Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts in waters 1999

Methods for the Examination of Waters and Associated Materials

This booklet is based on discussions held at an international workshop on *Cryptosporidium* in January 1997, jointly organised by the Environment Agency and Drinking Water Inspectorate and funded by the Environment Agency.

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About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommend methods of sampling and analysis for determining the quality of drinking water, groundwater, river and seawater, waste water and effluents as well as sewage sludges, sediments and biota. In addition, short reviews of the more important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated, with results from performance tests reported for most parameters. These methods should be capable of establishing within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated individual results encompassing at least ten degrees of freedom from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors), systematic error (bias), total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available. An indication of the status of the method is shown at the front of the publication on whether or not the method has undergone full performance testing.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets in the series 'Methods for the Examination of Waters and Associated Materials' and their continuous revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency. At present, there are nine working groups, each responsible for one section or aspect of water quality analysis. They are:

1.0 General principles of sampling and accuracy of results
2.0 Microbiological methods
3.0 Empirical and physical methods
4.0 Metals and metalloids
5.0 General non-metallic substances
6.0 Organic impurities
7.0 Biological monitoring
8.0 Sewage treatment methods and biodegradability
9.0 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members associated with these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. An index of methods and the more important parameters and topics is available from HMSO (ISBN 0 11 752669 X).

Every effort is made to avoid errors appearing in the published text. If, however, any are found please notify the Secretary.

Dr D Westwood
Secretary

October 1998

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and any regulations made under the Act, and Control of Substances Hazardous to Health Regulations 1988 (SI 1988/1657). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety, and these should be consulted and be readily accessible to all analysts. Amongst such publications are those produced by the Royal Society of Chemistry, namely 'Safe Practices in Chemical Laboratories' and 'Hazards in the Chemical Laboratory' 5th edition, 1992; by Member Societies of the Microbiological Consultative Committee, 'Guidelines for Microbiological Safety', 1986, Portland Press, Colchester; and by the Public Health Laboratory Service 'Safety Precautions, Notes for Guidance'. Another useful publication is produced by the Department of Health entitled 'Good Laboratory Practice'.

Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts in waters

1 Introduction

1.1 ***Cryptosporidium parvum.*** *C. parvum* is a coccidian protozoan parasite. It parasitises the small intestine of humans and other mammals and has a worldwide distribution. Its life cycle is direct, and the infectious stage is the sporulated oocyst which is voided in faeces and contains four, naked, motile sporozoites. The banana-shaped sporozoites are released through the oocyst wall following exposure to body temperature, acid, trypsin and bile salts. They attach themselves intimately to the surface of adjacent enterocytes (the epithelial cells which line the gastro-intestinal tract). Sporozoites invade enterocytes to initiate the asexual cycle of development. Sporozoites and all subsequent endogenous asexual and sexual stages develop within a parasitophorous vacuole which is intra-cellular, but extra-cytoplasmic. In sexual multiplication, the male gametes fertilise female gametes to produce a zygote which in turn develops into an oocyst. The zygote differentiates into four sporozoites within the oocyst, and fully sporulated oocysts (each containing four sporozoites) are released into the lumen of the intestine and pass out of the body in faeces where they are infectious for other susceptible hosts.

In the last 15 years, *Cryptosporidium* has been identified as one of the most common causes of acute self-limiting diarrhoeal illness in immuno-competent individuals. In immuno-compromised individuals, such as those with Acquired Immune Deficiency Syndrome (AIDS), cryptosporidiosis can be a life-threatening condition causing profuse intractable diarrhoea with severe dehydration, malabsorption and wasting, with spread to other organs.

1.2 ***Giardia duodenalis (Giardia intestinalis, Giardia lamblia).*** *G. duodenalis* is a flagellated protozoan parasite. It parasitises the small intestine of humans and other vertebrates. Its life cycle is direct, requiring no intermediate host, and the parasite exists in two distinct morphological forms, namely, the reproductive trophozoite (which parasitises the enterocytes of the upper small intestine) and the environmentally resistant cyst, voided in faeces, which is the infective and disseminating stage. Discovered by van Leeuwenhoek some 300 years ago, *G. duodenalis* is also recognised as one of the most common protozoan parasites causing diarrhoea in humans worldwide.

1.3 **Transmission.** Transmission can occur by any route where infective *C. parvum* oocysts or *G. duodenalis* cysts are ingested by a susceptible host. Both oocysts and cysts can survive for prolonged periods in cool, moist environments. Person-to-person transmission is the most commonly documented route. Secondary cases and possibly asymptomatic excreters can also be a source of infection for other susceptible persons. Food-borne transmission has been reported for both *Cryptosporidium* and *Giardia*, while air-borne transmission has been documented for *Cryptosporidium*. Zoonotic transmission, from infected animals such as livestock, pets and wildlife, has also been documented. Cryptosporidiosis has been reported in a variety of domesticated animals, livestock and wildlife, including companion animals which may be reservoirs of human infection. Water-borne transmission is also a major publicised route, for both *Cryptosporidium* and *Giardia*.

1.4 **Analysis of water and associated matrices.** Water-borne outbreaks, especially of cryptosporidiosis, have led to considerable interest in monitoring water for the presence of oocysts. The methods described in this booklet for the isolation and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts in ground, surface, finished waters and associated matrices use a fluorescent antibody procedure. The methods can also be used to help identify sources of contamination of water supplies for catchment control, for evaluating the effectiveness of treatment practices and in potential outbreak situations. While the antibodies are genus specific, they will not identify the species of *Cryptosporidium* and *Giardia* detected. A method also describes how to determine the viability of isolated

oocysts. The methods can provide a quantitative indication of the level of *Cryptosporidium* oocysts and *Giardia* cysts in raw and treated drinking waters.

1.5 **Limitations.** Environmental monitoring is made problematical by the small size of oocysts and cysts, the low numbers normally found in many water types, and the inability to increase their numbers by *in vitro* culture. In addition, it is difficult to identify oocysts and cysts among other particles and debris found in water concentrates. The methodology for the recovery and identification of oocysts and cysts is often labour intensive and time consuming. The presence of turbidity may interfere with the concentration, clean-up and examination of the sample. Autofluorescent-contaminating organisms, such as algal cells and fungal spores, may be confused with oocysts or cysts when water concentrates are analysed by fluorescence microscopy, and can be recorded as false-positive results. The absence of, or failure to detect, oocysts or cysts in a sample does not ensure *Cryptosporidium*- or *Giardia*-free water. Appendix A summarises recovery data obtained using procedures similar to those described in this booklet.

1.6 **Validation of methods.** Several methods for the sampling, concentration, extraction, clean-up and identification of *Cryptosporidium* oocysts and *Giardia* cysts are described in this booklet. While the various stages may be used in a variety of combinations, any procedure should be performance tested and validated by individual laboratories before being adopted for routine laboratory use. It is of paramount importance that the procedures adopted are shown to be capable of recovering low levels of oocysts and cysts typically present in environmental and treated waters. Validation data for the method to be used should be generated using oocysts and cysts in a condition that reflects those recovered from the environment and for each sample matrix (for example surface waters, treated waters, filter backwash waters and sludges).

1.7 **Health and safety.** The methods described do not claim to address all the safety matters associated with their use. It is the responsibility of users of the method to establish appropriate health and safety practices. Appropriate practices should be discussed thoroughly and documented when large numbers of oocysts or cysts are anticipated in an analysis. Such analyses include backwash waters and the evaluation of the effectiveness of methods and treatment practices where seeding with infectious oocysts or cysts is undertaken. The analysis of raw sewage poses additional problems of assessing the potential risk from other pathogenic organisms and chemicals.

2 Sampling - concentration

There are four principal areas for which sampling for *Cryptosporidium* and/or *Giardia* would be undertaken. These are:

- water treatment process control;
- monitoring of catchments and sources for water abstraction;
- incident management;
- investigations of waterborne outbreaks.

Sampling strategies should be appropriate for the purpose for which the sampling is being undertaken.

2.1 **Choice of methods.** A number of factors will influence the choice of sampling procedures used. These will include the purpose for which the sample is taken, the water type and quality, and the subsequent analytical treatment of the sample. The selection of sampling sites, sample volumes and sampling procedures will impact upon the usefulness of the data generated. Key factors which should be considered in the development of effective sampling strategies include:

- the sampling sites and number and frequency of samples collected;
- the sample volume;
- the sampling procedures, especially when on-site concentration is employed;
- the quality of the water being sampled that may affect recovery efficiencies, particularly with regard to the presence of particulate matter;
- efficiency of the procedure for extraction and clean-up of oocysts and cysts from the sample or on-site concentration filter, and the 'volume equivalent' finally examined.

Basically, the choice includes large-volume (on-site concentration) samples or small-volume (grab) samples. For surface water environmental monitoring, grab samples of 10 - 20 litres might be suitable, and for water supply and distribution system monitoring, volumes up to 100, or even 1000 litres (employing on-site concentration) may be appropriate. For the investigation of suspected water-borne outbreaks, a balance should be maintained between generating data for decision making and sampling meaningful volumes. It may be appropriate to plan sampling so as to obtain results from large volumes (for example, 1000 litres) filtered at a slower filtration rate to cover a 12 to 24 hour period from strategic points, coupled with data from more frequent smaller volumes (for example, 10 - 100 litres) filtered at supplementary sampling points which enable rapid results to be obtained. Guidance on sample volumes for the four principal areas of monitoring is given in Table 1.

Where there is information to allow an estimate of the likely concentration of oocysts or cysts in the water to be sampled (for example, 0.1 oocysts/litre in pristine waters, or 1 - 100 oocysts/litre in lowland rivers) and a knowledge of the recovery efficiencies of the concentration, extraction and clean-up procedures being employed, then this information and knowledge can be used to estimate a volume of sample that might yield more meaningful data.

Factors such as access to the sample site, the logistics of sampling and sample transport may also influence the choice of sampling method. For monitoring of water treatment works, it may be appropriate to plumb in fixed filtration housings for depth-filter cartridge or membrane on-site concentration.

For some investigations, other types of samples (for example, sand from filters, slurries from coagulation plants and sediments from service reservoirs or distribution pipes) may yield oocysts or cysts, but the amounts that can be analysed will depend upon the extraction and clean-up procedures used and the amount of interfering substances present. Before being used routinely, the sampling procedures, selected by the laboratory for the matrices to be analysed, should be fully validated.

2.2 **Large-volume samples (on-site concentration).** Large-volume samples are typically concentrated on-site. A number of procedures for on-site concentration have been investigated. Two that have been widely employed, and for which there are substantial data published, are depth-filter cartridge filtration and flat-bed membrane filtration. The choice of method will depend primarily upon the desired volume of sample to be filtered and the water quality. Generally, larger volumes can be filtered with a cartridge filter, particularly for highly turbid waters, but recovery efficiencies can be higher with membrane filters.

2.2.1 **Principle.** The principle of filtration is to separate particulate matter, including *Cryptosporidium* oocysts and *Giardia* cysts, from water. This is achieved by pressure filtration either through a cartridge filter (typically consisting of layers of wound fibres or a pleated membrane) or through a membrane filter (typically 142 mm in diameter). The process of filtration is a balance between maximising the volume of water filtered (before resistance to flow becomes too prohibitive) and maximising the number of oocysts and cysts to be recovered from subsequent elution processes. Since some of these features are in conflict, the optimal choice will be a compromise dependent upon the type of water being analysed. The cartridge or membrane filter of choice should produce an optimal

retention of oocysts or cysts and subsequent release of oocysts or cysts, while allowing an adequate volume (and number) of samples to be analysed.

2.2.2 **Wound polypropylene or pleated membrane cartridge filters.**

2.2.2.1 **Equipment.** The following equipment is required:

- Wound polypropylene or pleated membrane cartridge filters with 1 μm nominal pore diameter.
- Filter holder.
- Polyethylene, nylon or silicone tubing, hose clips and connectors.
- Flow-restricting valve.
- Water meter.
- Pump and power source for remote sampling.
- Large forceps, plastic bags (for used filters), labels, markers (for example, waterproof pens).

2.2.2.2 **Apparatus preparation and assembly.** The apparatus should consist of the essential plumbing as shown, for example in Figure 1. The basic set-up consists of inlet and outlet hoses, a filter holder, a 1 μm nominal porosity filter, a flow-control valve or device, and a water meter. Prior to use, new housings should be pressure checked to ensure that bypass flow and leakage do not occur. Performance validation should be undertaken on the type and make of filter being used. The flow-restricting valve should be set to a flow appropriate for the make of filter being used to allow optimal entrapment of *Cryptosporidium* oocysts and *Giardia* cysts. Higher flows may disrupt the filter fibres and allow breakthrough. A pump will be necessary for unpressurised sources.

Before use, the filter holder should be thoroughly cleaned. If detergent is used, the residue should be removed by rinsing with tap water. Apparatus previously used with samples containing high concentrations of oocysts or cysts should be soaked, for example, overnight in a dilute sodium hypochlorite solution (normally a 10 per cent v/v solution to yield 1 per cent v/v available chlorine) or similar solution, and thoroughly rinsed before subsequent use. While procedures involving boiling or autoclaving are effective for ensuring that oocysts and cysts are rendered non-viable, these processes will not necessarily reduce the ability of oocysts and cysts to be stained. Soaking in hypochlorite solutions destroys the epitopes on the outside of the oocysts and cysts and therefore prevents staining. Sampling housings should be kept segregated and dedicated for use when analysing treated or untreated waters. Consideration should be given to dedicating sampling housings to individual water sources.

2.2.2.3 **Water sample concentration.** If sampling from a tap or sampling line is being undertaken, ensure that any residual debris is displaced from the line or pipe by adequate flushing, before connecting the on-site concentration apparatus. This does not apply if the residual debris is to be analysed.

When connecting the apparatus to the tap, ensure that it is assembled securely. The housing can be flushed through prior to placement of the cartridge filter. Once the cartridge filter is placed in the housing, check that the housing is sufficiently tightened to prevent bypass flow of the filter.

During filtration, the flow rates should be those recommended for the make and type of cartridge filter being used. Avoid high flow rates, and bleed off trapped air at the start of the filtration.

Filter the desired volume of water (typically 100 - 1000 litres). The turbidity of the water being filtered should be taken into account before deciding the volume of water to be sampled.

When the required volume of water has been filtered, shut off the water supply. Remove the cartridge filter in such a manner so as to avoid contamination and place it into a protective plastic bag, which is then sealed. It is advisable to wear disposable gloves when handling the filter. The filter is carefully handled so as not to dislodge any trapped particles. The plastic bag should, preferably, be placed into a second protective plastic bag and sealed. Alternatively, the whole sampling apparatus can be disconnected from the sample tap. The inlet and outlet hoses are then disconnected and the housing ports sealed. The housing, containing the filter, can then be sent to the laboratory.

Sample details that should be recorded at the time of sampling include:

- sample location;
- date of sampling;
- time filtration began and the time filtration ceased;
- nominal flow rate;
- metered volume of sample;
- identity of the person taking the sample.

Additional information, such as the condition of the sampling point, should also be recorded where this information may affect the interpretation of any results obtained.

Transport the sample to the laboratory (see section 2.4).

2.2.3 **Flat-bed membrane filters.**

2.2.3.1 **Equipment.** The following equipment is required:

- Membrane filters of up to 3 μm pore size (for example, polysulphone, cellulose acetate, cellulose nitrate or polycarbonate membranes) of large diameter (typically 142 mm).
- Filter holder suitable for membrane filters. Where holders are fitted with upper plates, these should be removed.
- Polyethylene, nylon or silicone tubing, hose clips and connectors.
- Flow-restricting valve.
- Water meter.
- Pump and power source for remote sampling.
- Forceps, plastic bags (for used filters), labels, markers (for example, waterproof pens).

2.2.3.2 **Apparatus preparation and assembly.** The apparatus used should consist of the essential plumbing as shown, for example in Figure 2. The basic set-up consists of inlet and outlet hoses, a filter holder, a membrane filter, a flow-control valve or device, and a water meter. New filter holders should be pressure checked before being used to ensure that bypass flow and leakage do not occur. Performance validation should be undertaken on the type and make of filter to be used. The flow-restricting valve should be set to a flow rate appropriate for the make of filter being used to allow optimal entrapment of *Cryptosporidium* oocysts and *Giardia* cysts. Higher flows may rupture the filter and lead to loss of oocysts or cysts. A pump will be necessary for unpressurised sources and grab samples. Before use, the filtration apparatus should be thoroughly cleaned. If detergent is used, the residues should be removed by rinsing with tap water. Apparatus previously used with samples containing high concentrations of oocyst or cysts should be soaked, for example, overnight in a dilute sodium hypochlorite solution (normally a 10 per cent v/v solution to yield 1 per cent v/v available chlorine) or similar solution, and thoroughly rinsed before subsequent use. High concentrations of chlorine may cause discolouration of stainless steel units. Membrane filter holders should be dedicated for use when analysing treated or untreated waters, and kept segregated.

2.2.3.3 **Water sample concentration.** If sampling from a tap or sampling line is being undertaken, ensure that any residual debris is displaced from the line, pipe or tubing by adequate flushing, before connecting the concentration apparatus. This does not apply if the residual debris is to be analysed.

When connecting the apparatus to the tap, ensure that it is assembled securely. If necessary, the filter holder can be flushed through prior to placement of the membrane filter. Care should be taken when placing the filter on to the supporting screen to avoid tearing the filter and to ensure that it is centrally placed. Ensure that the filter is correctly orientated with regard to the direction of flow (see manufacturer's instructions). Failure to do so can impair the entrapment efficiency of the filtration process. Some types of membrane need to be moistened before placement to prevent wrinkling and damage. Once the filter is placed on the supporting screen, ensure that the filter holder is sufficiently tightened to prevent leakage.

For filtration, the flow rates should be those validated for optimal recovery of oocysts and cysts for the make and type of membrane filter being used. Bleed off trapped air at the start of the filtration.

Filter the desired volume of water (typically 10 - 100 litres). The turbidity of the water being filtered should be taken into account before deciding the volume of sample to be used. For good quality waters, larger volumes of sample (up to 1000 litres) may be filtered.

When the required volume of water has been filtered, shut off the water supply. Remove the membrane filter in such a manner so as to avoid contamination and place it into a protective plastic sample bag which is then sealed. It is advisable to wear disposable gloves when handling the filter to ensure the filter is not damaged and that trapped particles are not dislodged. The bag should, preferably, be placed into a second protective bag and sealed. Alternatively, the whole sampling apparatus can be disconnected from the sample tap. The inlet and outlet hoses are then disconnected and the housing ports sealed. The housing, containing the filter, can then be sent to the laboratory.

Sample details that should be recorded at the time of sampling include:

- sample location;
- date of sampling;
- time filtration began and the time filtration ceased;
- nominal flow rate;
- metered volume of sample;
- identity of person taking the sample.

Additional information, such as the condition of the sampling point, should also be recorded where this information may affect the interpretation of any results obtained.

Transport the sample to the laboratory (see section 2.4).

2.2.4 **Other sample concentration devices.** A number of other sampling and sample concentration devices are currently being evaluated in various laboratories. These include compressed foam filters, polysulphone membrane cartridges and vortex flow filtration systems. These, and others that may be developed, might prove to be suitable for the recovery of oocysts and cysts, but their performance should be validated prior to use.

2.2.4.1 **Compressed foam filters.** Basically, the apparatus consists of a module containing a set of open-cell reticulated foam rings compressed between two plates. This is placed into a filter housing and connected to the water source and a flow meter fitted to the outlet. The desired volume of water (typically 10 to 1000 litres) is filtered according to the manufacturer's instructions. When the required volume of water has been filtered, the module is removed from the housing and placed in an appropriate container with sample water to maintain moisture of the compressed foam. Alternatively, the whole housing containing the module can be disconnected (ensuring that water is kept within the housing) and the ports sealed prior to being sent to the laboratory.

The requirements for the dedicated use of housings to water types, the flushing of tap or sampling lines, and the recording of sample details, etc are as described in sections 2.2.2.2 and 2.2.2.3.

2.2.4.2 **Pleated polysulphone filters.** The apparatus consists of a sealed polypropylene capsule containing a pleated polysulphone filter of nominal pore size of 1 µm and effective filtration area of 1300 cm^2. The filter has a straight barbed hose inlet and outlet and is supplied complete by the manufacturer. The minimum filter volume is 127 ml. The filter is connected in line together with the fittings as described in 2.2.2.2 and 2.2.2.3. The bleed valve is opened while the capsule fills and the required volume of water is sampled. Once sampling has been completed, the outlet hose is disconnected, any water discarded and the end sealed. The inlet hose is then removed (taking care not to spill any water) and the end sealed. The capsule is then sent to the laboratory for extraction. While samples of up to 1000 litres of clean water can be sampled, the volume of turbid waters may be restricted.

2.3 Small-volume samples (grab samples). Small-volume samples, normally consisting of 10 litre volumes, are transported back to the laboratory prior to concentration. Larger volumes may be handled in this way but will depend upon the practicalities of transporting large volumes of water.

2.3.1 **Grab sample collection.** Samples from water supply distribution systems should be collected with the same consideration as for flushed chemical or bacteriological samples, though tap disinfection is not required. Further guidance can be found in "Microbiology of Water 1994 - Part 1 - Drinking Water". Dedicated sample lines and domestic taps should be flushed to purge residual debris from the line or pipe. It is important to ensure that any residual sediment in the sample line or pipe is thoroughly cleared before sampling. This would not apply if the residual debris is to be analysed.

Ten litre grab samples can be collected in 10 litre containers, or two 5 litre containers for ease and safety in carrying and handling. Polyethylene "jerry cans" have been found to be suitable since they are free from contamination and are sufficently inexpensive to be classed as 'disposable'. Low-cost disposable containers may be preferable since their surfaces are hydrophobic when new, which minimises adhesion of cysts and oocysts. Where non-disposable containers are used, consideration should be given to segregating those containers used for raw water and those used for water treatment works' final waters and distribution systems. Such containers should be thoroughly washed between use. Prior to collection of the sample, the container should be rinsed out with the water to be sampled.

2.3.2 **Grab sample concentration by flat-bed membrane filtration.** Grab samples can be filtered through flat-bed membrane filters at the laboratory using the procedures described in section 2.2.3.

2.3.3 **Calcium carbonate flocculation.** Usually, a small-volume (grab) sample is treated and the resulting suspension of particulate matter is centrifuged to produce a concentrate. This concentrate is then examined microscopically. If a viability assessment (see Appendix B) is to be undertaken, this technique should not be used.

To 10 litres of sample add approximately 100 ml of 1 molar calcium chloride solution. Mix well. Add approximately 100 ml of 1 molar sodium hydrogencarbonate solution and mix well. Add approximately 100 ml of 1 molar sodium hydroxide solution. Ensure that a floc has formed and if not, add more sodium hydroxide solution. The pH should be at least 10. Allow to stand until the precipitate has settled. This may take a minimum of 3.5 hours or up to overnight standing.

When the calcium carbonate floc has settled, aspirate the supernatant liquid until approximately 300 ml of liquid remains. Add approximately 200 ml of a 10 per cent m/v solution of sulphamic acid. Mix well to dissolve the precipitate. Add approximately 500 ml of an 0.01 per cent v/v solution of polyoxyethylene sorbitan mono-oleate (3.1.1.1). Mix well. Adjust the pH of the resulting solution to 6.0 - 6.5 using 1 molar sodium hydroxide solution.

The resulting suspension is centrifuged, either as a single individual unit, or repeatedly as several units to produce a single concentrated suspension of particulate matter containing oocysts and cysts.

2.4 **Transport and storage of samples.** Samples (cartridge and membrane filters or small-volume grab samples) may be transported at ambient temperature or refrigerated. The sample should be transported to the laboratory as quickly as possible and should not be allowed to freeze. Freezing results in the production of ice crystals within the oocyst or cyst. This can cause a change to the buoyant density and/or lead to disruption of organelles within the oocyst or cyst which may interfere with the detection and/or identification of oocysts or cysts present in the sample.

Analysis should commence as soon as possible after arrival at the laboratory. Samples that cannot be processed immediately should be refrigerated (preferably in the dark) between 2 - 8 °C.

On receipt at the laboratory, all the sample details should be recorded and the sample (or filter) allocated a sample reference number which should be unique and be used to identify the sample during subsequent concentration, extraction, clean-up and identification.

2.5 **Safety considerations.** All operators should be made aware of their obligations and the risks involved in sampling. Risks may be classified into three areas: the risks of injury associated with the sampling site (for example, deep water, risk of tripping); the need for good hygiene to minimise risks of infection (for example, no smoking during sampling, washing hands after sampling); and the need to protect the sample from contamination. It should be borne in mind that infectious agents other than *Cryptosporidium* and *Giardia* may also be concentrated using these procedures.

Further guidance can be found in appropriate booklets in this series, for example "General Principles of Sampling Waters and Associated Materials (second edition) 1996".

3 Extraction and clean-up

3.1 **Choice of methodology.** The choice of procedures used to extract and clean-up a sample or material trapped on a filter may depend upon the technique used to collect the sample. Any particulate matter, including oocysts and cysts, adhering to the filter should be removed and collected to produce a small concentrated suspension, the volume of which is recorded. Portions of this suspension, with or without clean-up, are then examined microscopically.

Before being used routinely, appropriate performance testing should be carried out on the extraction and clean-up procedures in order to provide validated performance data for the different sample types being examined. For example, surface waters, groundwaters, treated waters, filter backwash waters and sludges.

3.1.1 **Reagents.**

3.1.1.1 **Polyoxyethylene sorbitan mono-oleate solution.** Prepare a 1 per cent v/v polyoxyethylene sorbitan mono-oleate solution (for example Tween 80) in deionised water. Similarly, prepare 0.1 per cent v/v and 0.01 per cent v/v solutions in deionised water.

3.1.1.2 **Phosphate buffered saline solution, 0.01M.** Dissolve 1.07 g of anhydrous disodium hydrogen phosphate (Na_2HPO_4) and 0.39 g of sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) in 1 litre of aqueous sodium chloride solution containing 8.5 g of sodium chloride (NaCl). The pH of the resulting solution should be 7.2 - 7.5.

3.1.1.3 **Sucrose solution, d_{20} 1.18.** Dissolve 256 g of sucrose in 300 ml of deionised water and make up to 500 ml. Check the specific gravity and adjust if necessary. Store at refrigeration temperature and use straight from the refrigerator.

3.1.1.4 **Potassium citrate solution, d_{20} 1.18.** Dissolve 308 g of tripotassium citrate in approximately 800 ml of deionised water. Make up to 1 litre with water. A final density of 1.18 should be achieved at room temperature by the addition of water or tripotassium citrate.

3.1.1.5 **Polyoxyethylene sorbitan mono-oleate in phosphate buffered saline solution.** Prepare a 0.01 per cent v/v polyoxyethylene sorbitan mono-oleate solution (for example Tween 80) in phosphate buffered saline solution (3.1.1.2).

3.1.2 **Equipment.** The following equipment is required:

- Beakers, 5 litre capacity.
- Centrifuge tubes, conical or round-bottom (not flat-bottom), various sizes.
- Sharp knife, such as a "Stanley knife" or equivalent. Scalpels may not be suitable as the blades tend to break easily. The provision of butchers' "chain mail" style gloves should be considered for use during cutting of the filter.
- Gloves, disposable.
- Stainless steel tray to accommodate the filter.
- Wash bottle.
- Vacuum source.
- Vortex mixer.
- Centrifuge, with swing-out head and accepting large capacity tubes.
- Microcentrifuge, accepting microcentrifuge tubes of up to 2 ml capacity.
- Homogeniser, such as a "Colworth Stomacher" or equivalent and suitable polythene bags to contain filter fibres.
- Syringes, needles or stainless steel cannulae.

3.2 Extraction procedures.

3.2.1 **Samples collected from wound polypropylene or pleated membrane cartridge filters.** Wearing protective gloves, remove the filter from the housing, cut lengthwise and remove the plastic former. When cutting, work away from the body. The filter should be cut into sections which can be homogenised together or separately. In either case, tease apart the fibres or pleated membrane before homogenisation.

Depending upon the capacity of the homogeniser, place some or all of the filter fibres or pleated membrane into a plastic bag, which is contained inside another plastic bag. Depending upon the size of the bag, add 750-1500 ml of 0.1 per cent polyoxyethylene sorbitan mono-oleate solution (3.1.1.1) and homogenise for 10 minutes. Pour the washings into a beaker. Repeat the washing. Add the second amount of washings to that already contained in the beaker. If the washing solution is still discoloured, repeat the procedure as necessary.

Repeat this procedure until all the fibres or membrane have been treated. At this stage, the total volume of washings may be between 2 and 4 litres.

Adequately centrifuge the whole of the washings to produce a small concentrated suspension of the particulate matter. This can be achieved, using appropriate portions, by centrifuging the washings, for example at 1500 g for 10 minutes, aspirating the supernatant liquid, combining the suspensions and centrifuging again. If fitted, do not use the centrifuge brake. Carefully aspirate the supernatant liquid, using negative pressure applied at the meniscus only. To minimise the disturbance to the surface of the pellet of particulate matter, leave a column of liquid of about 3 cm above the pellet.

3.2.2 **Samples from flat-bed membrane filters.** Typically, the membrane filter is transferred from the filtration unit to a resealable polyethylene bag together with 20 ml of 1 per cent polyoxyethylene sorbitan mono-oleate solution (3.1.1.1). To minimise foaming, exclude as much air as possible.

Particulate matter is then removed from the surface of the filter. This can be achieved by rubbing the filter inside the plastic bag in such a manner that the bag is not damaged. This is carried out repeatedly as necessary. Pour the resulting suspension into a suitable container, for example a large centrifuge tube. Rinse the bag with more 1 per cent v/v polyoxyethylene sorbitan mono-oleate solution (3.1.1.1) and transfer the washings to the container. Repeat the procedure as necessary.

Alternatively, a rubber "windscreen wiper" blade may be used to wipe the particulate matter from the surface of the membrane filter. However, this may only be effective with track-etched polycarbonate membranes where surface adhesion is very low.

The combined washings are then centrifuged to generate a concentrated suspension of particulate matter, the volume of which is recorded (see section 3.3).

3.2.3 **Samples collected from other concentration devices.**

3.2.3.1 **Extraction from compressed foam filters.** The compressed foam filter module is transferred to the upper 'elution tube' of the wash unit and the retaining screw removed as per the manufacturer's instructions. The water from the housing or transportation container is poured into the lower 'concentrator tube' which has a membrane filter and closed drain-tap in its base. Approximately 600 ml of extraction solution, ie 0.01 per cent polyoxyethylene sorbitan mono-oleate in phosphate buffered saline solution (3.1.1.5) is added to the concentrator tube and the wash station assembled. The compressed foam rings expand inside the elution tube and the extraction solution is drawn through and expelled from the rings by a pumping wash action. After extraction, the concentrator tube is removed and placed on a magnetic stirrer and the extraction solution is drained out through the membrane via the drain port until approximately 20 ml remains. This is decanted into a suitable container. A further quantity of 600 ml of 0.01 per cent polyoxyethylene sorbitan mono-oleate in phosphate buffered saline solution (3.1.1.5) is added to the concentrator tube and the process repeated. Before reducing the volume of the second extraction solution, the 20 ml concentrate from the first extraction is added to the second quantity of 600 ml of solution after extraction is completed. The combined solutions are then reduced to approximately 20 ml and decanted into a suitable (centrifuge) tube. The membrane filter is then removed from the base of the concentrator tube, washed in 5 ml of extraction solution and added to the concentrate. The concentrate can then be subjected to an appropriate clean-up procedure.

3.2.3.2 **Extraction from pleated polysulphone filters.** Oocysts and cysts are removed from the capsule by shaking with an extraction buffer. If the inlet chamber of the filter is more than half full of water, this should be decanted carefully into a suitable container. Approximately 120 ml of extraction buffer are added to the capsule and the filter placed horizontally into a wrist-action shaker. The bleed valve should be at the top of the filter. The filter is shaken, for example at approximately 600 rpm for 10 minutes and the eluate decanted into the container. The process is repeated with a further 120 ml of extraction buffer with the filter positioned such that the bleed valve is now at 90 ° to the position of the first extraction. The eluate from the second extraction is combined with that of the first and concentrated by centrifugation and, if necessary, subjected to an appropriate clean-up procedure. The formulation of the extraction buffer is provided by the manufacturer.

3.3 **Clean-up procedures.** Where the volume of particulate matter is large, there may be advantage at this stage in carrying out a clean-up step. If a clean-up step is not used, then excess detergent should be removed before spotting onto microscope slides or membrane filters as the presence of detergent may interfere with subsequent processing. Aspirate the supernatant detergent solution to leave a small volume, resuspend the particulate matter and transfer the material to micro-centrifuge tubes. Centrifuge, for example at 10000g for 1 minute, remove the supernatant detergent and wash the deposit with phosphate buffered saline solution (3.1.1.2). Repeat the procedure until excess detergent has been removed, resuspend the pellets and, if necessary, combine the suspensions into one tube. Record the volume and store in a refrigerator until ready for examination. At this stage the particulate matter is ready for examination by staining on slides or filters or by flow cytometry.

3.3.1 **Flotation techniques.** A flotation technique is used to facilitate the separation of oocysts and cysts from other particulate matter present in the concentrated suspension, see Figure 3. The suspension to be cleaned is floated on a high density liquid. This enables oocysts and cysts to remain in suspension, mostly at or above the interface of the two liquids, while denser matter is centrifuged to the bottom of the tube. The additional manipulations can, however, lead to the loss of oocysts and cysts. Independent validation of this technique to establish the range of these losses should be undertaken by spiking different types of concentrates. The decision whether or not to apply a flotation stage is subjective and should be made with knowledge of the water source. If there is any doubt, the suspension can be split into two identical portions, typically 10 ml each, and processed with and without a flotation stage. A flotation technique can be applied to a concentrated suspension derived from a cartridge filter, membrane filter, compressed foam filter or flocculation process.

Vortex the known volume of concentrated suspension until it is evenly dispersed. Split the suspension so that, typically, 10 ml is clarified at any one time. Vortex the suspensions again if there is a delay before the flotation process begins.

Typically, 10 ml of a solution of sucrose (3.1.1.3) or tripotassium citrate (3.1.1.4) can be used to underlay the sample. This can be achieved with 10 ml of solution delivered by a syringe and cannula. Ensure that the sucrose (3.1.1.3) or tripotassium citrate (3.1.1.4) solution is introduced gently and that air is not injected into the suspension. It is best to rest the cannula near the bottom of the tube. A clear layer should be produced, and oocysts and cysts with some sediment should remain in the upper layer.

Centrifuge, for example at 1000g for 5 minutes with swing-out rotor and no brakes, the resulting layers. Carefully remove the liquid, for example with a pipette, leaving the pellet of dense particulate matter. Combine all recovered suspensions of oocysts and cysts into centrifuge tubes. Dilute with phosphate buffered saline solution (3.1.1.2) and centrifuge, for example at 1500g for 10 minutes. Carefully aspirate the supernatant liquid using negative pressure applied

at the meniscus and without disturbing the sediment. If necessary, combine the sediments into one tube, rinsing the second tube with phosphate buffered saline solution (3.1.1.2) and centrifuge again as above. Complete the process as described in section 3.3.

3.3.2 **Immuno-magnetic separation.** The immuno-magnetic separation (IMS) technique is an alternative procedure for separating oocysts and cysts from water concentrates. The water concentrates can be prepared as described in sections 3.2 to 3.4. Manufacturer's products are available and both paramagnetic colloidal magnetite particles (40 nm) and iron-cored latex beads have been assessed for their abilities to concentrate oocysts and cysts selectively from water concentrates. An antibody which is reactive with *Cryptosporidium* oocysts or *Giardia* cysts is covalently bound onto the outer surfaces of magnetisable beads. Antibody-coated beads are then added to a water concentrate and the suspension is mixed (in order to increase the collisions between antibody-coated beads and any oocysts and cysts present in the concentrate). Surface-exposed epitopes on oocysts and cysts become attached to antibody paratopes bound to beads and form bead-oocyst and bead-cyst complexes. These complexes are then concentrated in a reaction vessel by a magnetic field. Particulate matter which has not bound to the beads, as well as any suspending fluid, are then aspirated to waste while the bead-oocyst and bead-cyst complexes are magnetically retained to the wall of the reaction vessel. The bead-oocyst and bead-cyst complexes are then resuspended in buffer and the complexes dissociated to release the oocysts and cysts which are then enumerated (see section 4).

3.3.3 **Flow cytometry.** Flow cytometers are instruments designed for detecting, analysing and sorting particles, based on size, shape and fluorescence. They can be used to separate oocysts and cysts from debris in sample concentrates.

3.3.3.1 **Principles of flow cytometry.** A flow cytometer consists of two basic systems. A fluidics system guides the particles from a sample as a narrow stream through an optical system which illuminates the particles and detects their light scatter and fluorescence characteristics. As the stained oocysts and cysts pass through the flow cytometer they are illuminated by an argon laser, and separate detectors identify the light that is scattered forwards or sideways and the fluorescent light emitted by the FITC-conjugated monoclonal antibody. If the analysing software detects that a particle gives the light scatter and fluorescence typical of oocysts or cysts, it is sorted out of the concentrate directly onto a microscope slide or membrane filter. This slide or filter is then screened for the presence of oocysts and cysts by epifluorescence microscopy.

Before flow cytometry is used, the concentrated suspension is stained with a fluorescein isothiocyanate (FITC) conjugated monoclonal antibody against *Cryptosporidium* oocysts and *Giardia* cysts. The volume of sample analysed by the flow cytometer ranges from hundreds of microlitres to several millilitres. The concentrate is passed through a filter with a pore diameter of 30-50 μm to remove particles that may block the fluidics system.

3.3.3.2 **The application of flow cytometers for sorting oocysts and cysts in water.** Several flow cytometer instruments have been tested, optimised and used for sorting oocysts and cysts from water concentrates. Some have been developed for research applications while others have been developed for more routine applications.

The instruments usually sort particles by a flow-in-air system. The fluid stream is divided into small droplets and if a particle shows the typical optical characteristics of an oocyst or cyst, that droplet becomes electrically charged and diverted from the stream. Flow-in-air systems may generate aerosols. Due consideration should, therefore, be given to aerosol containment or inactivation

of oocyst or cyst (for example by heat pasteurisation) where samples containing large numbers of oocysts and/or cysts are to be sorted.

Flow cytometer systems were not specifically designed to enumerate oocysts and cysts in water concentrates. Since the light scatter and fluorescence intensity of these organisms vary, a range of light scatter and fluorescence characteristics should be set to obtain the maximum recognition of oocysts and cysts. Unfortunately, the sort regions used for the detection are not sufficiently narrow to exclude fluorescing debris while including all oocysts and cysts. Hence, confirmation of the sorted particles by fluorescent microscopy is necessary.

The flow cytometers currently in use to detect *Cryptosporidium* and *Giardia* in water samples use either a combination of forward scatter signal and fluorescence signal, or a combination of side scatter signal and fluorescence signal. Stained suspensions of the parasites are examined in the instrument which is optimised for recognition and discrimination with optimal recovery and minimum sorting of unwanted debris. Once these conditions are established, stained suspensions are examined and counted and compared with manual counting using water concentrates appropriately seeded with oocysts and cysts.

Flow cytometer instruments are used in the 'recovery mode' and not in the 'purification mode' which is designed to prepare suspensions of typical particles (of high purity) and exclude particles with atypical properties.

Different flow cytometer instruments sort particles at different rates. Those that sort by flow-in-air produce small volumes (μl) of purified concentrate that can be sorted directly onto a microscope slide. The catcher-tube instruments produce a much larger volume (ml) and the purified concentrate is sorted directly onto a membrane filter.

Prior to use, the performance of the selected flow cytometer instrument should be validated by performance testing on the various sample matrices that are analysed.

3.3.3.3 **Instrument alignment and optimisation.** To recover *Cryptosporidium* oocysts and *Giardia* cysts consistently from water samples, it is essential to ensure that the instrument is set up correctly. Air bubbles and debris can disrupt the hydrodynamic focusing, and it is important that the fluidics system is kept clean.

Flow cytometers are complex instruments and the manufacturer's instructions should be referred to for detail of their alignment, optimisation and operation.

3.3.3.3.1 **Alignment.** Manufacturers usually recommend that their instruments are switched on for 30 minutes before the alignment procedures begin. This is to allow the fluidics system and the optics system to stabilise. The aim of alignment is to align the flow of particles with respect to the laser beam and to ensure that the detectors produce maximum, reproducible signals. This is carried out by passing fluorescent spheres of a known size through the instrument and by the use of dot histograms, achieving a maximum detector sensitivity. In practice, the instrument settings should not vary significantly from day to day, and alignment should be achieved with a minimum of effort. Prior to use with each batch of samples, and when new batches of staining reagents are used, this alignment should be established.

The detail of the procedure varies between manufacturers and between instrument models. For particular instruments, the manufacturer's instrument handbook should be consulted. When the instrument is correctly aligned, the settings can be recorded and, together with the dot histograms, filed as a record of correct alignment.

3.3.3.3.2 **Optimisation of the fluidics system.** When the alignment of the instrument has been confirmed, the fluidics system should be optimised. The detail of the adjustment is specific to the model of the instrument, and for particular instruments, the manufacturer's instrument handbook should be consulted. When the system is set at its optimum and the instrument is correctly aligned, the settings can be recorded.

3.3.3.4 **Controls.**

3.3.3.4.1 **Instrument validation.** Before an instrument can be used for routine analysis, it should be validated to ensure that it is capable of detecting and sorting oocysts and cysts from suspensions and in material concentrated from water samples. Suspensions should be run through the instrument, sorted and counted and the results compared with those produced using direct microscopy. Three sets of suspensions can be used, for example, with each 10 µl portion containing approximately 5 - 10, 20 - 25 and 50 oocysts and cysts respectively. There should be no significant differences between the data obtained from both procedures.

In addition, it is important to establish that low levels of oocysts and cysts can be recovered from raw water samples, since these samples are likely to contain "aged" oocysts and cysts that may vary in shape and in their ability to take up the stains. Water samples known to contain oocysts and cysts should be processed and the concentrated material divided into portions for comparative analysis using flow cytometry and direct microscopy. Where samples containing oocysts and cysts are not available, suitable concentrates may be seeded with fresh and "aged" oocysts and cysts and the recovery process validated.

3.3.3.4.2 **Positive controls.** To ensure that the instrument is properly aligned, a stained suspension of oocysts and cysts should be run through the machine for sorting and counting (see 3.3.3.4.4). This will also ensure that oocysts and cysts are suitably stained and fall within the designated sort region. Additional checks can be made by using a suspension of green latex spheres, 6 µm in diameter. A 10 µl aliquot of this suspension (containing 2,000 - 3,000 spheres per ml) is added to approximately 500 µl of "sheath fluid" as specified by the instrument manufacturer, and run through the instrument. The spheres are sorted onto a slide or membrane, as appropriate, and counted. The count of the suspension should then be compared to the value determined by direct microscopy. This comparison should be undertaken with each batch of samples.

3.3.3.4.3 **Negative controls.** To ensure that there is no carry-over of oocysts and cysts from the preceding sample examined, a negative control can be run after each positive sample, or a thorough washing procedure can be validated. The validation of the instrument procedure (3.3.3.4.2) should ensure that oocysts and cysts are being counted properly and accurately.

3.3.3.4.4 **Stains.** Solutions of stains should be checked regularly to ensure that they stain the oocysts and cysts correctly and the fluorescence intensity recorded. In addition, the stain should also be checked regularly to ensure that it has not become contaminated. *Cryptosporidium* oocysts and *Giardia* cysts from a control suspension should be stained in suspension with fluorescein isothiocyanate conjugated monoclonal antibody (4.1.1) and either run through the instrument to ensure that they fall into the sort region and sorted onto a microscope slide, or washed in phosphate buffered saline solution (3.1.1.2), spotted onto a microscope slide and dried. The slide is stained with 4',6-diamidino-2-phenylindole (DAPI) (4.3), washed and dried. The slide is examined to ensure that the oocysts and cysts are typically stained and that nuclear material has been stained. These results, together with the monoclonal stain batch number, should be recorded. In addition, each new batch of stain should be checked to ensure that it does not contain fluorescent material that the cytometer will sort, and the data recorded. Stain preparations which contain large amounts of particulate fluorescent material can be filtered using, for example a 0.2 µm pore diameter filter.

3.3.3.4.5 **Routine sphere sorting.** A small number of spheres that should be sorted in the sort region should be added to each sample after staining is complete This ensures that the alignment and fluidics system are optimised during the sample analysis. Each slide should be examined to ensure that the spheres have been sorted correctly. Failure of the instrument to sort the spheres may imply that the analysis is invalid, and the sample should be re-examined.

3.3.3.5 **Staining.** Concentrates from water samples can be conveniently stained in the flow cytometer sample tube by mixing with an equal volume of fluorescein isothiocyanate conjugated monoclonal antibody (4.1.1) and incubating at 37 °C for 30 minutes.

4 Identification

In this section, staining with 4',6-diamidino-2-phenylindole solution assists only in the identification of oocysts and cysts, since reliance cannot be placed on any distinguishing features. In a later section (Appendix B) this stain is used under different conditions as an aid to assessing the viability of oocysts.

4.1 Reagents.

4.1.1 **Monoclonal antibodies.** Fluorescein isothiocyanate (FITC) conjugated monoclonal antibody kits can be purchased. They should be stored as specified by the manufacturer and should not be used after the "expiry date".

4.1.2 **4',6-diamidino-2-phenylindole solution (2 mgml^{-1}).** Prepare a stock solution in absolute methanol containing 2 mgml^{-1} of 4',6-diamidino-2-phenylindole (DAPI). Store in the dark at refrigeration temperature.

4.1.3 **4',6-diamidino-2-phenylindole in phosphate buffered saline solution (2 µgml^{-1}).** A working solution containing 2 mgml^{-1} of DAPI (4.1.2) is prepared in phosphate buffered saline solution (3.1.1.2).

4.1.4 **1,4-diazabicyclo[2.2.2]octane/glycerol mounting medium.** Warm 95 ml of glycerol in a beaker on a stirring hot plate and add 2 g of 1,4-diazabicyclo [2.2.2]octane (DABCO). Continue stirring until dissolved. The pH should be less than 8. Store at room temperature. Discard after 6 months. (DABCO is hygroscopic and can cause burns; avoid inhalation, skin and eye contact.)

4.2 Equipment.

Equipment. The following equipment is required:

- Warm-air oven, fan assisted, at a temperature between 28 - 40 °C.
- Epifluorescence and bright field microscope. With appropriate filters and light sources, suitable objectives, Nomarski differential interference contrast (DIC) optics, eye-piece graticule and calibration slide. Detailed specifications and procedures for establishing correct light paths are given in Appendix C.
- Multispot microscope slides. Polytetrafluoroethylene-coated, with several wells each of 10 mm diameter.
- Single channel automatic variable pipette. 5-50 µl, with disposable polythene tips.
- Cellulose nitrate membrane filters, 13 mm diameter, 3 µm pore size, and suitable filter holder.

4.3 Procedure.

Procedure. The sample should be concentrated using the techniques described in sections 2 and 3.

The prepared concentrate should be thoroughly mixed, and without delay, suitable aliquots (of known volume) carefully applied to a microscope slide, such that, for example newsprint can just be read through the wet suspension. If the concentrate contains very high levels of particulate matter, there is a possibility

that oocysts and cysts may be lost by detachment of the material from the slide. Mark the microscope slide with the sample identification number. Only one sample should be applied to each slide, but up to four replicates may be placed on a single slide. Dry the slide in the warm-air oven. The material on the microscope slide may be 'fixed' with methanol, and dried in a warm-air oven for five minutes. Acetone may be used as an alternative fixing agent.

Apply fluorescein isothiocyanate conjugated monoclonal antibody (4.1.1) according to the manufacturer's instructions. All mixing can be carried out by carefully tilting the slide. Incubate the slide at 37 °C for 30 minutes in a humidified chamber in the dark. After incubation, all surplus reagent should be removed by gentle suction and discharged to waste. Use a fresh tip on the suction device for each slide.

Apply, with a dropping pipette, sufficient DAPI in phosphate buffered saline solution (4.1.3) to cover the material in the well of the microscope slide. Allow to stand for 2 minutes at room temperature. All surplus reagent should be removed by gentle suction and discharged to waste. Using the same techniques, add phosphate buffered saline solution (3.1.1.2) for 10 seconds and then remove excess liquid.

Dry the slide in the warm-air oven and mount in DABCO-glycerol mounting medium (4.1.4).

Apply a coverslip (72 mm x 22 mm) and, if necessary, seal the edges with, for example clear nail varnish. Negative slides need not be sealed if they are to be examined immediately after being prepared and if they are to be discarded immediately after examination.

Sealed slides can be stored in a dry box in the dark at refrigeration temperature. Allow slides to reach room temperature before examining them microscopically. Slides may be stored for up to several days after preparation. If properly stored, this period may be extended for as long as three weeks. Positive and negative control slides should be included with each batch of samples examined.

For concentrates to be examined by staining on membrane filters, resuspend the concentrated suspension (section 3.3, or 3.3.1 in the remaining recorded volume). Prior to placement in the filter holder, moisten the membrane filter in phosphate buffered saline solution (3.1.1.2). Filter a suitable aliquot of the concentrate (the volume of which has been recorded) and flush through with air to ensure no excess of remaining fluid. This is typically done using a syringe. Wash the entrapped particulates with 10 ml of phosphate buffered saline solution (3.1.1.2) by gently pushing through with a syringe. For samples cleaned up by potassium citrate flotation, distilled water may be used. Gently displace any excess wash solution by flushing with air.

Ensure the filter holder is secured and that the outlet is closed off. Add an appropriate aliquot of fluorescein isothiocyanate conjugated monoclonal antibody (4.1.1) according to the manufacturer's instructions. Incubate for 30 - 60 minutes at 37 °C.

After antibody staining, wash with 10 ml of phosphate buffered saline solution (3.1.1.2) and add an appropriate aliquot of DAPI in phosphate buffered saline solution (4.1.3). Allow to stand at room temperature for 2 minutes. Wash with 10 ml of phosphate buffered saline solution (3.1.1.2) and ensure all excess fluid is displaced by gently flushing with air.

Remove the membrane filter from the filter holder. Place it on a microscope slide and mount in DABCO-glycerol mounting medium (4.1.4). Apply a coverslip (72 mm x 22 mm) and, if necessary, seal the edges with, for example clear nail varnish. Negative filters need not be sealed if they are to be examined

immediately after being prepared and if they are to be discarded immediately after examination.

Sealed slides can be stored in a dry box in the dark at refrigeration temperature. Allow slides to reach room temperature before examining microscopically. Slides may be stored for up to several days after preparation. If properly stored, this period may be extended for as long as three weeks. Positive and negative control slides should be included with each batch of samples examined.

4.4 **Examination of slides.** Instructions on how to set up and calibrate the microscope are contained in Appendix C. Scan each slide in a systematic manner. Start at one edge and cover the entire coverslip in an up-and-down and side-to-side pattern. Use 'landmarks' to ensure that no area is missed or that no area is counted twice. First examine the negative and positive controls. The negative control should show no oocysts or cysts and the oocysts and cysts in the positive control should be well stained and easily distinguished from the background material. Any unusual result should be investigated before slides derived from 'real' samples are examined. Using an appropriate filter, scan each slide, initially using the 20x or the 40x objective.

4.4.1 ***Cryptosporidium* oocysts.** Any presumptive oocysts should be examined in more detail using the 100x objective. Oocysts are 4 - 6 µm in diameter, and as a result of the application of the fluorescein isothiocyanate conjugated monoclonal antibody, appear bright apple-green with an enhanced brightness of the rim, which, generally, are smooth and unbroken. A typically stained oocyst is shown in Figure 4 (left) and Figure 5. It should be noted that the suture is a surface fold and is an artefact. It is no longer considered to be a diagnostic feature, although it is characteristic of *Cryptosporidium* oocysts. The effect of DAPI stain on the oocysts should also be examined using ultraviolet light. An oocyst of typical appearance is shown in Figure 4 (right). The morphology of oocysts should also be verified by examination with DIC illumination (Figure 6) or phase contrast (Figure 7). It should be borne in mind that, as a result of age, environmental stress or sample preparation, oocysts may be deformed, or the brightness of fluorescence modified.

4.4.2 ***Giardia* cysts.** Cysts are approximately 10 µm in diameter and appear similar to the stained oocysts. Typically stained cysts are shown in Figures 5 and 8.

Figures 9 - 11 show yeast and algal cells which may interfere with the identification of oocysts.

4.4.3 **Enumeration.** Taking into account the volume of the original sample; the volume of concentrated suspension and, where appropriate, any subdivisions; the final volume of concentrated suspension; and the volume and number of aliquots microscopically examined, the number of oocysts and cysts observed in each well should be recorded and the number of oocysts or cysts per litre (O) of the original sample calculated using the formula:

$$O = C / (V \times F \times E)$$

where C is the number of oocysts or cysts counted;
V is the volume in litres of the water sample;
F is the fraction of the initial concentrate which is further concentrated; and
E is the fraction of final concentrate examined.

An example of this calculation is presented in Appendix D.

When the whole of the concentrated suspension is not used for the microscopic examination, there is the potential for significant variations to occur in the estimation of the oocyst and/or cyst concentrations determined from different

subsamples. This should, therefore, be considered when interpreting results from fractions of the concentrated suspension, and periodic checks should be undertaken.

5 Quality assurance and quality control

5.1 **Quality assurance.** It is essential that a laboratory undertaking analysis for *Cryptosporidium* and *Giardia* should demonstrate that all results generated are reliable and are fit for the purpose for which they are to be used. This is achieved by implementing an appropriate programme of quality assurance. Over a period of time, the quality assurance programme should produce a database which can be used to provide valuable information regarding the accuracy and likely range of recoveries for the analytical techniques employed. Because of the potential significance of small numbers of oocysts and cysts in treated water supplies, it is essential to ensure that any oocysts and cysts isolated have originated from the original sample and have not been introduced inadvertently during sampling or analysis.

Any effective quality assurance programme should, ideally, cover the whole process from sample collection to reporting of results. It should also include a system of internal quality control, and participation in appropriate proficiency testing schemes.

Internal quality control should include procedures for monitoring and recording of working practices, equipment and reagents. These procedures should include the use of negative controls, positive controls, reference materials if available, replicate analysis of samples and control charts. It is important to establish the impact of losses of oocysts and cysts at the different stages of analysis such as sampling, concentration, clean-up and identification.

5.2 **Terms.** Quality assurance includes all those planned and systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality.

Quality control includes the operational techniques and activities that are used to fulfil requirements for quality.

External quality control, (proficiency testing schemes) includes the system of objectively checking laboratory results by an external agency. It includes comparison of a laboratory's results with those of other laboratories, the main object being to assess accuracy and bias.

Internal quality control includes the set of procedures undertaken by a laboratory for the continuous monitoring of operations and results, in order to ascertain whether results are sufficiently reliable. Internal quality control procedures primarily enable batch-wise trueness of results of quality control samples and precision on replicate analysis to be monitored.

A quality assurance system is a combination of a laboratory's activities aimed at achieving the required standard of analysis. While internal and external quality control are essential components, a quality assurance system should also include appropriate staff training, administrative procedures and management structures. Laboratory accreditation is normally assessed on the basis of the quality assurance system.

5.3 **Laboratory equipment.** The quality assurance requirements associated with laboratory equipment used in sampling and analysis are described in Appendix E.

5.4 **Internal quality control.** All batches of slides should be processed with a positive control (to check the staining procedure) and a negative control of oocyst- and cyst-free material (to check that cross contamination has not occurred). The positive controls should show sufficient fluorescence to allow

oocysts and cysts to be easily recognised. If not, and assuming the microscope has been set up correctly, the results should be considered unreliable.

Quality control procedures should include the analysis of a positive control sample and a negative control sample at an appropriate frequency commensurate with the number of samples being analysed. Positive control samples are normally prepared by spiking a water sample with a concentrated suspension of oocysts and cysts. The water samples chosen should include the various types of samples analysed, ie surface waters, groundwaters and treated waters. Negative control samples that are free from the target organisms should be taken through the whole analytical procedure.

The analysis of these control samples should reflect the whole method. In addition, positive and negative control checks should be undertaken on individual sections of the method, for example staining. Where the method includes procedures that may be selectively employed, for example flotation and clean-up, this too, should be checked with an appropriate positive control sample.

The failure to detect oocysts and cysts in a positive control sample, or to detect the presence of oocysts and cysts in a negative control sample, should invalidate the results of any sample being analysed at the same time. Remedial investigations should be taken to establish the cause of these failures.

Laboratories should calculate the recovery of the number of oocysts and cysts spiked into positive control samples and record these values. Where the recovery falls significantly below the range established by performance validation tests, this should be stated on the sample report.

5.5 **Reference materials.** The implementation of internal and external quality control procedures is greatly assisted by the provision of suitable reference materials. Reference materials should, ideally, consist of a known number of oocysts and/or cysts of defined age and viability, each originating from a common source. The provision of such materials would reduce the variability of the methods, especially when caused by factors associated with the age and viability of oocysts and cysts.

5.6 **Reporting of results.** When results of an analysis are reported, the following details should also be recorded:

(a) date and time sample taken;
(b) sample volume;
(c) sampling-concentration procedure (cartridge/membrane/grab, etc);
(d) extraction and clean-up procedure (flotation/flow cytometry, etc);
(e) the equivalent per cent of the original sample examined;
(f) the number of oocysts and cysts detected;
(g) the calculated concentration of the number of oocysts and cysts per litre of the original sample; and
(h) information on the viability of any oocysts detected, if established.

Table 1 Sample volumes for *Cryptosporidium* and *Giardia* monitoring

Sampling environment	Sample volumes
Environmental waters	
- surface waters	10 - 20 litres, or 100 litres for good quality waters.
- sewage works effluent	0.5 - 5 litres depending upon the particulate matter present.
- groundwaters	10 - 1000 litres.
Treatment works	
- post-filter and final waters	10 - 1000 litres.
- backwash waters, etc	1 - 10 litres.
Distribution systems	100 litres.
Outbreak investigation	10 - 1000 litres at strategic points and supplementary sampling points.

Figure 1 Apparatus for collecting large volume samples using wound polypropylene cartridge filters

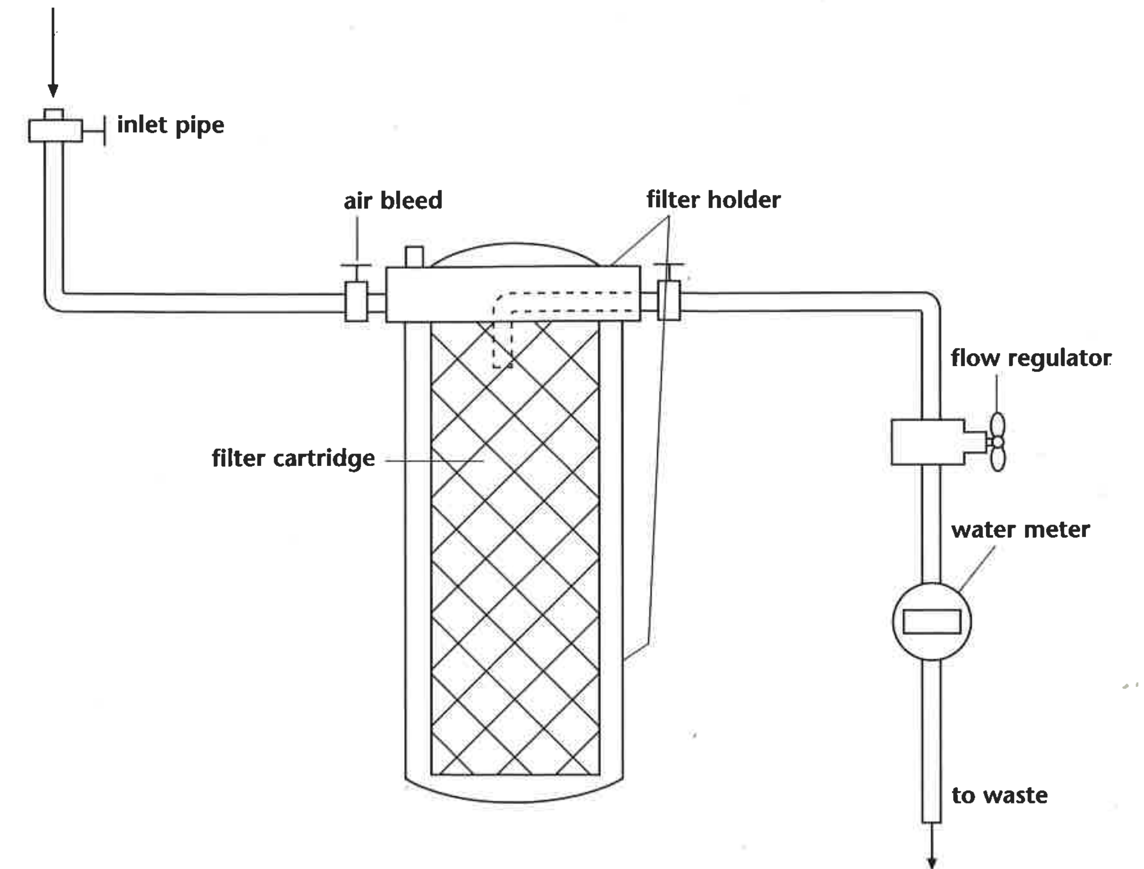


Figure 2 Apparatus for on-site concentration of samples by membrane filtration

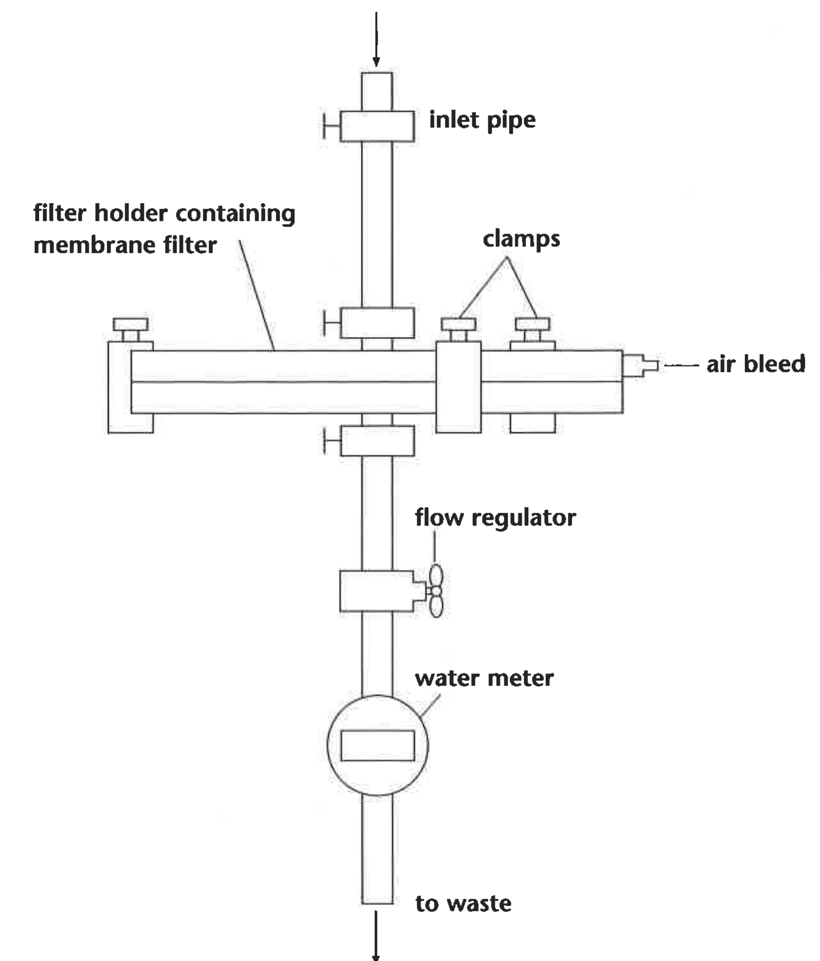


Figure 3 **Flotation clean-up of oocysts and cysts**

Left: Sample after addition of high density liquid before centrifugation

Right: Sample after centrifugation

Figure 4 ***Cryptosporidium parvum*** **oocyst stained with FITC-conjugated monoclonal antibody (left) and DAPI stain (right)**
The oocyst is shown after staining, illuminated by 488 nm (left) and ultraviolet light at 410 nm (right).

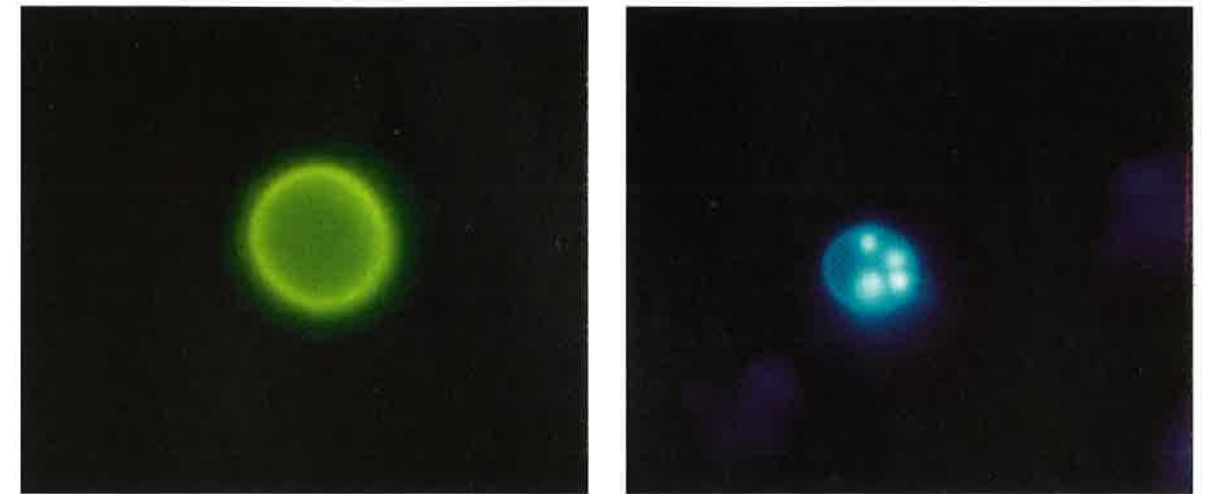


Figure 5 ***Cryptosporidium parvum*** **oocyst (C) and** ***Giardia*** **cyst (G) stained with FITC-conjugated monoclonal antibody**

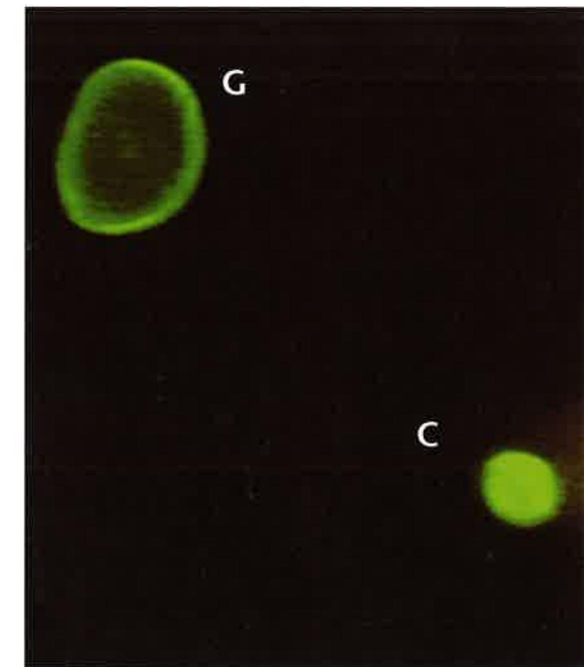


Figure 6 *Cryptosporidium parvum* oocysts with DIC illumination

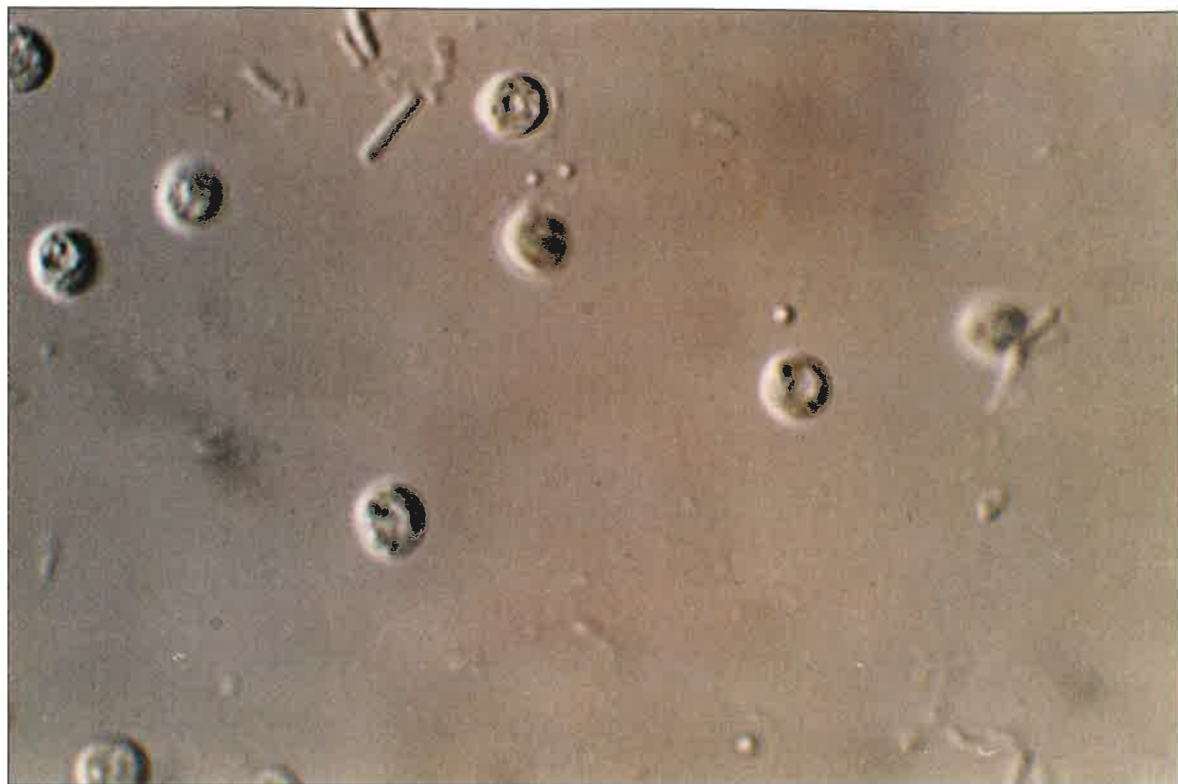


Figure 7 *Cryptosporidium parvum* oocysts with phase contrast illumination

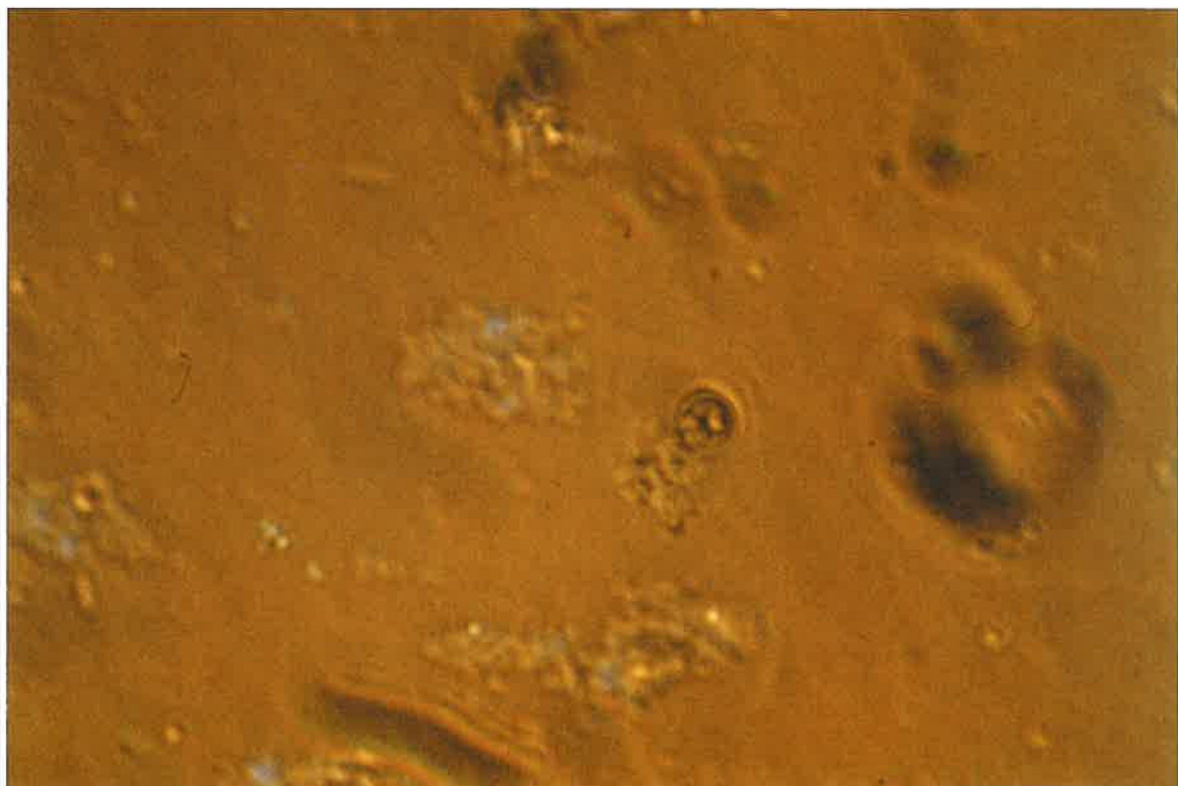


Figure 8 *Giardia* cysts stained with FITC-conjugated monoclonal antibody

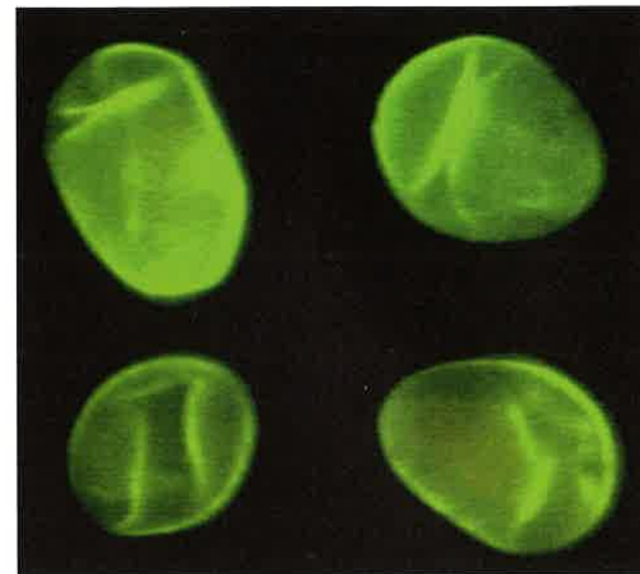


Figure 9 Example of autofluorescing yeasts which may interfere with the identification of *Cryptosporidium* oocysts, shown under DIC illumination (left) and UV illumination (right)

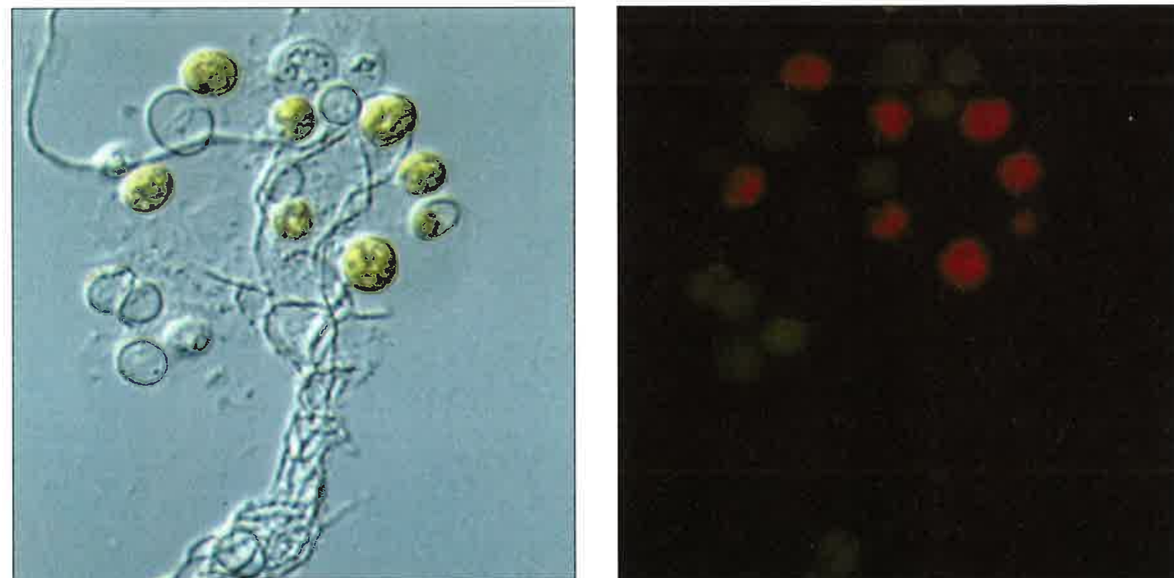


Figure 10 Comparison of a *Cryptosporidium* oocyst with other environmental organisms which have also been stained by FITC-conjugated monoclonal antibody

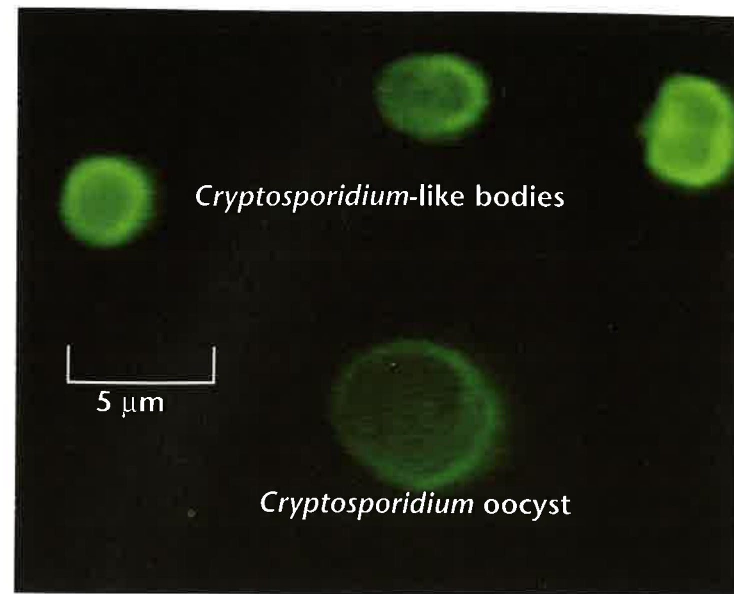
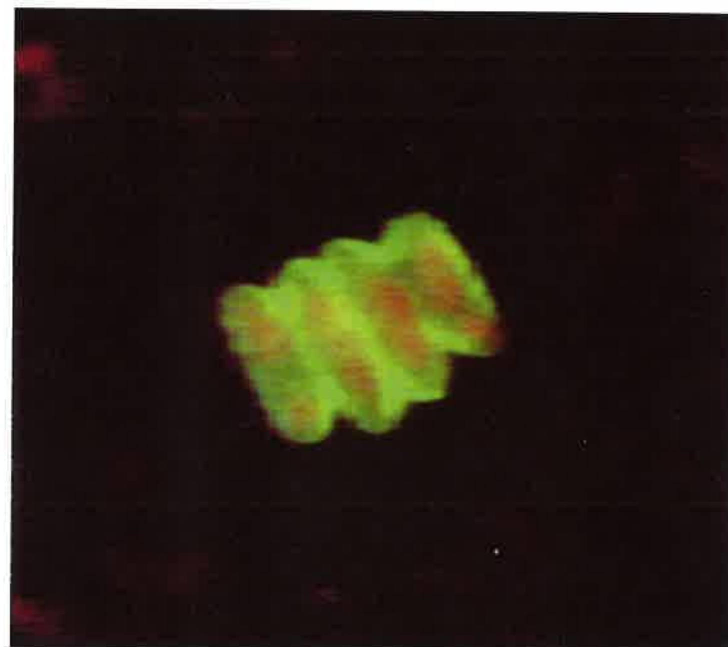


Figure 11 Algal cell stained with FITC-conjugated monoclonal antibody



APPENDIX A

Summary of recovery data. The following tables contain summaries of typical recovery efficiencies for methods for the enumeration of *Cryptosporidium* oocysts and *Giardia* cysts in waters.

Table A1 Typical recovery efficiencies quoted for methods for the enumeration of *Cryptosporidium* oocysts in water

Water type	Concentration method	Sample clean-up	Recoveries (%)	Reference
Various	Polypropylene wound filters	Various	9-59	2
Tap	Polypropylene wound filter	Sucrose gradient	14-21	9
Raw and treated	Polypropylene wound filter	Percoll-sucrose gradient	8 (mean)	6
Tap	Polypropylene wound filters	Sucrose gradient	0-12	7
		None	2-17	7
River	Polypropylene wound filters	Sucrose gradient	0-3	7
		None	3-17	7
Tap	Polypropylene wound filter	Sucrose gradient	3-29	14
Tap	Polypropylene wound filter	Sucrose gradient	0-32	16
Tap plus particulates	142 mm acrylic copolymer membrane 1.2 μm pore	Citrate gradient	20-37	4
Raw and treated	142/293 mm polycarbonate membrane 2.0 μm pore	Percoll-percoll gradient	9 (mean)	6
Tap	142 mm cellulose acetate membrane 1.2 μm pore	None	85-96	9
Distilled water	142 mm cellulose nitrate membrane 1.2 μm pore	None	31-52	5
Tap	142 mm cellulose acetate membrane 1.2 μm pore	None	25-56	7,8
	142 mm cellulose nitrate membrane 3.0 μm pore	None	19-36	7,8
	142 mm acrylic copolymer membrane 1.2/3.0 μm pore	None	10-36	7,8
	142 mm polycarbonate membrane 2.0 μm pore	None	4-32	7,8
River	142 mm cellulose acetate	Sucrose gradient	1-12	7,8
	membrane 1.2 μm pore	None	2-43	7,8
	142 mm cellulose nitrate	Sucrose gradient	5-8	7,
	membrane 3.0 μm pore	None	25-36	7,8
	142 mm acrylic copolymer membrane 1.2/3.0 μm pore	None	19-41	7,8
	142 mm polycarbonate	Sucrose gradient	4-7	7,8
	membrane 2.0 μm pore	None	32-39	7,8
Not stated	293 mm cellulose acetate membrane 1.2 μm pore	None	61-87	1
Tap	142 mm cellulose nitrate	None	55-130	13
	membrane 1.2 μm pore	Sucrose gradient	38-60	13
	142 mm cellulose acetate	None	90-124	13
	membrane 1.2 μm pore	Sucrose gradient	30-57	13
River and tap	142 mm acrylic copolymer membrane 3.0 μm pore	Citrate gradient	1-97	15
Tap	142 mm cellulose nitrate membrane 1.2 μm pore	None	38-56	16
River and Tap	Calcium carbonate flocculation	Flow cytometry	69-79	11
Tap	Calcium carbonate flocculation	None	66-93	3
Tap				
Fresh oocysts	Calcium carbonate flocculation	None	61-71	9
Aged oocysts	Calcium carbonate flocculation	None	19-32	9
Tap	Calcium carbonate flocculation	Flow cytometry	20-64 (means)	10

Table A2 Typical recovery efficiencies quoted for methods for the enumeration of *Giardia* cysts in water

Water type	Concentration method	Sample clean-up	Recovery	Reference (%)
Raw and treated	Polypropylene wound filter	Percoll-sucrose gradient	12 (mean)	6
Tap	Polypropylene wound filters	None	12-23	8
River	Polypropylene wound filters	None	10-20	8
Tap	Polypropylene wound filters	Sucrose gradient	27-60	14
Raw and treated	142/293 mm polycarbonate membrane 2.0 µm pore	Percoll-percoll gradient	49 (mean)	6
Distilled water	142 mm cellulose nitrate membrane 1.2 µm pore	None	61-106	5
Tap	142 mm cellulose acetate membrane 1.2 µm pore	None	31-64	8
	142 mm cellulose nitrate membrane 3.0 µm pore	None	57-78	8
	142 mm acrylic copolymer membrane 1.2/3.0 µm pore	None	22-51	8
	142 mm polycarbonate membrane 2.0 µm pore	None	35-67	8
River	142 mm cellulose acetate membrane 1.2 µm pore	None	37-62	8
	142 mm cellulose nitrate membrane 3.0 µm pore	None	47-60	8
	142 mm acrylic copolymer membrane 1.2/3.0 µm pore	None	37-52	8
	142 mm polycarbonate membrane 2.0 µm pore	None	46-51	8
Stream	110 mm polycarbonate membrane 5.0 µm pore	Sucrose gradient- or none	~20- ~90	12
Tap	142 mm cellulose nitrate membrane 1.2 µm pore	None	53-102	13
		Sucrose gradient	16-65	13
	142 mm cellulose acetate membrane 1.2 µm pore	None	57-114	13
		Sucrose gradient	49-98	13
River and tap	142 mm acrylic copolymer membrane 3.0 µm pore	Citrate gradient	2-105	15
Tap	Calcium carbonate flocculation	Flow cytometry	33-66 (means)	10

APPENDIX B Assessment of viability of oocysts

B1 **Principle.** This fluorogenic assay is based on the use of 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) as fluorogenic vital dyes. The sporozoite nuclei of all oocysts are stained by the inclusion of DAPI which show a sky-blue fluorescence (DAPI+). In addition, non-viable oocysts are stained by PI and these show a red fluorescence (PI+). The contents of viable oocysts are not stained by PI (PI-). Examples of DAPI+, PI+ and PI- oocysts are shown in Figures B1 and B2. Occasionally, oocysts will fail to stain with DAPI or PI (DAPI-/PI-). This may be because the oocysts do not contain sporozoites (ie are non-viable) or alternatively, the oocyst may contain sporozoites but have failed to stain (ie are potentially viable). The presence or absence of sporozoites can be demonstrated by using DIC or phase contrast illumination to observe internal contents (see Figures 6 and 7). Empty oocysts are non-refractile whereas oocysts containing sporozoites are highly refractile. Staining with DAPI may be maximised by pre-incubation in acid conditions before staining with the vital dyes. Occasionally, the cytoplasm and sporozoite nuclei will include DAPI (DAPI+*/PI-) and these appear as a diffusely stained oocyst which should be considered as non-viable.

Oocysts are pre-incubated in acidified Hanks Balanced Salt Solution (HBSS) before being washed and stained with the vital dyes. Towards the end of staining, FITC conjugated anti-*Cryptosporidium* monoclonal antibody is added to stain the oocyst wall. Stained oocysts are washed before being viewed by epifluorescence and DIC or phase contrast illumination using a x100 oil immersion objective. Each oocyst is examined and assessed as DAPI+/PI- (ie viable); DAPI-/PI+ (ie non-viable); DAPI-/PI- (ie either as potentially viable, or if empty ghosts as non-viable); or DAPI*+/PI- (ie non-viable).

B2 **Reagents.** Both fluorogenic dyes are toxic and disposable gloves should be worn throughout the procedure.

B2.1 **Hanks' balanced salts solution (HBSS).** Prepare a solution of the following compounds in water, containing per litre:

Calcium chloride dihydrate	0.185 g
Potassium chloride	0.40 g
Potassium dihydrogen phosphate	0.06 g
Magnesium chloride hexahydrate	0.10 g
Magnesium sulphate heptahydrate	0.10 g
Sodium chloride	8.0 g
Disodium hydrogen phosphate	0.48 g
D-glucose	1.0 g

Alternatively, this solution may be obtained commercially.

B2.2 **Acidified Hanks' balanced salts solution.** Adjust Hanks' balanced salts solution (B2.1) to pH 2.75 with 1 molar hydrochloric acid.

B2.3 **4',6-diamidino-2-phenylindole solution.** (2 mgml^{-1}). Prepare a solution of absolute methanol containing 2 mgml^{-1} of 4',6-diamidino-2-phenylindole (DAPI). Store in the dark at refrigeration temperature.

B2.4 **Propidium iodide solution.** (1 mgml^{-1}). Prepare a solution of phosphate buffered saline (3.1.1.2) containing 1 mgml^{-1} of propidium iodide (PI).

B3 **Equipment.** The following equipment is required:

- Microscope. See appendix C. This should be fitted with suitable filters, an ultraviolet filter (350 nm excitation, >450 nm emission) for DAPI staining, and a green filter (535 nm excitation, >590 nm emission) for PI staining.
- Incubator. This should be set to 37 ± 0.5 °C.
- Vortex mixer.

B4 **Procedure.** Incubate 100 µl of oocyst-containing deposit with 1 ml of acidified HBSS (B2.2) at 37 °C for 1 hour in a 1.5 ml microcentrifuge tube.

Wash the suspension three times in HBSS (B2.1) by centrifugation, for example at 10000g for 1 minute. Aspirate the supernatant liquid to just less than 100 µl.

Resuspend the deposit in the residual liquid by vortex mixing and adjust the volume to 100 µl with HBSS (B2.1).

Add 10 µl of DAPI stain (B2.3) and 10 µl of PI stain (B2.4). Mix, by vortex mixing, and incubate at 37 °C in the dark for 1.5 hours.

Add 100 µl of *Cryptosporidium* monoclonal antibody (4.1.1) and incubate at 37 °C in the dark for 30 minutes.

Make the volume up to 1.5 ml with HBSS (B2.1) and centrifuge.

Aspirate the supernatant to 100 µl, resuspend in 1 ml of HBSS (B2.1) and centrifuge. Repeat this step two more times.

Resuspend the pellet in 100 µl of HBSS (B2.1). Dispense the 100 µl of HBSS (B2.1) in 10 µl aliquots to a series of microscope slides. Cover with a coverslip. Avoid trapping air bubbles and seal, if necessary, the edges of the coverslip, for example with nail varnish.

The 10 µl aliquots of suspension are then examined using DIC and epifluorescence. From their reaction with the stains the oocysts may be described as:

Ghost oocysts	These are readily visible under DIC or phase contrast illumination as ruptured oocysts that are non-refractile apart from the residual body;
PI+ oocysts	Positive oocysts fluoresce bright red under the green light. The fluorescence varies with distinct intense points corresponding to the locations of sporozoite nuclei, to a more diffuse fluorescence within the oocyst (Figure B1);
PI- oocysts	Negative oocysts do not show any fluorescence under green light.
DAPI+	The nuclei of the sporozoites of DAPI positive oocysts fluoresce a distinct sky-blue under the UV light (Figure B2);
DAPI-	These oocysts show either a rim of fluorescence or an absence of fluorescence under UV light;
DAPI+*	Occasionally, the cytoplasm of the oocyst as well as the sporozoite nuclei takes up the DAPI stain and fluoresces a sky-blue colour under the UV light.

Record the numbers of oocysts that are:

ruptured (ghost)	ie those that are readily visible as ruptured oocysts,
PI+	ie those that fluoresce bright red under green light,
DAPI+ and PI-	ie those that fluoresce sky-blue under UV light and those that do not show any fluorescence under green light,
DAPI- and PI-	ie those that do not show any fluorescence under green light,
DAPI+* and PI-	ie those where the cytoplasm fluoresces sky-blue and those that do not show any fluorescence under green light.

Where possible, examine at least 100 oocysts. Using the data collected, calculate the proportion of viable oocysts and the proportion of potentially viable oocysts. The proportion of the oocyst population considered viable is calculated by dividing the number of oocysts that are (DAPI+/PI-) by the total number of oocysts observed, including 'ghosts'.

The proportion of potentially viable oocysts is calculated by taking the sum of those oocysts that are (DAPI+/PI-) and those that are (DAPI-/PI-) and dividing this combined value by the total number of oocysts. It is recognised that not all those classed as (DAPI-/PI-) will convert (under the conditions used) to (DAPI+/PI-) but are considered as potentially likely to convert. Hence all (DAPI-/PI-) are considered as potentially viable. It is noted that those classed as (DAPI+*/PI-) are not included. An example of these calculations is shown in Appendix D.

Figure B1 *Cryptosporidium parvum* oocysts stained by propidium iodide (PI+)

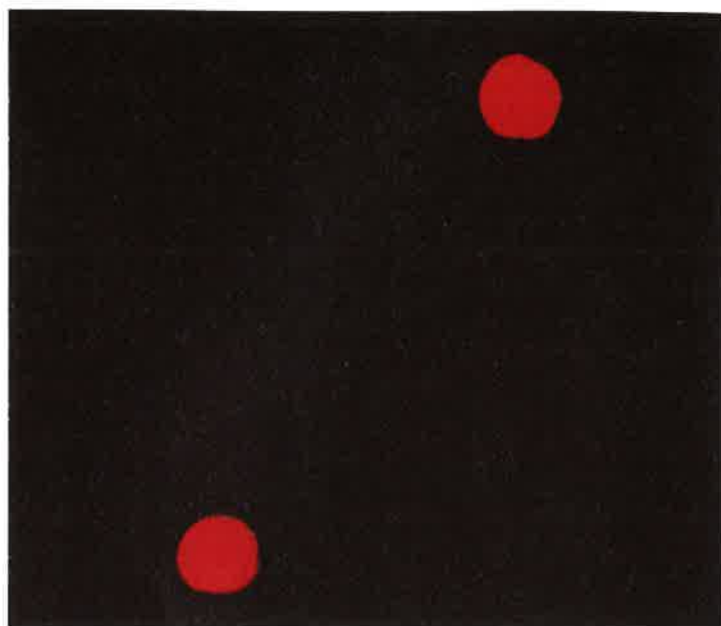
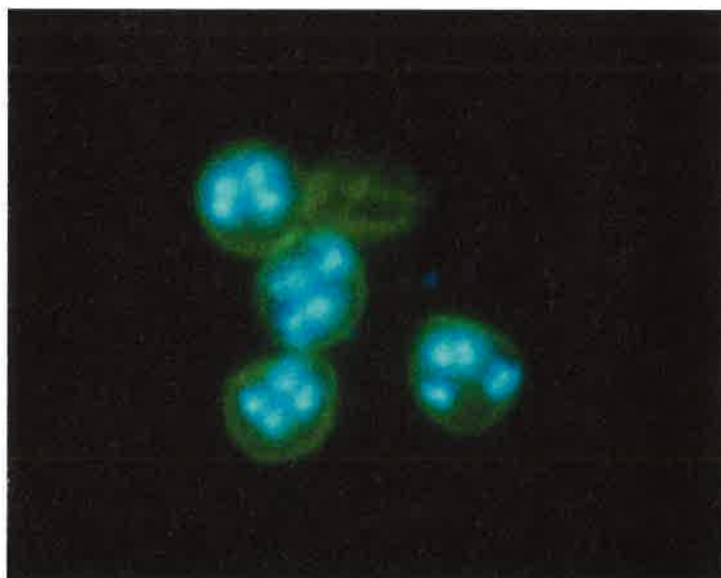


Figure B2 *Cryptosporidium parvum* oocyst stained by 4',6-diamidino-2-phenylindole (DAPI+)



APPENDIX C Microscope specification and alignment.

C1 **Equipment.** The following equipment is required:

- A microscope capable of epifluorescence and differential interference contrast (DIC) illumination and equipped with objectives of 20x and 40x magnification, of long working length and with a minimum numerical aperture (NA) of 0.5, for scanning the slide, as well as 100x magnification (NA 1.25-1.3) oil immersion objective for detailed examination of objects.
- The microscope should be fitted with appropriate excitation and band pass filters for examining fluorescein isothiocyanate-labelled specimens. A proven practice is to use a choice of long pass, band pass and interference filters which, while limiting excitation (475 - 495 nm; 505 dichroic; 515 nm long pass) and emission (20 nm band pass) wavelengths, produce a characteristic 10-15 nm wavelength which, with the appropriate choice of interference filters, optimises specificity and transmission of FITC.
- Eyepiece and stage micrometer kit.

C2 **Adjustment of the instrument.** The effective use of the microscope depends upon very sophisticated optics. Without proper alignment and adjustment the instrument will not function at maximum efficiency. Consequently, it is imperative that the light path from the light source to both eyepieces is properly adjusted.

Although microscopes from various manufacturers differ in detail, they all operate on the same general principles. Therefore, slight deviations or adjustments may be required to make these guidelines work for particular instruments and reference should be made to the manufacturer's handbook. The controls of a typical microscope are shown in Figure C1.

C2.1 **Light bulb filament adjustment.** The purpose of these procedures is to ensure even field illumination.

C2.1.1 **Mercury bulb adjustment.** Neither the ultraviolet light emanating from the mercury lamp house, nor the ultraviolet light image should be directly observed without a barrier filter in place. Ensure that no glass portion of the mercury bulb is touched with bare fingers while it is being installed.

These instructions assume the condenser of the microscope is adjusted to produce Köhler illumination (section C2.4).

There is, usually, a diffuser lens between the lamp and the microscope which should either be removed or swung out of the light path.

Using a prepared microscope slide, adjust the focus so that the image in both eyepieces is sharply defined.

Replace the slide with, for example, a business card or a piece of lens tissue. Close the field diaphragm (iris diaphragm in the microscope base) so that only a small point of light is visible on the card or tissue. This dot of light provides information on where the centre of the field of view is located.

Mount the mercury lamp house on the microscope without the diffuser lens in place and turn on the mercury bulb.

Remove the objective in the light path from the nosepiece. A primary (brighter) and secondary (dimmer) image of the mercury bulb arc should be observed on the card or tissue after focusing the image with the appropriate adjustment.

Using the other lamp house adjustments, adjust the primary and secondary mercury bulb images so they are side by side (parallel to each other) with the transmitted light dot between them.

Re-attach the objective to the nosepiece.

Insert the diffuser lens into the light path between the mercury lamp house and the microscope.

Turn off the transmitted light, remove the card or tissue from the stage, and replace it with a slide of fluorescent material. Check the field for even fluorescent illumination. Adjustment of the diffuser lens will most likely be required.

Maintain a log of the number of hours the mercury bulb has been in use. The bulb should not be used for longer than it is rated. For example, a 50 watt bulb should not be used longer than 100 hours and a 100 watt bulb should not be used longer than 200 hours.

C2.1.2 **Transmitted light bulb adjustment.** Ensure that no glass portion of the transmitted light bulb is touched with bare fingers while installing it. These instructions assume the condenser has been adjusted to produce Köhler illumination (section C2.4).

There is, usually, a diffuser lens between the lamp and the microscope which should either be removed or swung out of the light path. Re-attach the lamp house to the microscope.

Using a prepared microscope slide and a 40x (or similar) objective, adjust the focus so that the image in both eyepieces is sharply defined.

Without the eyepiece or Bertrand optics in place, the pupil and filament image inside can be seen at the bottom of the tube.

Focus the lamp filament image with the appropriate adjustment on the lamp housing.

Similarly, centre the lamp filament image within the pupil with the appropriate adjustment(s) on the lamp housing.

Insert the diffuser lens into the light path between the transmitted light lamp and the microscope.

C2.2 **Adjustment of eyepieces.** These adjustments are necessary, to reduce eye strain to a minimum and should be made for each individual using the microscope.

C2.2.1 **Inter-pupillary distance**

Place a prepared slide on the microscope stage, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.

Using both hands, adjust both eyepieces until a single circle of light is observed while looking through both eyepieces (with both eyes).

C2.2.2 **Eyepiece adjustment for each eye.** Persons with astigmatic eyes should wear their contact lenses or glasses when using the microscope.

This section assumes a focusing eyepiece is fitted to the microscope and that it is the right eyepiece.

Place a prepared slide on the microscope stage, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment.

Place, for example, a business card or lens tissue between the right eyepiece and right eye, but keep both eyes open. Using the fine adjustment, focus the image for the left eye to its sharpest point.

Transfer the card or tissue between the left eye and left eyepiece. Keeping both eyes open, bring the image for the right eye into sharp focus by adjusting the eyepiece collar at the top of the eyepiece, without touching the coarse or fine adjustment.

C2.3 **Calibration of eyepiece micrometer.** This section assumes that an eyepiece micrometer or reticule has been installed and that a stage micrometer is available for calibrating the eyepiece reticule. Once installed, the eyepiece reticule should be left in place. This calibration should be done for each objective in use on the microscope.

Place the stage micrometer on the microscope stage, turn on the transmitted light, and focus the micrometer image using the coarse and fine adjustment for the objective to be calibrated. Continue adjusting the focus on the stage micrometer until it is possible to distinguish between the large (0.1 mm) and the small (0.01 mm) divisions.

Adjust the stage and eyepiece with the micrometer so the zero-line on the eyepiece micrometer is exactly superimposed on the zero line on the stage micrometer.

Without changing the stage adjustment, find a point as distant as possible from the two zero lines where two other lines are exactly superimposed.

Determine the number of eyepiece micrometer spaces and the number of millimetres on the stage micrometer between the two points of superimposition.

Calculate the size, M, (in µm) of each eyepiece micrometer space using the equation:

$$M = S \times 1000 / E$$

where S is the stage micrometer reading in mm; and
E is the number of eyepiece micrometer spaces.

It is helpful to record this information for each objective.

C2.4 **Köhler illumination.** This section assumes that Köhler illumination will be established for only the 100x magnification oil immersion objective which is used to identify internal morphology of the *Cryptosporidium* oocysts.

If more than one objective is to be used for DIC optics then Köhler illumination should be re-established each time the objective is changed. See previous sections for adjustment of eyepieces and light sources. If Köhler illumination is not properly established, then the DIC optics will not work to their maximum potential.

Place a prepared slide on the microscope stage, place oil on the slide, position the 100x magnification oil immersion objective into place, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment.

Both the radiant field diaphragm in the microscope base and the aperture diaphragm in the condenser should be wide open. Close the radiant field diaphragm in the microscope base until the light field is reduced to a small opening.

Using the condenser screws, move the light portion of the field to the centre of the visual field.

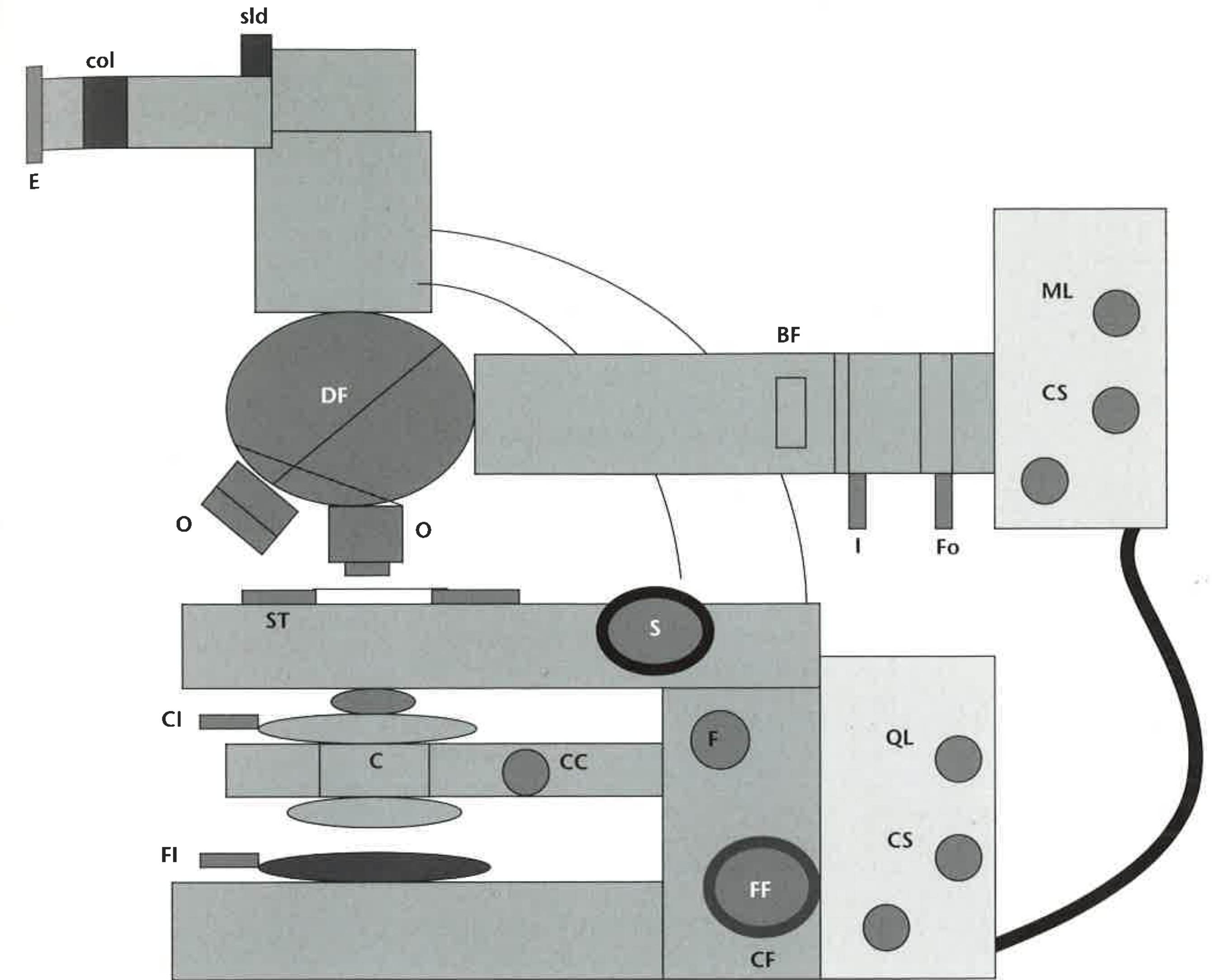
Looking through both eyepieces, adjust the condenser focusing so that the leaves of the iris field diaphragm are sharply defined. Open the radiant field diaphragm until the leaves just disappear from view.

The aperture diaphragm of the condenser is adjusted to make it compatible with the total numerical aperture of the optical system. This is done by removing an eyepiece, looking into the tube at the rear focal plane of the objective, and closing the aperture diaphragm iris until the leaves are just visible inside the rear plane of the objective.

Return the eyepiece to its tube and proceed with the adjustments required to establish DIC illumination.

C2.5 **Phase contrast illumination.** With the microscope already set up for Köhler illumination, select the phase contrast objective. Select the substage phase ring to match the objective until the number matching the objective magnification is selected. Remove an eyepiece and replace with a phase telescope. Adjust the phase telescope to focus sharply on the phase rings. Centre the substage ring by careful adjustment of the condenser phase ring adjusters until the two rings are perfectly superimposed. This series of operations will need to be repeated for each magnification required. Remove the phase telescope and replace the eyepiece. The microscope is now ready for phase contrast microscopy. The entire procedure will need to be repeated at regular intervals to check the set-up and ensure that the quality of phase contrast has not deteriorated.

Figure C1 **Typical controls of an incident light fluorescent microscope**



E = eyepiece (col = eyepiece collar: sld=eyepiece slide adjustment)
O = objectives on nosepiece
ST = stage (S = stage movement control)
C = condenser (Cl = condenser iris: CC = condenser centering screws)
F = condenser focus knob
Fl = field iris
FF = microscope fine focus adjustment
CF = microscope coarse focus adjustment
QL = quartz-iodine lamp (normal illumination)
ML = mercury lamp for incident fluorescence
DF = dichroic filter (BF = blanking and filter slide: I = iris: Fo=focus lens)
CS = centering screws for lamps and mirrors

APPENDIX D Calculations

D1 Number of oocysts per litre of sample. Calculate the fraction, E, of the final concentrate examined from:

$$E = (V_a \times N_a) / V_c$$

where V_a is volume of aliquot;
N_a is number of aliquots of equal volume (Va) placed on slide or membrane; and
V_c is volume of final concentrate.

Calculate the fraction, F, of the initial concentrate used to prepare the final concentrate from:

$$F = V_f / V_t$$

where V_f is volume of initial concentrate subject to flotation; and
V_t is total volume of initial concentrate.

If the initial concentrate is not further concentrated or subjected to a flotation stage, or if all the initial concentrate is concentrated, the value of F is 1.

Calculate the number of oocysts per litre, O, of the original sample from:

$$O = C / (V \times F \times E)$$

where C is the total number of oocysts counted on the microscope slide;
V is the volume (in litres) of the water sample;
F is the fraction of the initial concentrate which is further concentrated; and
E is the fraction of final concentrate that is examined on the slide or membrane.

If, for example, 350 litres of water are passed through a filter and the entrapped material is washed out and concentrated to 4 ml; and 2 ml of this concentrate is stored and 2 ml concentrated by a sucrose gradient to 1 ml; and four aliquots, each of 20 µl, are examined microscopically; and a total of 15 oocysts are observed, then

$$E = (0.02 \times 4) / 1 = 0.08$$

$$F = 2 / 4 = 0.5$$

Using C = 15 and V = 350,

$$O = 15 / (350 \times 0.5 \times 0.08) = 1.07 \text{ oocysts per litre}$$

D2 Proportion of viable oocysts. The proportion of viable oocysts is calculated by dividing the number of DAPI+/PI- oocysts by the total number of oocysts observed, including 'ghosts'.

The proportion of potentially viable oocysts is calculated by taking the sum of DAPI+/PI- and DAPI-/PI- oocysts and dividing by the total number of oocysts.

If, for example, a total of 81 oocysts is observed comprising two 'ghost' oocysts, 13 PI+ oocysts, 62 DAPI+/PI- oocysts, three DAPI-/PI- oocysts, and one DAPI+/PI- oocyst, then the percentage of viable oocysts is calculated from

$$\frac{\text{DAPI + /PI-}}{\text{total observed}} \times 100 = \frac{62}{81} \times 100 = 76.5\ \%$$

and the percentage of potentially viable oocysts is calculated from

$$\frac{\text{(DAPI+/PI-) + (DAPI-/PI-)}}{\text{total observed}} \times 100 = \frac{62 + 3}{81} \times 100 = 80.2\ \%$$

APPENDIX E Laboratory equipment

Thermometers. The accuracy of thermometers or other temperature-measuring instruments require periodic checking against certified reference thermometers. Thermometers should be capable of recording differences of 0.5 °C.

pH meters. pH meters will require regular standardisation and should be calibrated before use, using standard buffer solutions. Buffer solutions with pH values in appropriate ranges are commercially available.

Balances. Balances should have a range of sensitivity appropriate for the amount of substance to be weighed. Balances should be kept clean, serviced periodically and be subjected to calibration checks prior to use.

Refrigerators. Refrigerators should have their operating temperatures (normally, between 2 - 8 °C) checked regularly and de-frosted at regular intervals. Stored items should be inspected periodically and discarded at the end of their storage life.

Freezers. Freezers require regular temperature checks to ensure that they comply with the manufacturer's specifications and should be de-frosted at regular intervals. They may be fitted with over-temperature alarms. Their contents should be inspected periodically and unwanted items discarded.

Microscopes. The optics and stage should be cleaned regularly. Fluorescence microscopes require regular calibration and maintenance. Appropriate procedures are described in Appendix C.

Glassware and reagents. All glassware should be cleaned thoroughly before use. In many cases, the use of pre-sterilized disposable plastic alternatives is appropriate to prevent potential cross contamination. The accuracy of volumetric equipment such as pipettes should be traceable to national standards. Water used in the preparation of reagents should be of appropriate quality. Chemicals used in the preparation of reagents should also be of appropriate analytical quality.

Water meters. Water meters should have appropriate calibration certificates and be subject to regular calibration checks.

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Address for correspondence

However well a method is tested, there is always the possibility of discovering a hitherto unknown problem. Users with information on these procedures are requested to write to the address below:

The Secretary
Standing Committee of Analysts
Environment Agency
11 Tothill Street
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Members assisting with this booklet

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P Jiggins
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