

1.5M = 51.35g/100mL (Relative density, $\rho_{20} = 1.2$).
3.0M = 102.69g/100mL ($\rho_{20} = 1.38$).

Stored tightly sealed. Replacement of 3.0M solution may be necessary if it begins to crystalize.

Fluorescent-labelled antibodies

Materials from MERIFLUOR-GIARDIA and MERIFLUOR-CRYPTOSPORIDIUM kits (kits for clinical diagnosis from Meridian Diagnostics, Cincinnati, USA), or from Meridian HYDROFLUOR-Combo kit ("environmental kit" intended for tests for both organisms).

The kits contain **Primary Antibody** and **Fluorescent Monoclonal Antibody**, linked to the fluorochrome fluorescein isothiocyanate (FITC) and sachets of PBS. The clinical kits also contain precoated microscope slides which are not used in these tests. Similar materials may be available from other manufacturers.

NOTE: These materials are very heat-sensitive. Heat damage during transport can result in complete failure of labelling. Immediately on receipt of a new batch, a positive control was prepared to test its integrity (see **APPENDIX 1, Stage 3**).

Primary Concentration

A preliminary assessment was made of crossflow microfiltration, during limited access to units from two suppliers: Memtec (Memcor Model 1MSLX) and Membrane Filtration Industries Limited (Crossflow Microfiltration System).

Secondary Concentration

Clements 2000 bench centrifuge, with swing-out buckets to hold 50mL tubes.

50mL graduated polypropylene centrifuge tubes (Disposable Products 24222S).

Microscopy

An Olympus BH-2 microscope with accessories for epi-fluorescence was used in this study. Filters required for fluorescence of FITC were:

1] Barrier Filter 0515
2] Exciter filter EY455
3] Exciter filter FITC 490 (IF-490)

with the exciter filters mounted in the G-B filter bank.

Procedures

The procedures used for concentrating, separating and visualising *Giardia* and *Cryptosporidium* are presented as a flow diagram in Figure 1. Detailed laboratory protocols for **secondary concentration and separation** and **microscopic examination** appear in Appendix 1. Procedures for primary concentration are still regarded as tentative.

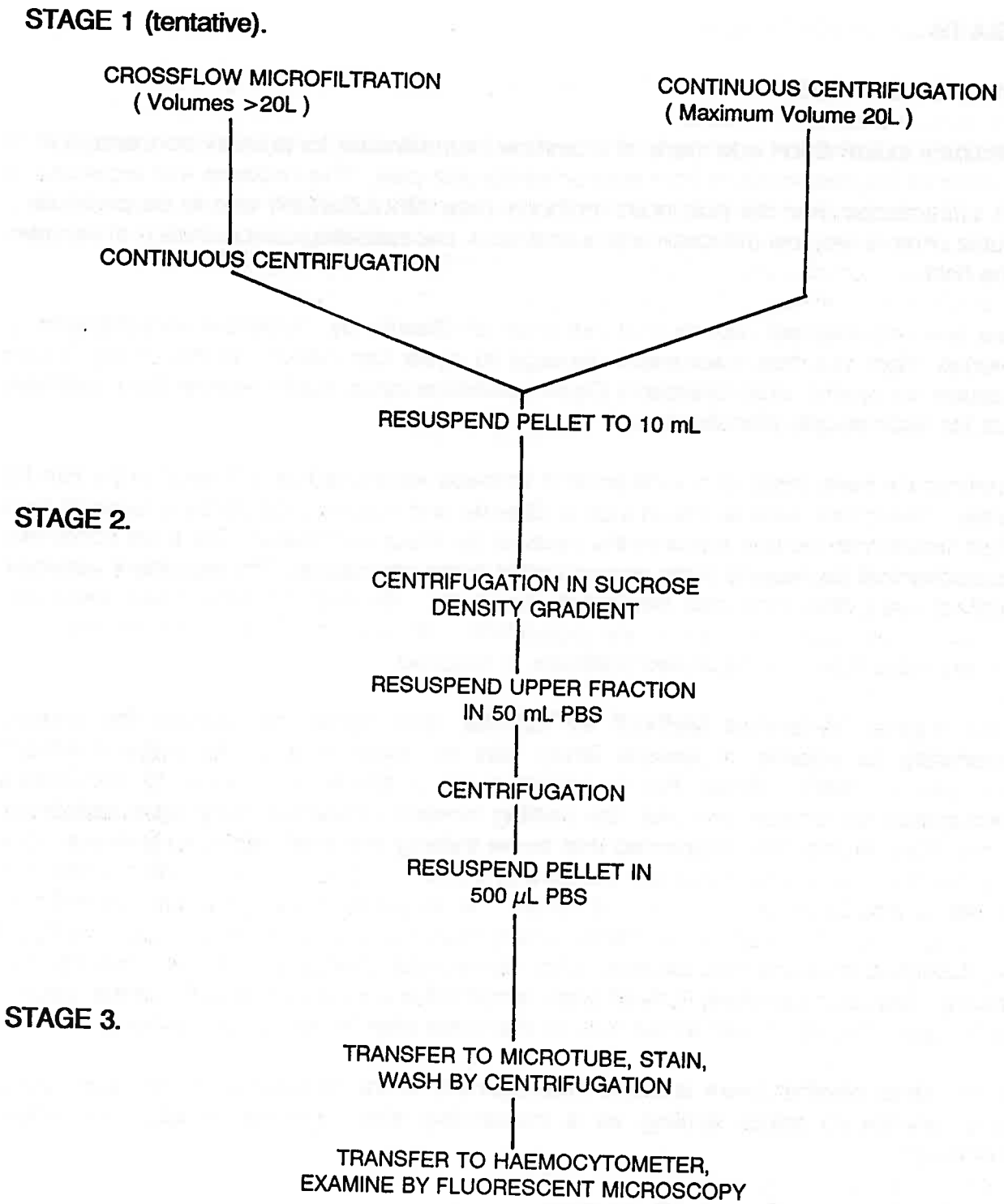

Figure 1. Flow Chart of Steps in the Detection of *Giardia* and *Cryptosporidium* in Water.

RESULTS

Primary Concentration

Preliminary examination was made of crossflow microfiltration for primary concentration of *Giardia* and *Cryptosporidium* from environmental samples. This process was expected to have advantages over the published methods (see **DISCUSSION**) and to be particularly suitable when a very low detection limit is desirable, necessitating concentration of samples in the field.

There are unpublished reports that retention of *Giardia* by crossflow microfiltration is extremely high, but that mechanical damage to cysts can occur. In this study, it was important not only to retain *Giardia* or *Cryptosporidium* cysts, but to recover them relatively intact for microscopic identification.

In preliminary trials, cysts of a vahlkampfiid amoeba were used as a "proxy" organism for *Giardia*. The cysts were similar in size to *Giardia* and recovery could be assessed by a plaque count method that requires the cysts to be intact and viable. The trials confirmed that mechanical damage to cysts occurs under some conditions. The important variables identified were flow rate and backwash pressure. By manipulating these variables, recovery of 56% was achieved in one experiment with *Giardia*. Further comparison with recovery rates from the published methods is required.

In the original "Reference Method" for *Giardia*, one option for treating the primary concentrate (a volume of several litres) was to allow material to settle overnight (Jakubowski, 1985). Since this is attractive as a simple alternative to continuous centrifugation for smaller samples, the settling kinetics of *Giardia* cysts were examined. A preliminary experiment suggested that some settling occurred within 60 minutes. In a more careful experiment, a cylinder containing a 330 mm column of deionised water was allowed to equilibrate to 20°C for 16 hours. A suspension of cysts was added and dispersed using a magnetic stirrer (final density 1400 mL^{-1}). After mixing ceased, samples were collected hourly at two depths. After some initial changes in density, settling was relatively slow: approximately 70% of cysts remained in the upper half of the water column after 5 hours (Figure 2) and about 10% of the cysts after 90 hours (not shown).

It is not clear whether there is some heterogeneity in the buoyancy of the cysts, but it seems unwise to adopt settling as a processing step until the kinetics are better understood.

Secondary Concentration and Separation

The primary concentrate from a field sample is certain to include particulate material of a mineral and organic nature that may obscure *Giardia* or *Cryptosporidium* cysts, or confound their identification. The purpose of the secondary step is to concentrate the sample further to a volume that can be examined under the microscope and to separate *Giardia* and *Cryptosporidium* from material that would interfere with recognition and counting. The most effective method for separation of cells and other particles, used in

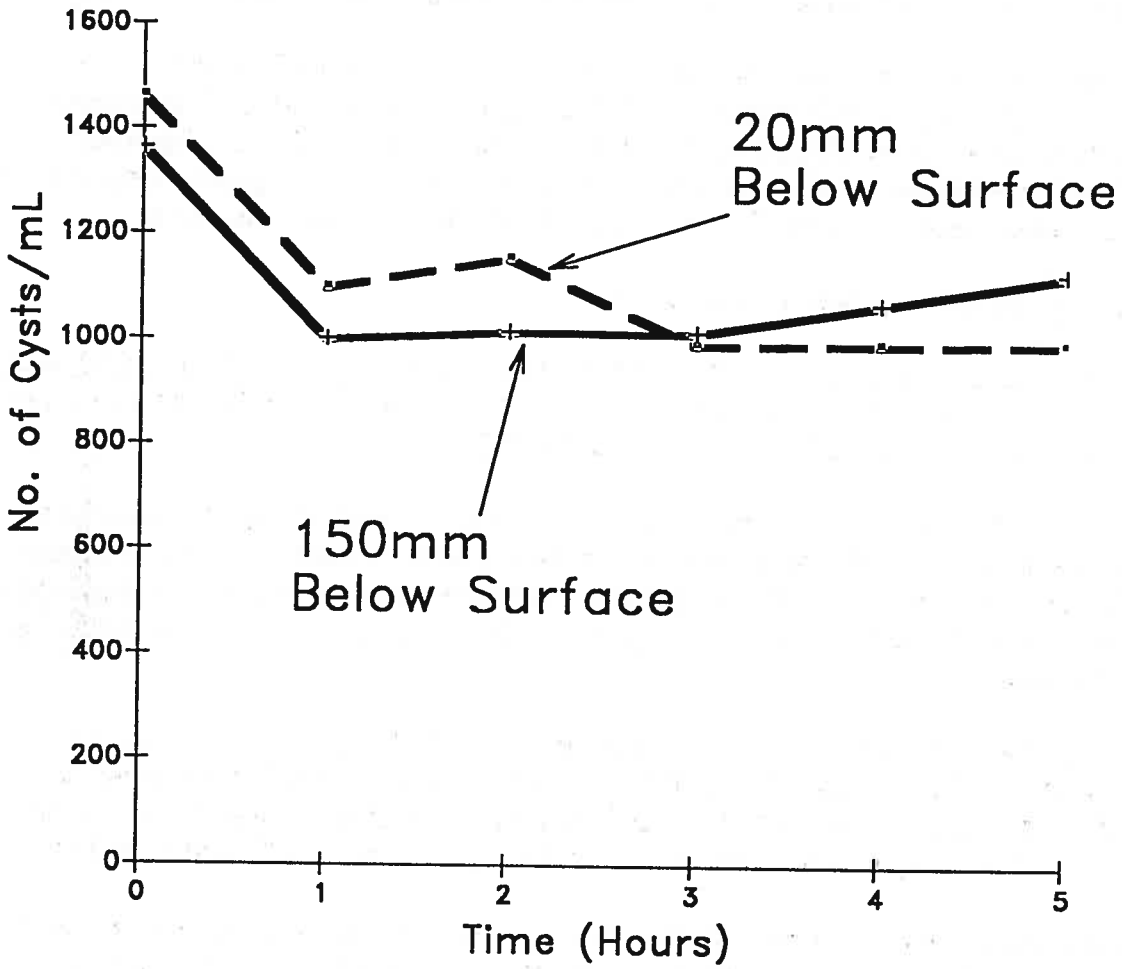

Figure 2. Natural Sedimentation of *Giardia* Cysts in Water (undisturbed at constant 20oC)

examination of clinical specimens for parasites, is centrifugation in a medium of precise relative density (ρ_{20}), or a gradient of relative density.

A comparison was made between the recovery of *Cryptosporidium* in discontinuous gradients of $ZnSO_4$ and sucrose. The density interface, ρ_{20} 1.2/1.38, was based on the density of *Cryptosporidium* oocysts (ρ_{20} approximately 1.2). In the sucrose gradient, the *Cryptosporidium* remained above the interface, predominantly in the uppermost fraction (Figure 3), accounting for 91% of the original oocysts. In the $ZnSO_4$ gradient, the *Cryptosporidium* did not segregate as predicted from their relative density, but collected in the bottom fraction, apparently through co-sedimentation with micro-crystals of ZnSO4. Counting all fractions accounted for only 63% of oocysts (Figure 3); it appears that a significant proportion were obscured by the crystals, which had a mean length of 60 μm.

In further experiments with sucrose gradients, *Cryptosporidium* were dispersed more evenly in the ρ_{20} = 1.2 fractions. Recoveries of *Giardia* and *Cryptosporidium* were generally above 90% (Figure 4). In experiments with natural water "spiked" with *Giardia* or *Cryptosporidium*, mineral particles and much of the other organic material sedimented to the lower fractions, providing partial purification of the parasite cysts.

Staining and Microscopic Examination

The fluorescent-labelled monoclonal antibodies were highly successful in labelling the *Giardia* cysts and *Cryptosporidium* oocysts, highlighting them against background particles that would otherwise obscure them (Figures 5,6).

Staining and washing the cysts in a microcentrifuge tube is an important variation on some published methods. If the staining is performed on a microscope slide, the washing step, which is important in reducing background fluorescence, results in a significant loss of cysts. Recovery trials of the staining and washing steps gave losses below 3% during centrifugation.

Early in the study, there were difficulties with materials which had been delivered during hot weather. The monoclonal antibodies are sensitive to heat during transport or storage, which can result in complete failure of labelling. A practice of preparing a positive control to test the integrity of each new batch was adopted (see **MATERIALS AND METHODS**).

Tests showed that monoclonal antibodies against both *Giardia* and *Cryptosporidium* could be used simultaneously without interference. The lack of size overlap makes the organisms easy to distinguish. This approach would be useful in a prospective study of water contamination, when either organism might be present. A commercial kit for a combined test has recently become available, but has not yet been assessed (see **MATERIALS AND METHODS**).

Adding **n-propyl gallate** increases the stability of the FITC fluorescence, which otherwise bleaches on exposure to UV-excitation, improving recognition of the organisms under any circumstances. The use of this agent is essential for photography and for any use of video Image Analysis (see **DISCUSSION**).

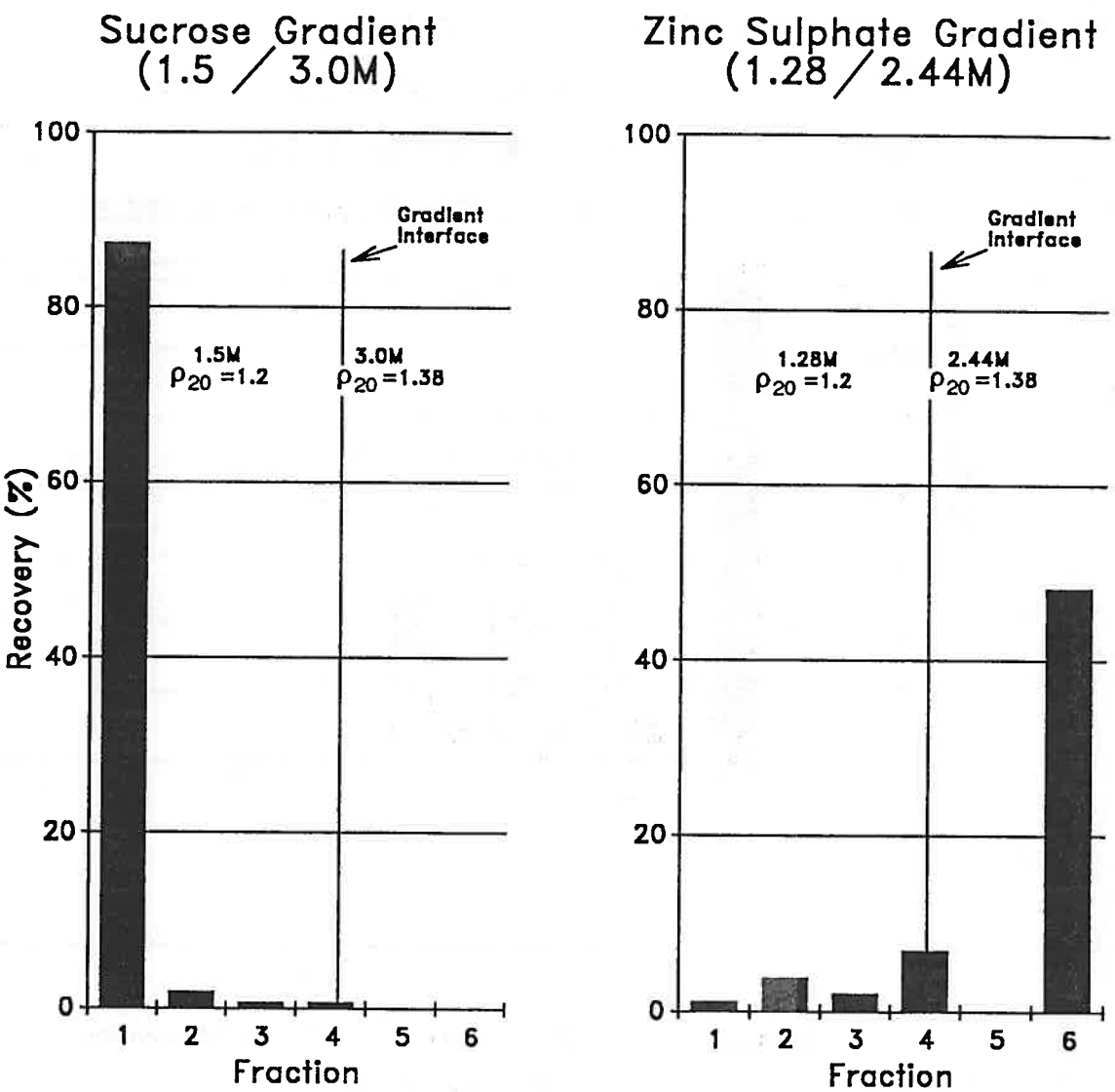

Figure 3. Recovery of *Cryptosporidium* Oocysts from Sucrose and Zinc Sulphate Gradients.

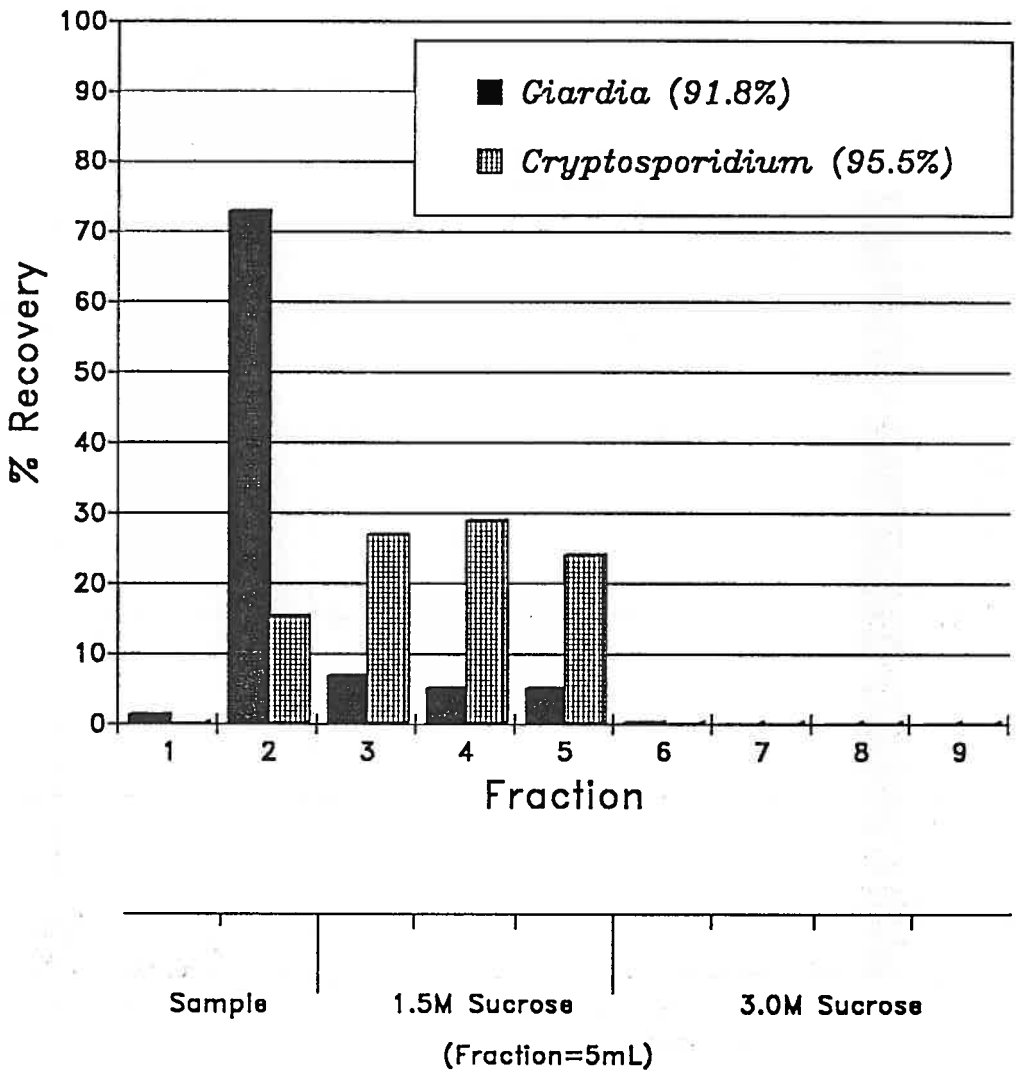

Figure 4. Distribution of *Giardia* and *Cryptosporidium* Cysts after Centrifugation in a Sucrose Density Gradient.

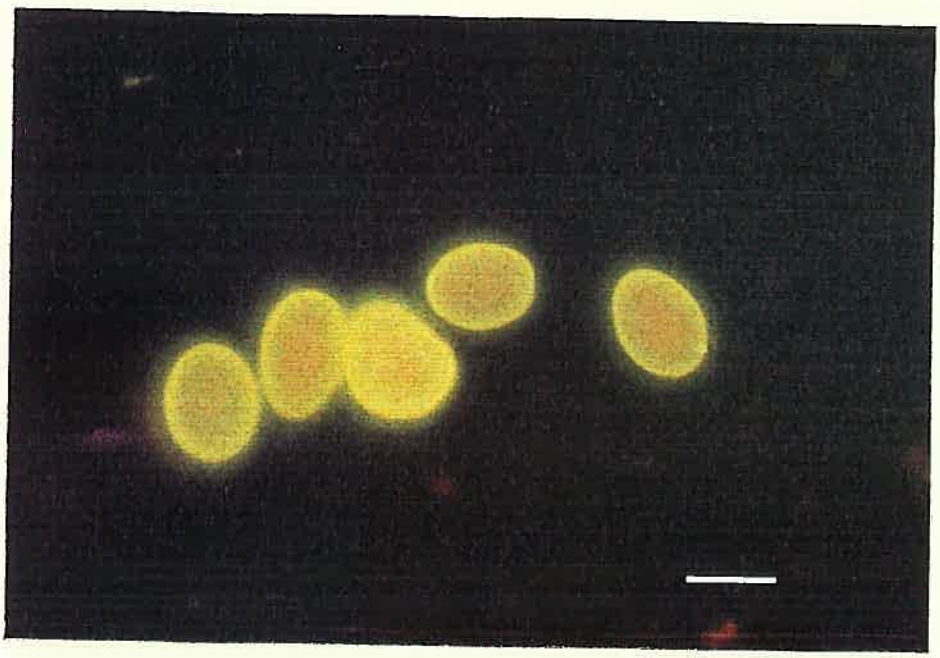

Figure 5. *Giardia* cysts stained using FITC-labelled monoclonal antibodies. Scale = 10 μm.

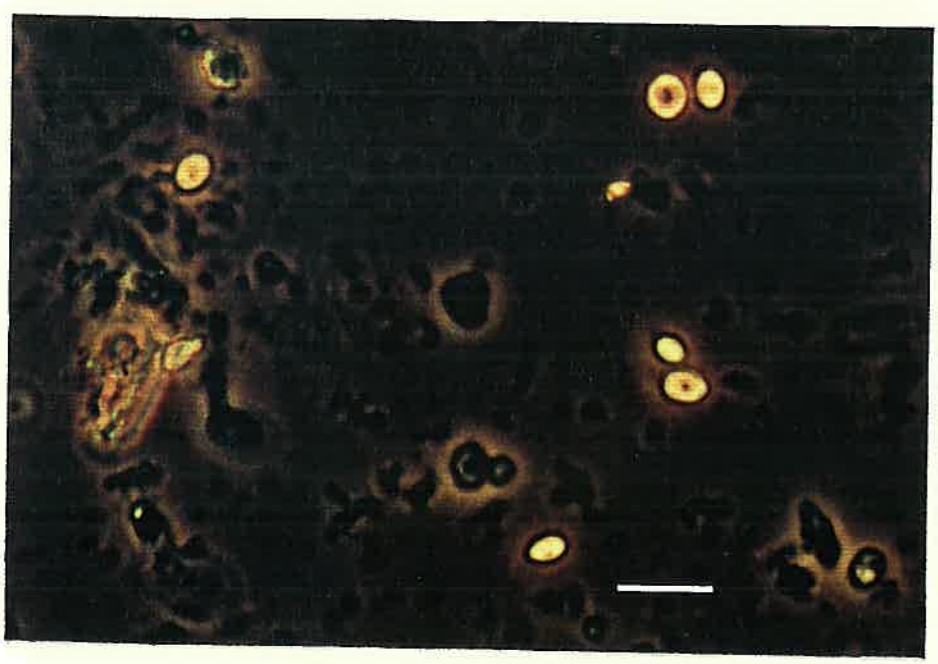

Figure 6. *Cryptosporidium* oocysts, showing the specificity of FITC-labelled monoclonal antibody (unstained organisms and inorganic material illuminated by low-light phase contrast). Scale = 10 μm

SURVEY OF POTENTIAL USERS

Australian interest in *Giardia* and *Cryptosporidium* and perceptions about the role of water in their transmission were canvassed. Questionnaires were circulated to the water industry, to state health authorities and to laboratory organisations that analyse clinical or veterinary specimens, using somewhat different questions for each group. Distribution to the largest group, the clinical laboratories, was facilitated by RCPA Quality Assurance Programs (an agency of the Royal College of Pathologists of Australia), which circulated questionnaires with its newsletter.

There was significant interest in the method development from water laboratories. Most of those that responded had been asked to analyse water samples for *Giardia* in the past; there had been few requests for analysis for *Cryptosporidium*. Health authorities in three States considered that water is a likely source of *Giardia* infections in Australia; only one considered this likely for *Cryptosporidium*.

A significant number of clinical and veterinary laboratories had been asked to examine water or wastewater for *Giardia* or *Cryptosporidium* in the past. Among clinical laboratories (124 responses), 91% were interested in having a method available for detecting *Giardia* in water; 69% were interested in a method for *Cryptosporidium*. Among those that were involved in epidemiological interpretation of the laboratory results, 89% have suspected waterborne transmission in at least some *Giardia* infections, and 57% have suspected water of being involved in transmission of *Cryptosporidium*. A number of veterinary laboratories suspected that water is responsible for some transmission of these organisms between animals.

DISCUSSION

Published investigations of giardiasis in Australia have shown little evidence of transmission by water except perhaps for direct contact with septic tank effluent (Boreham et al., 1981, 1986; Walker et al., 1986; Cameron, 1989). The influence of water temperature on the viability of *Giardia* cysts suggests that any involvement of water in disease transmission may be periodic or localised to cooler areas. However, relatively few incidents have been studied, and the epidemiological methods employed are rather indirect. A method for detecting *Giardia* in water would permit more specific investigation of this infection.

There is little evidence concerning transmission of *Cryptosporidium* in Australia. At present, for example, it would be difficult to determine whether drinking water or exposure to farm wastes contribute to a high rate of human infection in a dairying area, or whether infections should be attributed solely to zoonosis. Since approaches to preventing the infections would be quite different in these cases, a method for detecting *Cryptosporidium* in the environment would be be particularly useful.

Despite the lack of epidemiological evidence, there is a general perception among clinical laboratories in all States that water is involved in some of the infections that they diagnose. This view is more strongly held for *Giardia* than for *Cryptosporidium*, which may partly reflect the strong emphasis of the North American medical literature on waterborne outbreaks of giardiasis and the "newness" of the literature on *Cryptosporidium* in water.

Water authorities do not readily accept that these infections are attributable to water, but their laboratories are likely to find the methods useful, since most receive requests for analysis of water for *Giardia*.

From this study, there appears to be scope for simplifying the primary concentration step in analysing a sample for *Giardia* or *Cryptosporidium* and probably for improving its recovery rate and reproducibility. Two techniques appear to be suitable alternatives to the published methods for this step: crossflow microfiltration for large volumes processed in the field and continuous centrifugation for volumes that can be transported to the laboratory.

Crossflow microfiltration has several potential advantages over yarn-wound and other cartridge filters for large samples that must be concentrated in the field. The physical characteristics of the membrane should provide absolute retention of both *Giardia* and *Cryptosporidium*, which is not possible for example with yarn-wound filters. Backwashing the membrane is a mechanical process, programmed as part of the filtration cycle, which should greatly reduce the labour involved in recovering the concentrate. Among the published methods, the labour at this step is greatest (and most critical to the outcome) for yarn-wound and other cartridge filters. In the two instruments examined in this study, the air-pulse used to backwash the membrane minimises the volume required, although a centrifugation step is still necessary before the concentrate is ready for final preparation.

Retention of *Giardia* cysts by crossflow microfiltration is high, with the reported penetration of the membrane below 1 in 10^4, but optimal conditions for recovery of cysts from the membrane need to be explored more thoroughly.

Continuous centrifugation is a potential alternative to membrane-based methods for samples that can be carried to the laboratory, usually 10 to 20 litres. The detection limit provided by samples of this size is likely to be adequate for many purposes. Recovery of *Giardia* and *Cryptosporidium* from membranes with large surface areas is laborious and the recovery variable (Ongerth, 1989). Experience with the cysts of other protozoans suggested that continuous centrifugation may be simpler and more reliable. The important variables that will be examined as the project proceeds are the relative centrifugal force (RCF) and the "feed rate" for introducing the sample.

In the secondary concentration and separation step, recovery of *Giardia* or *Cryptosporidium* exceeding 90% is possible. The level of recovery may vary depending on the number and sizes of other organisms present in a sample, but the use of monoclonal antibodies and fluorescent microscopy in the final step improves the recognition and discrimination of these organisms significantly.

There are potential advantages in employing Image Analysis in the microscopic examination, but this method has not yet been tested. In particular, simultaneous detection of *Giardia* and *Cryptosporidium* would be facilitated by the ability of Image Analysis to measure randomly-oriented cells automatically, since there is no size overlap between the organisms. The facility to subtract images from each other may also be useful where fluorescent methods are available for distinguishing viable and non-viable organisms (Schupp and Erlandsen, 1987).

The methods being developed in this project are regarded as an investigative tool for prospective studies or investigating disease outbreaks in which water may have played a role. Despite the scope for simplification of the published methods, the time required for individual analyses will make it inappropriate to use these tests for general monitoring of the microbiological quality of water. There has been little discussion of the importance of detection thresholds for *Giardia* or *Cryptosporidium* in recent literature. Provided that recovery rates can be made reproducible, analysis of samples of 10 to 20 litres is likely to be adequate for an assessment of the immediate risk of infection. Larger samples will be useful for other purposes, including the study of seasonal variation in contamination by these organisms and assessment of water treatment processes.

CONCLUSIONS

Giardia and *Cryptosporidium* are significant causes of human gastroenteritis in Australia, where outbreaks or isolated infections are usually interpreted in terms of transmission between individuals. There has been little investigation of the contribution of water to transmission of these organisms, despite strong perceptions that water is a source of infection, at least for *Giardia*.

Detecting *Giardia* and *Cryptosporidium* in water is possible using materials and equipment readily available in Australia. There is scope for simplifying the primary concentration step, which in the published methods is time-consuming and gives variable recovery of the organisms. Crossflow microfiltration is the most promising alternative for large volumes that must be concentrated in the field, but further trials are needed to establish the optimum conditions for this process. Continuous centrifugation is likely to be useful for smaller sample volumes. Comparison with the reported recovery efficiency from large area filters is still required.

Secondary concentration and separation by centrifugation in a sucrose density gradient can recover more than 90% of *Giardia* or *Cryptosporidium* in the primary concentrate, while segregating mineral material and much of the organic material into separate fractions.

Differentiation of *Giardia* and *Cryptosporidium* from other organisms is greatly facilitated by use of monoclonal antibodies and fluorescent microscopy. There are potential applications for Image Analysis to counting, size differentiation and viability tests which should be explored.

Methods for detecting *Giardia* and *Cryptosporidium* would permit direct investigation of contamination of water in epidemiological or prospective studies and should be useful to laboratories throughout Australia.

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APPENDIX 1: PROCEDURES

Stage 1: Primary Concentration

Procedures for primary concentration are regarded as tentative and require further comparison with published methods and calibration of recovery rates.

Stage 2: Secondary Concentration and Separation

Fractionate the primary concentrate using a discontinuous sucrose gradient, 1.5M (ρ_{20} 1.2) to 3.0M (ρ_{20} 1.38), prepared immediately before use[1], as follows. This protocol is for a 50mL tube (volume of concentrate, 10 mL).

1. Dispense 20mL of 3.0M Sucrose into the tube.

2. Carefully layer 15mL of 1.5M Sucrose over the first volume.

3. Layer 10mL of the sample on top of the gradient.

4. Centrifuge for 30 min at 1500g (3000rpm in a Clements 2000 centrifuge).

[1] **Fresh preparation is necessary for sharp gradients.**

For *Giardia*[2], proceed as follows.

5. Remove the upper fraction until the **sample/1.5M interface** is reached (approximately 10mL - see Figure 4) and transfer it to a clean tube. Discard the lower fraction.

6. Add PBS to a total of 50mL and mix gently.

7. Centrifuge for 15mins at 1500g.

8. Remove supernatant until 500μL of fluid remains.

Proceed with staining **(Stage 3)**.

[2] **This procedure assumes that the primary concentrate will be rather "dirty" and sacrifices some recovery for improved separation. The fraction retained contains 70% of *Giardia* cysts. If the primary concentrate seems fairly clean, the larger fraction collected for *Cryptosporidium* (see below) can also be used for *Giardia*, giving approximately 90% recovery from the primary concentrate (see Figure 4).**

For *Cryptosporidium*, proceed as follows.

5. Remove the upper fractions until the **1.5M/3.0M interface** is reached (approximately 25mL - see Figure 4) and transfer it to a clean tube. Discard the lower fraction.

6. Add PBS to a total of 50mL and mix gently.

7. Centrifuge for 15mins at 1500g.

8. Remove supernatant until 500μL of fluid remains.

Proceed with staining **(Stage 3)**.

Stage 3: Detection by Microscopic Examination

Preparation

1. Add 7μL of Primary Antibody (undiluted) to 50-100μL of sample in a microtube (1.5mL).

2. Incubate for 30-40mins in the dark at room temperature.

3. Centrifuge in a microtube centrifuge for 20 sec.

4. Remove supernatant and resuspend pellet to original volume with PBS.

5. Add 7μL of Fluorescent monoclonal antibody (undiluted) to the sample in a microtube (1.5mL).

6. Incubate for 30-40mins in the dark at room temperature.

7. Centrifuge in a microtube centrifuge for 20 sec.

8. Remove supernatant and resuspend pellet to original volume with PBS.

Microscopy

9. Turn the fluorescence lamp on 5 minutes before use.

10. Introduce sample (from 8, above) to a haemocytometer or other counting chamber.

11. Focus on the plane of the slide using phase-contrast microscopy.

12. Select the appropriate filters for FITC fluorescence (see p 10).

13. Scan or count fields as required. *Giardia* cysts or *Cryptosporidium* oocysts show "yellow-green" fluorescence typical of FITC.

NB. Any report of density should take account of the likely precision of the estimate, which will be low when the total number of cells counted is low. There are published procedures for estimating such errors.

To inhibit Photobleaching of FITC;

Add 100μL n-propyl gallate/10mL sample during **Stage 2**

Alternatively, if counting a high-density preparation (e.g. for method calibration), add 1μL n-propyl gallate/100μL sample before the last centrifugation (Step 7).

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