Isolation and Identification of Giardia Cysts, Cryptosporidium Oocysts and Free Living Pathogenic Amoebae in Water etc 1989

Methods for the Examination of Waters and Associated Materials

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Isolation and
identification of giardia
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Methods for the Examination of Waters and Associated Materials

1. This booklet contains 2 sets of provisional procedures. The first is for detecting parasites chiefly intestinal in origin: Giardia, Cryptosporidium and *Entamoeba histolytica.* The second is for detecting certain other waterborne amoebae harmful to man.

2. Proprietary products used in the development and testing of these methods have been mentioned. Other products of proven similar or superior quality may be substituted after thorough examination.

3. Although well tested, these methods are only rated as Provisional because further research may lead to considerable improvement in the future.

4. Bear in mind that for Cryptosporidium and Entamoeba, and sometimes for Giardia, water is only one of the possible means of infection. If an outbreak of disease is being investigated, a thorough epidemiological study should be made, investigating all means of infection. For Cryptosporidium and all amoebae, strict attention to laboratory cleanliness is essential, to prevent air- or apparatus-borne contamination of the samples during examination

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About This Series

This booklet is part of a series intended to provide recommended methods for determining the quality of water and associated materials. In addition short reviews of the more important analytical techniques of interest to the water and sewage industries are included.

In the past, the Department of the Environment and its predecessors, in collaboration with various learned societies, have issued volumes of methods for the analysis of water and sewage culminating in 'Analysis of Raw, Potable and Waste Waters'. These volumes inevitably took some years to prepare so that they were often partially out of date before they appeared in print. The present series is published as a series of booklets on single or related topics; thus allowing for the replacement or addition of methods as quickly as possible without need of waiting for the next edition. The rate of publication will also be related to the urgency of requirement for that particular method.

Although ideally, all methods published should be fully tested, this is not often possible without delay in publication. Furthermore, the limit of detection, range, precision and interference effects applying to instrumental methods can be dependent on the actual instrument used, as well as on sample type, reagent purity, operator skill, etc. Even methods tested in many laboratories have been known to acquire problems when a new domestic product appeared (introducing a new substance into effluents), changes in production methods altered reagent quality, or the method was used to analyse a new type of sample (despite apparent similarity to samples already evaluated). As a guide, the following categories have been given to methods:
tested, usually in five or more laboratories
– no grade indicated;
tested in one to three or four laboratories
– Tentative;
evaluated, but not fully tested, but publication is urgently required
– Note;
tested and found to be satisfactory by several laboratories, but in the opinion of experts requires a high degree of skill or has some other difficulty such that the method would be replaced if a better method were discovered
– Provisional.

The aim is to provide as complete and up to date a collection of methods and reviews as is practicable, which will, as far as possible, take into account the analytical facilities available in different parts of the Kingdom, and the quality criteria of interest to those responsible for the various aspects of the water cycle. Because both needs and equipment vary widely, where necessary, a selection of methods may be recommended for a single determinand. It will be the responsibility of the users, the senior technical staff, to decide which of these methods to use for the determination in hand. Whilst the attention of users is drawn to any special known hazards which may occur with the use of any particular method, responsibility for proper supervision and the provision of safe working conditions must remain with the user.

The preparation of this series and its continuous revision is the responsibility of the Standing Committee of Analysts (to review Standard Methods for Quality Control of the Water cycle). The Standing Committee of Analysts is a committee of the Department of the Environment set up in 1972. Currently it has nine Working Groups each responsible for one section or aspects of water cycle quality analysis. They are as follows:

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 Nonmetallic Substances
6.0 Organic Impurities
7.0 Biological Monitoring
8.0 Sewage Works Control Methods
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 the main committee. The names of those associated with this method are listed inside the back cover.

Publication of new or revised methods will be notified to the technical press, whilst a list of Methods in Print is given in the current HMSO Sectional Publication List No 5.

Whilst an effort is made to prevent errors from occurring in the published text, a few errors have been found in booklets in this series. Correction notes and minor additions to published booklets not warranting a new booklet in this series will be issued periodically as the need arises. Should an error be found affecting the operation of a method, the true sense not being obvious, or an error in the printed text be discovered prior to sale, a separate correction note will be issued for inclusion in that booklet.

L R PITTWELL
Chairman & Secretary

1 February 1990

Warning to Users

The analytical procedures given in this booklet should only be carried out by competent trained persons, with adequate supervision when necessary.

Local Safety Regulations must be observed.

Laboratory procedures should be carried out only in properly equipped laboratories.

Field Operations should be conducted with due regard to possible local hazards, and portable safety equipment should be carried.

Care should be taken against creating hazards for one's self, one's colleagues, those outside the laboratory or workplace, or subsequently for maintenance or waste disposal workers. Where the Committee have considered that a special unusual hazard exists, attention has been drawn to this in the text, so that additional care might be taken beyond that which should be exercised at all times when carrying out analytical procedures. Reagents of adequate purity must be used along with properly maintained apparatus and equipment of correct specifications. Specifications for reagents, apparatus and equipment are given in manufacturers' catalogues and various published standards, if contamination is suspected, reagent purity should be checked before use.

The best safeguard is a through consideration of hazards and the consequent safety precautions and remedies well in advance. Without intending to give a complete checklist, points that experience has shown are often forgotten include: laboratory tidiness, stray radiation leaks (including ultra violet) use of correct protective clothing and goggles, removal of toxic fumes and wastes, containment in the event of breakage, access to taps, escape routes, and the accessibility of the correct and properly maintained first-aid, fire-fighting and rescue equipment. Hazardous reagents and solutions should always be stored in plain sight and below face level. Attention should also be given to potential vapour and fire risks. If in doubt, it is safer to assume that the hazard may exist and take reasonable precautions, rather than to assume that no hazard exists until proved otherwise.

There are numerous handbooks on first aid and laboratory safety. Among such publications are: 'Safe practices in Chemical Laboratories' and 'Hazards in the Chemical Laboratory', issued by the Royal Society of Chemistry, London: 'Safety in Biological Laboratories' (Editors Hartree and Booth), Biochemical Society Special Publication No 5, The Biochemical Society, London, which includes biological hazards; and 'The Prevention of Laboratory Acquired Infection', Public Health Laboratory Services Monograph 6, HMSO, London.

It cannot be too strongly emphasised that prompt first aid, decontamination, or administration of the correct antidote can save life; but that incorrect treatment can make matters worse. It is suggested that both supervisors and operators be familiar with emergency procedures before starting even a slightly hazardous operation, and that doctors consulted after any accident involving chemical contamination, ingestion or inhalation, be made familiar with the chemical nature of the injury, as some chemical injuries require specialist treatment not normally encountered by most doctors. Similar warning should be given if a biological or radiochemical injury is suspected. Some very unusual parasites, viruses and other micro-organisms are occasionally encountered in samples and when sampling in the field. In the latter case, all equipment including footwear should be disinfected by appropriate methods if contamination is suspected. If an amulance is called or a hospital notified of an incoming patient, give information on the nature of the injury especially if poisoning is suspected, as the patient may be taken directly to a specialized hospital.

Safety while Sampling

Prior consideration must be given, especially when sampling in confined spaces or where access is difficult, to guard against suffocation, drowning, falls, and poisoning or infection by ingestion, inhalation, or skin contact.

Good Laboratory Practice

The Department of Health issue a booklet entitled: Good Laboratory Practice; the United Kingdom Compliance Programme, 1989.
This can be obtained by writing to that Department in London. It deals chiefly with toxicity studies, but much can be applied to analytical chemistry.

Methods for Isolating and Identifying *Giardia* Cysts, *Cryptosporidium* Oocysts and some Free-living Amoebae from Water, Sewage Effluent, Slurry and Sand

1. Introduction

1.1 Several protozoan parasites, including *Cryptosporidium*, *Giardia intestinalis* (= *lamblia* = *duodenalis*) and a number of amoebae, can be transmitted in water and may give rise to infections in man. The life cycle of each parasite is different; the stage which is detectable in water is the cyst for Giardia and the oocyst for *Cryptosporidium*. Tentative methods for the isolation and identification of *Giardia* cysts, *Cryptosporidium* oocysts and some amoebae from a variety of waters are given in this booklet.

1.2 Waterborne outbreaks of *Cryptosporidium* infection have been defined by epidemiological studies of clusters of cases of human cryptosporidiosis which have shown a common water source, either potable or recreational, while waterborne *Giardia* infection is well recognised. Other sources of these and related infections are known and can complicate the epidemiology. Because the presence of cysts or oocysts in water is sought well after the onset of an outbreak, and therefore after the probable peak in cyst or oocyst numbers, the number of cysts or oocysts in the water may be low. To enable detection of as little as one cyst or oocyst per litre of treated water it is necessary to concentrate up to 500 litres of water.

1.3 The methods described here are provisional. The methodology is continuously being refined, and amendments can be expected.

2. Equipment

2.1 Filter holder (AMF Cuno 1N1-FC maximum working pressure 10 bars at 20°C) with associated polythylene, nylon or silicone tubing, clips, water meter and flow restricting valve.
Cartridge filters (Cuno, Europe SA, Super Microwynd Cartouche, DPPPY, polypropylene) 1μm nominal pore size.
Flow restrictor adjustable from one to ten litres per minute. (Most laboratories standardise on either 1.5 or 4L/min. Should the flow fall off markedly, the Flow Restrictor must be checked and, if necessary, cleaned.)
Large forceps
Polyethylene bags, to carry individual used filters
Labels, indelible pens
Powered pump and power source (for raw water samples)
Water meter BS 5728/1 TWA Kent Water Meters
Equipment must be clean and free from cysts or oocysts but it need not be sterile.

2.1.1 A different model of cartridge filter* is now available and has been tested, which is easier to use, and has a higher efficiency of recovery. With these filters, after the cartridge has been removed and its outer shell taken off, the filter fabric is unwrapped and the filter layer is washed as for the AMF Cuno filter. Somewhat higher filtration rates may also be permitted. Users should check recovery rates as they will be different from those using the Cuno filter.

2.2 Beakers, glass, steel or polyethylene, 5L Kilner jars, 1 litre, or equivalent
Centrifuges, capable of 3000rpm (1500g) and of accepting Centrifuge bottles, plastic, screw top, 500mL, and
A range of disposable centrifuge tubes, graduated, e.g. 250mL
50mL
20mL
15mL
10mL

*Several of the "Polyfil" range of cartridge filters from Vokes Microfilters are suitable.

Centrifuge tubes must not be reused for sequential analysis.

Stanley knife or similar, with blades
(NOT a scalpel – they break)
Stainless steel tray to accommodate 1 cartridge filter
Gloves, disposable
Strong polyethylene bags, to contain shredded filter
Vortex mixer
Wash bottle
Vacuum source
Colworth Stomacher, Stobhill washing machine or similar device
10 mL and 50 mL syringes and stainless steel cannulae, 15 gauge, 10 cm long
Microcentrifuge suitable for Eppendorf polyethylene tubes, 1.5mL, c. 10,000G
Incubator, set at 37° + 0.5°C
or Fan oven, set not to exceed 50°C.

2.3 Epifluorescent and bright field microscope, with appropriate filters and light source for FITC fluorescence, ×25, ×40 dry objectives, ×50 water immersion, ×100 oil or water immersion objectives, eyepiece graticule and calibration slide, and preferably with Nomarski Differential Interference Contrast (DIC) optics.
Fluorescence mounting medium
Multispot microscope slides, with at least four wells of about 10mm diameter per slide
Single channel automatic variable volume pipette, 5–50µL, with disposable polyethylene tips
Humid chamber

3. Reagents

3.1 The following reagents are required:
Tween 80 (polyoxyethylene sorbitan mono-oleate) solution, 0.1% v/v in (oocyst-free) distilled water
Sterile phosphate buffered saline (PBS) 0.01M pH 7.2 – 7.5†, 2% sodium dodecyl sulphate (lauryl sulphate) w/v / 2% Tween 80 v/v solution
Antifoam A (Sigma). This is not always necessary and is not recommended if *Giardia* cysts are being sought
Sucrose solution d_{20} 1.18
Dissolve 256g sucrose (Analar) in 300mL and make up to 500mL with distilled water. Check specific gravity and adjust if necessary. Store at 4°C.
Specific monoclonal antibody (Mab). Two suitable preparations for *Cryptosporidium* oocysts are available commercially, one from Northumbria Biologicals Ltd and one from Meridian Diagnostics Incorporated (supplied by C.C. Laboratories). These should be diluted before use according to the manufacturers' instructions, and stored at 4°C. There is good but not absolute agreement between these preparations, and when looking for oocysts the use of both is recommended. Meridian Diagnostics Incorporated also market a kit for the identification of *Giardia* cysts using monoclonal antibodies.
A positive control sample consisting of a suspension of cysts or oocysts air dried on multispot slides and stored at −20°C in polyethylene bags containing silica gel
A negative control sample consisting of the distilled water used to suspend the positive control cysts or oocysts, or a concentrate from a known negative water, air dried on monospot slides.

The working strength of the monoclonal reagents MUST be assessed initially and checked periodically by diluting the antibody in PBS and preparing a series of two-fold dilutions which are each tested against an individual well of a positive control slide. The dilution which highlights the cysts or oocysts and gives an intensity of fluorescence which is 50% of that given by the lowest dilution is the END POINT of the antibody, and the working dilution is two dilutions below the end point.

Double strength Lugol's iodine for *Giardia* cyst staining
1.0 g iodine
2.0 g potassium iodine
50 mL distilled water

Add the iodine to the solution of potassium iodine, mix and store at room temperature (keeps indefinitely). Make sure to avoid accidentally adding sample material to the stock.

† PBS formulation
Na_2HPO_4 anhydrous 1.07g
$NaH_2PO_4.2H_2O$ 0.39g dissolved in 1 litre of 0.145M(8.5gL^{-1}) NaCl.

Use one volume of double strength Lugol's iodine to 2 volumes of cyst suspension.

4. Sampling

4.1 Sampling points

On domestic premises, collect water from a tap off main, and a tap off tank. Also collect sediment from tanks. Whenever possible collect a hydrant wash-out sample, but do this only using a proper standpipe connection under the supervision of the water supplier. At water treatment works collect water from taps representing fully treated water and raw water. Source water, effluents etc can be sampled using a portable pump. It may be useful to sample the outputs from individual treatment filters before disinfection, backwash water from filters being cleaned, and settled effluent from coagulation plants.

4.2 Sample volumes

The volume sampled will depend on the type of water and the circumstances requiring investigation. 100 – 500 litre samples are used for disinfected water, and similar volumes are appropriate for good quality raw borehole water. 50 – 100L samples may be suitable for water from other abstraction points, and 10 – 50L for filter backwash waters, waste waters, sewage effluent and slurry. It is rarely satisfactory to sample less than 100L for treated waters. Sand samples should not be less than 100 grams.

The optimum flow rate through cartridge filters is 1.5L a minute, and filtrates from large volumes thus take some time to collect. For standard points, overnight sampling is often best. For non-standard points and consumer premises, the time and volume will be governed by other constraints, but as large a volume as practicable should be processed. Sampling apparatus should be fitted with flow restrictor valves and water meters to control the maximum flow and record the volumes passed. WARNING: it is better to collect smaller volumes at the prescribed flow rate than larger volumes at higher flow rates, as overall recovery of oocysts can be reduced by 20 – 50% at higher flow rates.

4.3 Procedure

Connect the filter equipment to the tap or standpipe using Jubilee clips. Ensure the water meter is downstream of the filter and that the flow through the meter is in the direction of the arrow on the top of the housing. Open the tap, expel air through the pressure release valve and run at the optimum flow rate, which is controlled automatically by the flow restriction valve. Record the meter reading in a logbook. After the required volume has passed, close the tap, record the meter reading, disconnect the filter and drain the apparatus. Return the filter to the laboratory for processing.

On some occasions it will be necessary to use the same filter holder to take another sample. In this case, disconnect the filter and drain the apparatus, unscrew the filter housing, remove the old filter cartridge and place in a clean polyethylene bag. Label clearly with sample number, location, meter readings, date and times. Wash the equipment through with at least 100 litres of water from the new sampling point and only then fit the new filter cartidge.

Collect tank sediments by suction with a clean plastic wash bottle or by syphonage – use a tube full of water to initiate a flow, and then place one end of the tube in the sediment and the other end in a sample container at a lower level. An alternative approach is to disturb the sediment in the tank with a dip jug and resample the tap off tank. For sampling raw waters and effluents a portable pump will be required. Samples of sand can be collected into clean polyethylene bags.

4.4 Safety

Sampling techniques are covered by stringent Health and Safety requirements, and the hazardous nature of some sampling procedures needs emphasis. Samplers must follow the general requirements in the Warning to Users at the front of this document. The routine collection and examination of water for parasites does not pose any particular or additional hazard extra to those recognised by water undertakers generally. In those instances where a site is under suspicion of contributing to an outbreak of waterborne disease the water undertaker, local authority or health authority responsible for the

investigation will provide any further safety advice prior to site attendance. However, care must be taken to avoid ingestion of any water suspected of containing the parasites. Wash water from filter beds at a water works, or sludge, slurry or effluent from agricultural or human waste discharge points, may contain high numbers of oocysts and other infective agents.

When sampling natural water bodies or fast flowing channels always work in pairs, with appropriate safety clothing and equipment, including ropes, harnesses and life jackets. When sampling standpipes which are located in highways take care to provide adequate hazard warnings for vehicles. Take particular care when sampling in enclosed spaces.

4.5 Sample transport and storage

Return all samples and equipment to the laboratory for processing, cleaning and disinfection. All samples must be clearly labelled.

Parasite cysts and oocysts are very resistant and can sometimes survive for very long periods in water. Advice on sample storage or transport conditions should be sought from the microbiologist who will receive the samples. In general, store samples at 4°C, and transport in a cool box if transport time is likely to be longer than four hours.

4.6 Disinfection

Conventional chemical disinfectants are not effective against all parasites. Some but not all equipment can best be disinfected by boiling or steaming. A wash in hot soapy water followed by prolonged flushing with large volumes of clean water may help.

5. Elution and concentration

5.1 On receipt of the sample, record the sample point, the date, the volume passed through the filter and any other information on the label, and assign the sample a unique laboratory number.

5.2 Wearing protective gloves, remove the filter from the housing, cut lengthwise and separate from the plastic core. When cutting, cut AWAY FROM your body, and remember that blades can snap. The filter can be cut into sections, which are tested and processed separately, or can be teased and processed as a single unit. In either event the fibres should be teased apart before elution.*

5.3 Place some or all of the filter fibres and other material into two plastic bags, one inside the other. Agitate mechanically using a Colworth stomacher for at least 10 minutes. If a Colworth stomacher is not available place the filter fibres and other material in a 5L beaker. Add a measured volume, about 750mL, of 0.1% Tween 80 and agitate mechanically with some other device. Drain the washings into a clean beaker or jar, add a further 750 to 1000 ml of 0.1% Tween 80 to the filter portion and repeat. This process can be repeated a third time if the fibres and material are still dirty or the Tween solution is discoloured. Repeat with any remaining portions of the filter until the whole filter has been eluted. Pool the eluates.

5.4 The total volume of the washing should be between 2 and 4 litres.

Centrifuge at 1500g for 10 minutes. Carefully aspirate the supernatant, using negative pressure and sucking at the meniscus only. Collect the pellets, resuspend in 0.1% Tween 80 solution and pool.

If large volume centrifugation is not available, then allow to settle at least overnight undisturbed at 4°C. Longer may be necessary.

5.5 If settling overnight at 4°C, after settling, aspirate the supernatant by negative pressure, sucking at the meniscus only, to leave about 200 mL of sediment. Suspend the sediment and decant into conical centrifuge tubes. Wash the beaker with about 40 mL of 0.1% Tween 80 and add to the sediment in the centrifuge tubes.

5.6 Concentrate the pooled pellets by centrifugation in conical centrifuge tubes at 1500g for 10 minutes.

5.7 Carefully aspirate supernatant from centrifuge tubes, sucking at the meniscus only, leaving about 3cm of fluid above the pellets.

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* See also Section 2.1.1.

5.8 Cap the tubes and mix using a vortex mixer to resuspend pellets. Transfer to a 50 mL centrifuge tube. Use a small amount of 0.1% Tween 80 solution, if necessary, to assist transfer. Top up to 50 mL with 0.1% Tween 80. Centrifuge at 1500g for 10 minutes.

5.9 Repeat 5.7 and resuspend to a final volume of 20 mL. Divide into two equal portions and store one sample in a sterile universal container at 4°C for confirmation, which may include submission later to independent expert assessment, if required, (see Quality Control, below).

5.10 At this stage, sufficient of the sample to represent the equivalent of 1L of the original sample can be examined for cysts or oocysts using the identification procedure described below in section 6. If cysts or oocysts are identified at this stage, this may be sufficient for the purpose for which the examination was undertaken, but it may give little information about cyst or oocyst numbers and distribution in the problem waters. Examination of a greater proportion of the original sample may be desirable, if so steps 5.11 onward should be followed.

5.11 If the examination at 5.10 is negative, at the discretion of the microbiologist in charge, further concentration and clarification can be used. Sucrose flotation is the procedure which has been used by most workers to date. However, the factors which most strongly influence recovery and detection of oocysts are the sample water quality and the nature of the deposited material. As these vary with the sample site, the effects of different clarification procedures differ with different samples. When sucrose flotation fails, expert advice should be sought (see 8.4 and 8.5 below).

Always record fully the procedure carried out.

5.12 The suggested procedure for suspected cryptosporidium oocysts is as follows:

Add 10 mL of 2% SDS/Tween 80 to the 10 mL pellet sample in the 50 mL centrifuge tube and vortex for 30 sec. Centrifuge at 1500g for 10 minutes.

If *Giardia* cysts are being sought, use 2% Tween 80 without SDS.

5.13 Proceed as in 5.7 above to leave 10 mL of fluid in the centrifuge tube. Vortex to resuspend the pellet.

5.14 **Either**

(a) Suck up 10 mL cold sucrose solution (d_{20} 1.18) into cannula and 10 mL syringe. Carefully underlay the suspension with the sucrose solution by inserting the tip of the cannula into the base of the centrifuge tube. Squeeze the syringe plunger slowly. The sediment will float on the sucrose layer. Do not inject air, as this will disrupt the interface.

Or

(b) Add approximately 1 volume of deposit to 4 volumes of 50% w/v sucrose solution. Mix well and adjust to d_{20} 1.18 using an appropriate hydrometer.

5.15 Centrifuge at 1000g for 5 minutes.

5.16 Recover all fluid (when using 5.14a, include the interface) by aspirating with a pipette or into a 50 mL syringe attached to a stainless steel cannula. Do not disturb the pellet. About 10 to 15 mL of fluid should be recoverable.

5.17 Place fluid from 5.16 into two clean 50 mL centrifuge tubes. Top up each to 50 mL with PBS or, when *Giardia* cysts are being sought, distilled water. Centrifuge at 1500g for 10 minutes.

5.18 Aspirate the supernatant as in 5.7. Resuspend the pellets by vortexing. Pool the resuspended pellets.

5.19 Top up to 50 mL with PBS, or, for *Giardia* cysts, distilled water, and centrifuge at 1500g for 10 minutes.

5.20 Repeat 5.18 and 5.19.

5.21 Reduce the fluid to an appropriate standard volume, usually 1 mL, by aspirating without disturbing the pellet. It is important to note this volume when it departs from the usual standard, as this will affect the final calculations of oocyst numbers. Resuspend the pellet and retain for the identification procedure.

5.22 Although it is usually possible to reduce the sample volume of treated waters to 1 mL by following steps 5.13 to 5.21, the volume of other samples such as raw waters and effluents may need to be further reduced as follows:

5.23 Reduce the volume of the suspension by further centrifugation in the same tube at 1500g for 10 minutes. Aspirate the supernatant carefully down to the 2 mL mark. Resuspend the pellet.

5.24 Transfer the suspension into 2 × 1.5 mL polyethylene Eppendorff tubes, placing equal volumes in each tube. Wash the 50 mL centrifuge tube with 0.5 mL distilled water. Transfer this to Eppendorff tubes. Cap the tubes.

5.25 Centrifuge at 10,000g for 1 minute in a microcentrifuge. Aspirate 50% of the fluid from each tube. Resuspend the pellets and pool into one tube.

5.26 Centrifuge again at 10,000g for 1 minute and aspirate to the 1 mL mark. Resuspend the pellet and retain for the identification procedure.

5.27 Liquid samples of effluents, slurry or raw waters.

Prescreen about 5 litres of sample through a coarse (50μm to 150μm) filter, but omit this step if the sample contains flocs.

5.28 Take a total volume of 4 litres of the sample and settle or centrifuge at 1500g as described in 5.4 above.

5.29 Proceed as in step 5.7, combining the deposits in up to four 50 mL centrifuge tubes, and continue.

5.30 Sand samples

Weigh up to 500g of the sample into a Kilner jar and record the weight. Add 1000 mL of 0.1% Tween 80 solution, seal the jar and shake vigorously for about one minute. Allow to stand until the sand has settled, then pour off the supernatant liquid and proceed to treat the supernatant as from 5.6 above.

6. Identification

6.1 It is important to test a representative portion of the concentrated suspension. For treated waters it is recommended that at least 25% of the original sample volume be tested for the presence of oocysts, unless these are readily seen at step 5.11.

6.2 Mark each slide with the sample number. Apply portions of only one sample to a slide. Dispense at least four replicates from the final concentrate, one per well, onto each slide with an automatic micropipette. The volume of each replicate, usually 10 to 25 μL, will depend on the type of slide used. Ensure even coverage of each well. Dry the slides in air in an incubator or fan oven. Include a positive and a negative control slide with each batch of test slides.

6.3 Follow the manufacturers' instructions when applying the Mabs. If both the Northumbria Biologicals Ltd and the Meridian Diagnostics Incorporated Mabs are used, two slides will be necessary for each sample, one for each Mab.

6.4 **Fixing.** Each manufacturer recommends a different fixing step. However, fixing by any method may distort the characteristic morphology of the oocysts. Best results are obtained by relying on adequate air drying alone, and omitting any further fixation. Fixation may be necessary for *Giardia* cysts, and the manufacturer's instructions should be followed.

6.5 Apply 10 to 25μL (depending on the size of the well) of the Mab at working strength to each spot. Ensure complete coverage of each well with the Mab. Incubate in

a humid chamber at 37°C for 30 minutes. Rinse each slide individually with a gentle stream of PBS to remove residual Mab. Immerse slides in a staining jar containing PBS. Wash in 3 changes of PBS, 5 minutes to each change. It is essential to wash thoroughly. Drain off excess moisture. The positive control slide must be washed in a separate jar to avoid possible carry-over of oocysts to the test samples. Slides from different samples must also be washed in different jars.

6.6 Remove the residual PBS on the wells of the multispot slides by allowing them to dry naturally in air, or by placing briefly in an incubator. Do NOT allow to dry out completely.

6.7 If the Northumbria Biologicals Ltd procedure is being followed, apply 1 drop of mounting medium to each sample. Avoid bridging between slide and dropper; allow the drop to fall freely. Cover with a 22 × 64 mm coverslip. Do not press the coverslip, but allow its weight to displace the mounting medium.

If the Meridian Diagnostics Incorporated indirect fluorescence procedure is being used, add the secondary antibody (FITC conjugate). Repeat steps 6.4 and 6.5, and then mount and cover as above.

6.8 Store the slides in a slide box to await microscopic examination. If the slides are not to be read immediately, mark the box with the date and time of preparation.

7. Viewing

7.1 Calibrate the microscope, using the eyepiece graticule and calibration slide, to facilitate measurement of the oocysts.

7.2 First examine the positive and negative controls to verify that the reagents as used have functioned correctly. The negative control shall not exhibit any fluorescence and the oocysts in the positive controls shall be well stained and easily distinguishable from the background material. Oocysts appear as apple green spherical objects 4 to 6 μm in diameter. If any control gives an abnormal result, report to the supervising microbiologist.

7.3 Examine each well of the multispot slide using the ×25 or ×40 objective or the ×50 water objective. Cover the whole area of the spot with vertical or horizontal sweeps. Ensure the whole area is scanned. Note any presumptive oocysts present. Look for the diagnostic suture or fold line with the ×50 or ×100 objective unless it has been seen clearly with the ×40 objective.

7.4 The criteria to be met before any viewed object is regarded as an oocyst are:

(a) Bright green fluorescence of the wall

(b) Size between 4 and 6 microns

(c) Presence of the diagnostic fold or suture line in the wall.

In many samples, not every likely object will show all the criteria. The microscopist's judgement is paramount, but no slide should be scored positive unless it contains at least one object meeting all three criteria. All relevant observations should be recorded. Fluorescence should be scored as +, ++ or +++, where + is a weak reaction and +++ is not less than that shown by the positive control. The limitations of this scoring method when comparative studies are being undertaken must be recognised.

Where practicable, examine the oocysts by phase or differential interference contrast (DIC), as further verification of structure.

7.5 The number of *Cryptosporidium* oocysts seen in each well should be recorded, and the number of oocysts per litre of the original sample calculated.

7.6 The modified (cold) Ziehl-Neelsen stain (Casemore et al 1985, Journal of Clinical Pathology **38** 137–41) may be used as an additional check on the Mab results.

7.7 *Giardia* cysts are ovoid in shape, have an obvious wall and are 8 to 14 μm in length and 7 to 10 um in width. They contain 2 to 4 nuclei, crescent shaped profile(s) and one or more longitudinal fibrils, flagellar axonemes. These features are visible in iodine stained material or under Nomarski DIC. The criteria for the identification of *Giardia* cysts are that they shall be the correct size and shape, show at least two different internal organelles by bright field (+ or − iodine) or DIC, and in the fluorescent antibody test show bright green fluorescence of the wall.

8. Quality control

8.1 The procedures described include positive and negative controls for the monoclonal antibody reagents. Other controls are essential to ensure that there is no cross-contamination from one sample to another in the field or in the laboratory. As a minimum these should include:

(1) the filtering, processing and examination of 100 litres of distilled water through the sampling equipment used. This constitutes a check on the procedures used to clean the equipment between samples.

(2) the processing and examination of an unused cartridge filter at the end of a batch of 'live' samples. This constitutes a check on the maintenance of a clean laboratory environment, and on all the techniques employed.

8.2 Any samples giving a positive fluorescence reaction with objects which do not meet the other criteria for cysts or oocysts should be referred for independent assessment.

8.3 The sample concentration and processing procedures should be validated by running experiments with 100 litre samples of water seeded with (oo)cysts. The recovery of the seed should be close to 50% of the total inoculum, and not less than 30%. The factor which most affects the efficiency of recovery is the nature of the sample water itself, so separate experiments must be undertaken for all types of water and other material samples which it is intended to process. Seeded control studies should be performed initially, before reporting any sample results, and at least twice a year thereafter.

8.4 At intervals, portions of positive and negative concentrates should be submitted for verification to one of the independent experts who have agreed to accept such material. This should be done initially when setting up the laboratory, and then at least twice a year. The independent experts are:

For *Cryptosporidium*

Dr D. Blewett

Moredun Research Institute
408 Gilmerton Road
Edinburgh
Scotland

Dr D. Casemore

Public Health Laboratory
Ysbyty Glan Clwyd
Bodelwyddan
Near Rhyl
Clwyd LL18 5UJ
Wales

For *Cryptosporidium* and *Giardia*

Drs H. Smith, R. Gilmour

Scottish Parasite Diagnostic Laboratory
Stobhill General Hospital
Glasgow G21 3UW
Scotland

Dr D. Warhurst

London School of Hygiene and Tropical Medicine
Keppel Street
London WC1E 7HT
England

Dr Joan Rose

University of Florida, USA

8.5 There are many practical issues that can arise when setting up a laboratory to perform these techniques, not least being the acquisition of specialist materials and equipment and the obtaining of representative samples from a wide range of sites.

Those who are not familiar with water treatment works, distribution systems, plumbing installations, swimming pools and the like should obtain advice from one of the water industry experts who have agreed to provide guidance. Currently, Dr C. Benton, of Strathclyde R.C. Water Dept., Dr J.S. Colbourne and Mr A. Evans, of Thames Water, and Mr J. Watkins, of Yorkshire Water, have agreed to act in this role.

9. A Note on the Dysentery Amoeba, *Entamoeba histolytica*

9.1 Introduction

The cysts of *E. histolytica* may be found in waters contaminated with sewage. Techniques for the detection of this organism in water supplies have not been properly validated, but it is the general consensus that methods concentrating *Giardia* will be effective for *Entamoeba* spp. The detection of cysts morphologically identical to those of *E. histolytica* does not have the same impact as the detection of *Giardia* or *Cryptosporidium* since species of *Entamoeba* found in wild, farm and domestic animals are not infective for man. Currently there is no commercially available antibody specific for *E. histolytica* cysts although specific antibodies reacting with the growing stages are available.

E. histolytica viability is easier to determine than that of *Giardia* or *Cryptosporidium* since reliable culture systems are available. Successful cultures can be analysed by zymodeme typing or fluorescent antibody to identify the organism. At present the culture is best done in centralised specialist laboratories – for example, the PHLS Malaria Reference Laboratory at the London School of Hygiene and Tropical Medicine will undertake culture of concentrated material routinely, and may undertake further analysis, depending on availability of staff time.

1.2 Identifying of cysts of *Entamoeba histolytica*

E. histolytica cysts are spherical with a clearly defined wall and are 10 to 15μm in diameter. They contain 1 to 4 spherical nuclei consisting of a ring of dense, iodine-staining material surrounding a clear area containing a small dot. They may contain blunt-ended bars of refractile material, not staining well with iodine but visible under a light microscope with partially closed condenser iris or DIC. Immature cysts (fewer than 4 nuclei) may contain a diffuse vacuole staining brown with iodine. When suitable antibodies become available, the green fluorescence of the cyst wall will be an additional identifying factor.

Methods for the Isolation of Pathogenic Free-Living Amoebae from Water Samples

1. Introduction

The methods recommended here were developed as a result of an evaluation of various protocols for the isolation of pathogenic free-living amoebae (FLA) from water samples (Department of the Environment contract number PECD 7/7/268).

2. Safety

Acanthamoeba and *Naegleria* include species potentially pathogenic for man. Samples thought to contain these organisms should be processed in a Containment Level 3 facility and all equipment and consumable items autoclaved before being discarded or reused. The culture manipulation of known or suspected members of these genera should be conducted in an approved biological safety cabinet. Specific information regarding the safety precautions are to be found in **Categorisation of pathogens according to hazard and categories of containment.** Advisory Committee on Dangerous Pathogens, 1984. HMSO London.

3. Equipment

3.1 Filter holders for 47mm diameter membranes, manifold unit, vacuum pump and silicone connecting tubing.

3.2 47mm diameter 0.45μm pore size cellulose acetate membranes.

3.3 Inverted light microscope with ×10 and ×20 objectives.

3.4 Vortex mixer (optional).

3.5 Incubators set at 30°C, 37°C, 42°C and 44°C.

3.6 90mm polystyrene Petri dishes, glass universal containers, plastic Pasteur pipettes, flat-bottomed 96-well microtitre plates.

3.7 Sterile swabs, wax crayons or marker pens, scalpel blades.

4. Materials and Reagents

4.1 Page's amoeba saline (PAS) at pH 6.8 – 6.9; 0.12g NaCl, 0.004g $MgSO_4 . 7H_2O$, 0.004g $CaCl_2$. 0.142g Na_2HPO_4, 0.136g KH_2PO_4, in 1 litre of distilled water. Autoclave at 121°C for 15 minutes and store at room temperature.

4.2 Non-nutrient agar – *Escherichia coli* (NNA – *E. coli*) plates; non-nutrient agar seeded with a lawn of *E. coli* on which the amoebae feed. These are prepared as follows:

4.2.1 Non-nutrient agar plates (NNA). 1.5% w/v bacteriological grade agar in PAS. Autoclave at 121°C for 15 minutes. Distribute approximate 25ml volumes into Petri dishes and dry at 37°C for 24 hours. Plates should not be dried uncovered as cysts of FLA may be present in the air of the laboratory. Plates are stored in sealed polyethylene bags at room temperature for up to 14 days.

4.2.2 *Escherichia coli* NCTC 10418 is grown on nutrient agar plates at 37°C for 24 hours. A stock culture plate can be stored at 4°C for up to 1 month. This is used to seed the entire surface area of several nutrient agar plates. Following incubation, the seeded plates are stored at room temperature in sealed polyethylene bags for up to 7 days, or at 4°C for two weeks.

4.2.3. A thick portion of *E. coli* is taken from a seeded nutrient agar plate using a sterile swab and spread over the entire surface of one NNA plate. The plates can then be stored at room temperature in sealed polythene bags for up to 7 days.

5. Sample material and collection

Surface water, mud, soil, acquatic plants, water treatment system and bathing pool filter deposits, swimming pool water and potable water samples are all suitable for the isolation of pathogenic FLA. Samples should be collected into sterile glass or polypropylene containers and the environmental temperature recorded. For chlorinated water samples, sodium thiosulphate as an 18g/L stock solution should be added to give a final concentration of 18 mg/L. Samples should be transported to the laboratory without refrigeration and processed on arrival. it is generally accepted that refrigeration is harmful to trophozoites of thermophilic amoebae and that delay in processing may lead to overgrowth of pathogenic FLA by non-pathogenic species.

6. Sample volume

The volume of water to be processed for the detection of pathogenic FLA is determined by the nature of the sample, the sample site and the purpose of the examination. A single 250ml sample of water may be sufficient for the routine monitoring of domestic drinking water or an adequately maintained chlorinated bathing pool, since if there are no FLA of any kind in 250ml there are unlikely to be any pathogenic species even in much larger volumes. Much larger volumes, up to several litres, may have to be examined in order to isolate pathogenic FLA from a site implicated epidemiologically as the possible source of a human infection. In such cases it is preferable to process the sample in several subsamples rather than as one single specimen. When examining surface waters, filterbed samples etc, the volume may need to be reduced if initial samples yield such high numbers of FLA that the presence of pathogenic species may be obscured.

7. Isolation methods

There are two methods which are recommended for the isolation of FLA. Which one should be used is determined by the nature of the sample to be examined.

7.1 Filtration

7.1.1 Thoroughly mix the water sample and filter through a 0.45μm pore size cellulose acetate membrane by suction at a flow rate not exceeding 30mL a minute. Do not allow the membrane to dry; stop filtration when 2 to 3mL of sample remains above the membrane.

7.1.2 Carefully wash the membrane **in situ** with the residual sample, using a plastic Pasteur pipette.

7.1.3 Transfer the whole of the residual sample used to wash the membrane into a sterile glass universal container. Place the membrane in the same universal, so rolled that the upper or sample surface is inward and not in contact with the wall of the universal.

7.1.4 Shake vigorously with a vortex mixer for 10 seconds.

7.1.5 Distribute the whole of the fluid from 7.1.4 over the surface of 2 or 3 NNA–*E. coli* plates and allow to absorb to dryness at room temperature. Alternatively, pour the fluid onto the surface of a single NNA–*E. coli* plate and leave at room temperature for 2 hours. Pipette off the excess fluid and allow to dry. Plates should not be dried uncovered, as cysts of FLA may be present in the air of the laboratory. Include uninoculated lawn plates as controls.

7.1.6 Divide the membrane into halves and place each, face down, on the surface of a single NNA–*E. coli* plate.

7.2 Direct plating of samples

7.2.1 Untreated water samples may contain large numbers of FLA. This can result in failure to obtain isolates of individual amoebae because of overcrowding on the plates. To avoid this possibility, unconcentrated sample volumes should be inoculated directly onto the surface of NNA–*E. coli* plates.

Pipette 1.0mL volumes of the water onto each of 3 NNA–*E. coli* plates and leave at room temperature for 2 hours. Pipette off the excess fluid and allow the plate to dry. Keep the plates covered while drying, as cysts of FLA may be present in the air of the laboratory. Include uninoculated plates as controls.

7.2.2 Inoculate **solid material** directly on NNA–*E. coli* plates.

7.2.3 Vortex **swab samples** in 2mL of PAS and culture the liquid as in 7.1.5.

8. Incubation temperatures

The upper limit (ceiling) temperatures for incubation are determined by the type of amoeba to be isolated.

Potentially pathogenic *Acanthamoeba* species will grow at 37°C. *N. australiensis* will not grow above 42°C. *N. fowleri* and *N. lovaniensis* will grow at 44°C. The following incubation temperatures are therefore recommended:

8.1 30°C. for total *Acanthamoeba* and *Naegleria*.

8.2 37°C. for pathogenic *Acanthamoeba*.

8.3 42°C. for *N. australiensis*, *N. lovaniensis* and *N. fowleri*.

8.4 44°C. for *N. lovaniensis* and *N. fowleri*.

8.5 Incubate plates inverted in sealed polyethylene bags.

9. Detection of Free-Living Amoebae

9.1 Examine plates daily for up to 7 days with the ×10 objective of the inverted light microscope.

9.2 Free-living amoebae are seen as feeding trophozoites producing tracks and clearings in the *E. coli* lawn.

9.3 Identify the areas of amoeba growth as they appear by marking the underside of the Petri dish with a marker pen or a wax crayon.

9.4 Cut out the marked areas of agar with a sterile scalpel blade and place seeded side down on a fresh NNA–*E. coli* plate.

9.5 Incubate the subcultures at the original temperature of isolation.

10. Provisional identification of *Acanthamoeba* and *Naegleria.*

It is beyond the scope of this document to detail the methods needed for the accurate identification of species of *Acanthamoeba* and *Naegleria*. Techniques such as antigenic analysis, iso-enzyme electrophoretic profiles and molecular DNA analysis are available only in a specialised laboratory. However, provisional identification may be attempted using the basic descriptions below.

10.1 Scrape off a small area of trophozoite growth using a wire loop and inoculate into one well of a microtitre plate containing 100μL of PAS.

10.2 Seal the plate and incubate at 30°C. for 30 minutes.

10.3 Observe the trophozoite morphology using the ×20 objective of the inverted microscope.

11. Morphological characteristics of *Acanthamoeba* and *Naegleria*

11.1 *Acanthamoeba* trophozoites are approximately 25 to 40μm in length and show numerous needle-like projections from the trophozoite body, termed 'acanthopodia'. A central contractile vacuole is present in the cytoplasm. Trophozoite movement is slow and polydirectional with a hyaline pseudopodium that slowly stretches out and widens.

11.2 *Acanthamoeba* cysts are formed on prolonged incubation on NNA–*E. coli* plates. Sizes range from approximately 15 to 28μm depending on the species, and are double-walled. The intermittent joining of the inner wall to the outer gives rise to a polygonal arrangement. This feature enables differentiation from other FLA.

11.3 *Naegleria* trophozoites are approximately 10 to 20μm in length and exhibit a high degree of motility. Movement is slug-like ('limax') by protrusion of distinct hyaline pseudopodia.

11.4 **Flagellation test**

A temporary flagellate phase is formed by *Naegleria* trophozoites, and can be induced by re-incubating the microtitre plate from 10.2 and observing the test wells every 30 minutes for up to 6 hours. Flagellates are seen as highly motile swimming forms which can revert to the trophozoite stage readily. It must be recognised that the ability to produce flagellate forms is not a feature unique to the *Naegleria*.

11.5 *Naegleria* cysts are produced during prolonged incubation on NNA–*E. coli* agar. They are round, double-walled and vary in size from approximately 7 to 18µm. The inner and outer cyst walls are parallel. Occasional pores, through which the trophozoite excysts, join the two walls. These pores are difficult to see using conventional light microscopy and are best observed under phase-contrast.

11.6 The maximum temperature of growth on NNA-*E. coli* agar may provide provisional species identification of *Naegleria*.

11.6.1 Growth at 37°C but not at 42°C, *N. gruberi*.

11.6.2 Growth at 42°C but not at 44°C, *N. australiensis*.

11.6.3 Growth at 44°C, *N. lovaniensis* or *N. fowleri*.

12. Quality Control

The ability of the NNA–*E. coli* plates to support the growth of reference strains of *Acanthamoeba* and *Naegleria* should be tested with every set of samples processed. Reference strains of *A. castellanii* CCAP 1501/1A and *N. Gruberi* CCAP 1518/1E are recommended and may be purchased from the Culture Collection of Algae and Protozoa, Fresh Water Biological Association, The Ferry House, Ambleside, Cumbria LA22 0LP. The strains are maintained by weekly subculture of the trophozoites on NNA–*E. coli* at 30°C. The cyst forms of the species may be stored on sealed NNA–*E. coli* plates at 4°C for at least 6 months before subculturing. This will also help familiarise the worker with the morphological characteristics of the members of these two genera. Uninoculated NNA–*E. coli* plates should also be included when processing environmental samples to serve as a negative control.

13. Reference Facilities

13.1 Isolates may be sent to Mr S. Kilvington, Public Health Laboratory, Royal United Hospital, Bath BA1 3NG or to Dr D. Warhurst, Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT for full identification.

Illustrations for Both Methods

Figure.

1	Layout of sample apparatus for Cryptosporidium and Giardia
2 a	Cutting open the filter for Cryptosporidium and Giardia
b	Teasing open the filter for Cryptosporidium and Giardia
3 a & b	Giardia cysts showing immunofluorescence with specific antibody
4	Giardia cysts stained with Iodine. Length c. 12μm Note nuclei and crescent shaped body
5	Giardia trophozoite
6 a & b	Giardia cysts under Nomarski D.I.C. Optics
7	Other material of similar size, but not Giardia or Cryptosporidium Note absence of cell contents
8 a to c	Cryptosporidium oocysts showing FITC fluorescence Note, Fig. 8 c shows numerous oocysts of *Cryptosporidium parvum* (bovine isolate) stained by immunofluorescence using the PHLS Mab/FITC conjugated stain, counterstained with Evan's Blue to quench background autofluorescence. The preparation has been photographed by epifluorescent illumination with a mercury vapour (HB050) lamp, a Leitz BG38 excitation filter and using a ×100 objective. The general appearance of intact oocysts is typical with uniformity of size (c. 4.5×5μm) and with a stained suture or fold line visible on the surface of some. On at least one oocyst the suture has opened and one oocyst has collapsed.
9 a & b	Cryptosporidium oocysts (5 μm). Stained with Modified Ziehl Neelsen (MZN) stain. Fig. 9 b is at higher magnification
10	Cryptosporidium parvum. Fluorescent sporozoites
11 a & b	Cryptosporidium oocysts. Electron Micrographs Note suture line shown clearly in Fig. 11 a
12	Entamoeba coli – the large cyst poorly focused, AND Entamoeba histolytica (12 μm) – the uninucleate cyst (in focus). Iodine stained.
13	Entamoeba histolytica trophozoite
14	Acanthamoeba cyst (16 μm)
15	Acanthamoeba trophozoite
16	Naegleria fowleri cyst (black and white photomicrograph)
17 a & b	Naegleria fowleri trophozoite Fig. 17 b black and white photomicrograph
18	Naegleria fowleri flagellate (black and white photomicrograph)
19	Stobhill Washing Machine. Container removed to show the ultrasonic vibrator

Acknowledgements

Stobhill General Hospital, Glasgow Figs 1, 3b, 6a, 6b, 8a, 10, 19.
London School of Hygiene and Tropical Medicine Figs 3a, 4, 5, 7, 12, 13, 14, 15, 17a.
Moredun Research Institute, Edinburgh Figs 9a, 9b.
Thames Water plc. Figs 2a, 2b, 8b, 11a, 11b.
PHLS Ysbyty Glan Clwyd Bodelwyddan, Wales Fig 8c.
PHLS Royal United Hospital North, Bath Figs 16, 17b, 18.

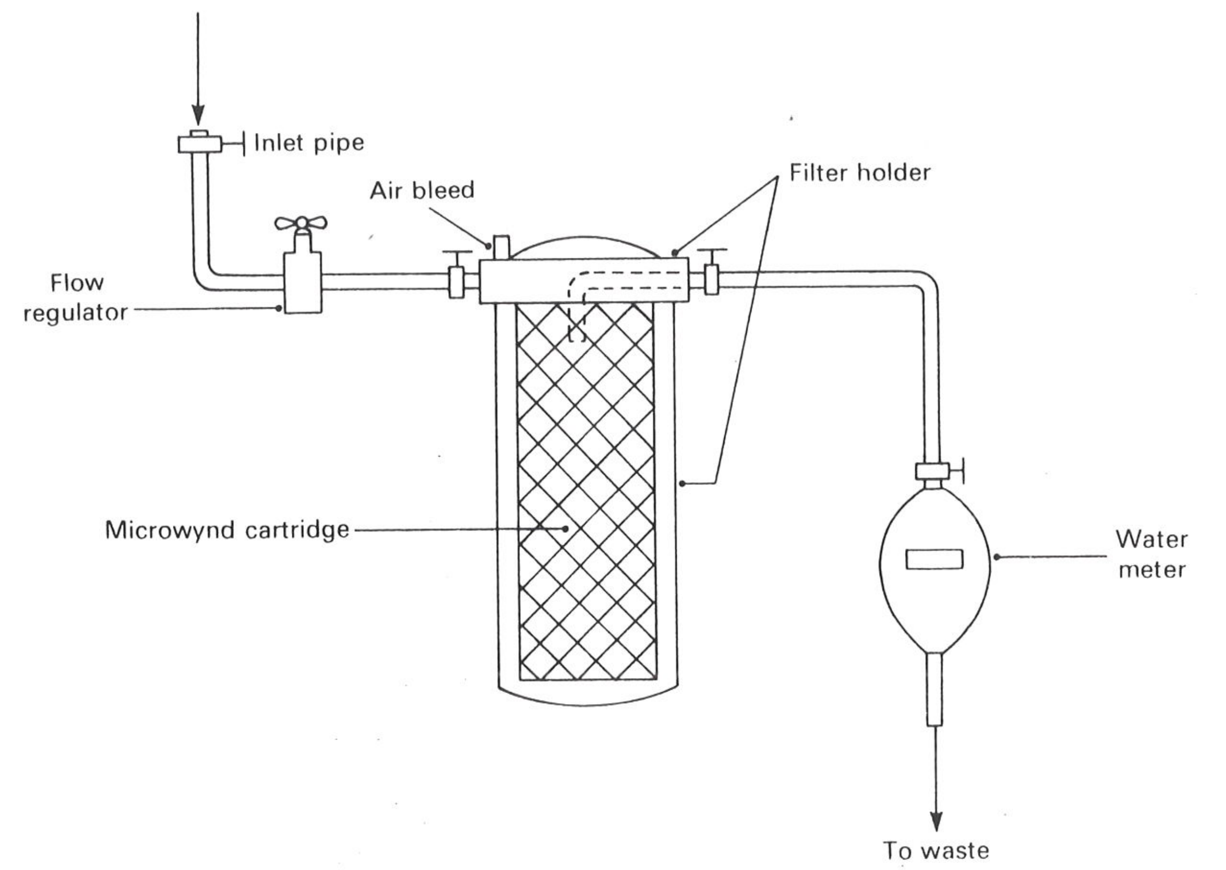

Fig. 1. Layout of sample apparatus for Cryptosporidium and Giardia.

Fig. 2a. Cutting open a Cuno filter for Cryptosporidium and Giardia.

Note, Vokes filters are of pleated fabric and are unfolded for washing, after removing the case.

Fig. 2b. Teasing open a Cuno filter for Cryptosporidium and Giardia.