Analysis of Wastewater for Use in Agriculture - A Laboratory Manual of Parasitological and Bacteriological Techniques

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1. Introduction

The use of wastewater for crop irrigation is becoming increasingly common, especially in arid and semi-arid areas. Crop yields are higher as the wastewater contains not only water for crop growth, but also plant nutrients (mainly nitrogen and phosphorus). However, there is the risk that wastewater irrigation may facilitate the transmission of excreta-related diseases. In the late 1980s, the World Health Organization, the World Bank and the International Reference Centre for Waste Disposal sponsored a series of studies and meetings of experts to examine these health risks (International Reference Centre for Waste Disposal, 1985; Shuval et al., 1986; Prost, 1988; World Health Organization, 1989). From an appraisal of the available epidemiological evidence, it was established that the major risks were:

- the transmission of intestinal nematode infections both to those working in the waste-water-irrigated fields and to those consuming vegetables grown in the fields; these infections are due to *Ascaris lumbricoides* (the human roundworm), *Trichuris trichiura* (the human whipworm), and *Ancylostoma duodenale* and *Necator americanus* (the human hookworms); and

- the transmission of faecal bacterial diseases - bacterial diarrhoea and dysentery, typhoid and cholera - to the crop consumers.

Table 1
Recommended microbiological quality guidelines for treated wastewater used for crop irrigation[a]

Category	Reuse conditions	Exposed group	Intestinal nematodes[b] (arithmetic mean no. of eggs per litre[c])	Faecal conforms (geometric mean no. per 100 ml[c])	Wastewater treatment expected to achieve the required microbiological quality
A	Irrigation of crops likely to be eaten uncooked, sports fields, public parks	Workers, consumers, public	≤1	≤1000[d]	A series of stabilization ponds designed to achieve the microbiological quality indicated, or equivalent treatment
B	Irrigation of cereal crops, industrial crops, fodder crops, pasture and trees[e]	Workers	≤1	No standard recommended	Retention in stabilization ponds for 8-10 days or equivalent helminth and faecal coliform removal
C	Localized irrigation[f] of crops in category B if exposure of workers and the public does not occur	None	Not applicable	Not applicable	Pretreatment as required by the irrigation technology, but not less than primary sedimentation

Source: World Health Organization (1989).

[a] In specific cases, local epidemiological, sociocultural and environmental factors should be taken into account, and the guidelines modified accordingly.
[b] *Ascaris* and *Trichuris* species and hookworms.
[c] During the irrigation period.
[d] A more stringent guideline (≤200 faecal coliforms per 100 ml) is appropriate for public lawns, such as hotel lawns, with which the public may come into direct contact.
[e] In the case of fruit trees, irrigation should cease two weeks before fruit is picked, and no fruit should be picked off the ground. Sprinkler irrigation should not be used.
[f] Also called drip or trickle irrigation.

In order to prevent the transmission of these diseases, it has been recommended (World Health Organization, 1989) that:

- only treated wastewaters should be used for crop irrigation; and

- the treated wastewaters should comply with the microbiological quality guidelines given in Table 1.

This laboratory manual describes procedures for the examination of treated wastewater samples to determine whether or not they comply with the guideline values given in Table 1. The procedures described have been selected because they are simple and require only the minimum of equipment; they can be readily carried out by laboratory technicians, even if they have no previous microbiological expertise. Section 2 describes a method of counting the number of intestinal nematode eggs in a wastewater sample, and section 3 describes two methods for counting the numbers of faecal coliform bacteria. These parasitological and bacteriological methods both focus on the analysis of samples at or near the quality guideline values given in Table 1, i.e. those containing about one intestinal nematode egg per litre and about 1000 faecal coliform bacteria per 100 ml, although they can be simply adapted to count much higher numbers. Finally, section 4 contains recommendations for routine monitoring programmes.

2. Sanitary parasitology

2.1 The modified Bailenger method

Selection of method and comparison with others

The development of medical parasitology has led to a wide range of techniques for the enumeration of intestinal helminth eggs and larvae in faeces, and the basic principles of these methods have been adapted to the enumeration of helminth eggs in sludge and compost. The enumeration of intestinal helminth eggs and larvae in wastewater, however, is much less straightforward. A great variety of human and animal parasite species, as well as free-living species, may be present, varying in size, specific gravity and surface properties, and at much lower concentrations than in faeces, sludge or compost.

Many methods for the enumeration of helminth eggs in wastewater are described in the literature. Each method has its own advantages and disadvantages: some have a high percentage recovery, but are very time-consuming; many are not reported in sufficient detail for replication to be possible, or their recovery rate is unknown; some require prohibitively expensive chemicals or are otherwise unsuitable for use in laboratories with limited equipment; and others only recover a limited range of species. It is clear that there is no one method that is universally useful, recovers all the helminth eggs of medical importance, and has a known rate of recovery.

All the available methods are based on one of two fundamental principles: either the parasites are floated away from other debris in a solution of comparatively high relative density, or the fatty and other matter is separated in an interphase solution (normally ether or ethyl acetate) while the parasites sediment into a non-miscible buffer below. Both processes rely on centrifugal force. The factors which determine whether or not the concentration of particular species of parasite is successful are thought to be mainly the hydrophilic-lipophilic balance of the organism itself and its relative density in relation to that of the separating reagent (Bailenger, 1979). In practice, this means that the pH or the presence of heavy metals or alcohols in the reagents used may change the surface properties of the parasite, and each species will respond differently to these changes: hence, no one method will concentrate all species with the same efficiency.

Bouhoum & Schwartzbrod (1989) compared a range of methods for faecal analysis with a view to adapting them for wastewater samples. Of the wide range of flotation solutions tested, they found that iodomercurate (Janeckso & Urbanyi, 1931) concentrated the greatest range of species of parasitic helminth eggs, but concluded that the reagent was too corrosive and expensive for routine use. Arthur's method (described in Faust et al., 1938), in which saturated saccharose is used as a flotation solution, was found to deform eggs rapidly, while zinc sulfate solution (Faust et al., 1938) did not concentrate *Trichuris* spp. or *Capillaria* spp. very well. Bouhoum & Schwartzbrod (1989) concluded that Bailenger's method (Bailenger, 1979), which they adapted for wastewater, was the best method overall: it requires relatively inexpensive reagents and successfully concentrates the full range of species routinely found in wastewater.

This modified Bailenger method is generally useful, simple and cheap. However, its limitations are well recognized (see below), and there is still a need for its further evaluation. Nevertheless, of all the methods available, it reliably recovers the eggs of the intestinal nematodes mentioned in Table 1, is replicable, and is already widely used in laboratories around the world. It is hoped that this manual will highlight the strengths and weaknesses of the method, standardize the way in which it is performed, and encourage the carrying out of the research still needed.

Advantages and disadvantages

The modified Bailenger method has the following advantages:

1. Sample collection and preparation are straightforward. Specialized containers are not required for sedimentation, and only the minimum of laboratory equipment is needed for sample processing. A few special chemical reagents are required but are usually both locally available and inexpensive. McMaster slides are routinely used in parasitology laboratories, and should be readily available from laboratory supply companies.

2. Long periods of time spent at the microscope are very tiring and can lead to errors. The time required to examine each McMaster slide is usually only 1-2 minutes, so that operator error is reduced.

3. A subsample of each processed sample is examined for eggs. For greater accuracy and to check the homogenization, replicate samples can be examined and a mean egg count used; 2-3 McMaster slides should be examined from each sample and the arithmetic mean count calculated.

The method has the following disadvantages:

1. The percentage recovery of eggs when this method is used is not known, but it has been shown that it compares favourably with that in all other techniques (Ayres et al., 1991; Bouhoum & Schwartzbrod, 1989). Bouhoum & Schwartzbrod showed that this method would successfully recover a wide range of helminth eggs including *Ascaris* spp., *Trichuris* spp., *Capillaria* spp., *Enterobius vermicularis, Toxocara* spp., *Taenia* spp. and *Hymenolepis* spp., and Ayres et al. also routinely recovered hookworm eggs.

2. The method is not suitable for many of the operculated or trematode eggs, including those of *Clonorchis sinensis, Diphyllobothrium latum, Fasciola hepatica, Fasciolopsis buski, Paragonimus westermani, P. pulmonalis,* and *Schistosoma* spp. These all have intermediate aquatic hosts and are important in aquacultural (but not agricultural) reuse systems. Some of these eggs may float in the zinc sulfate flotation solution but sink again quickly or become distorted, making accurate identification difficult.

3. Ether is *highly flammable* and *toxic.* Rude, Peeler & Risty (1987) have shown that ether can be replaced by ethyl acetate for the extraction of parasite eggs from faeces without any loss in efficiency. Ethyl acetate is much safer than ether; it has lower boiling and flash points and is less toxic. Its use is unlikely to affect the efficiency of the method for either raw or treated wastewater.

Equipment and consumables

Reagents

The reagents required are the following: zinc sulfate solution (33%, relative density 1.18); ether (or ethyl acetate); acetoacetic buffer (pH 4.5) (15 g sodium acetate trihydrate, 3.6 ml glacial acetic acid, made up to 1 litre with distilled water); detergent solution (1 ml Triton X-100 or Tween 80, made up to 1 litre with tapwater).

Equipment

The following will be required: plastic containers for sample collection; a centrifuge (capable of generating 1000 g) and centrifuge tubes with lids (50-ml and 15-ml tubes are preferable); Pasteur pipettes and teats; McMaster counting slides (1 or 2); a vortex mixer (not absolutely essential); a siphon; a 10-ml or 50-ml measuring cylinder or 10-ml graduated pipette.

Illustrated step-by-step guide

The method is very efficient for use with raw wastewater. However, the sample size must be

increased to at least 10 litres for the efficient recovery of eggs in treated waste-water effluents, since egg numbers are then much lower (see Note (1), p. 8). It consists of the following steps:

1. Collect a sample of wastewater of known volume (*V* litres), usually 1 litre for raw or partially treated wastewaters and 10 litres for final treated effluents.

2. Allow the sample to sediment for 1-2 hours, depending on the size of the container (see Note (2), p. 10). It is recommended that an open-topped, straight-sided container should be used for sedimentation, since this makes removal of the supernatant easier and permits thorough rinsing of the container (Fig. 1).

3. Remove 90% of the supernatant using a suction pump or siphon (Fig. 2).

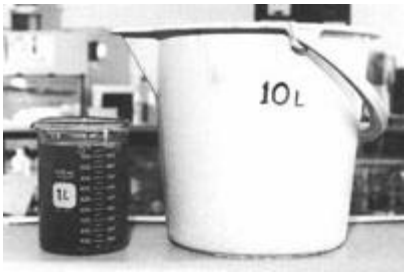

Fig.1 - Straight-sided containers suitable for sedimentation

Fig. 2. Removal of the supernatant with a suction pump

4. Carefully transfer the sediment to one or more centrifuge tubes, depending on the volume, and centrifuge at 1000 g for 15 min. Remember to rinse the container well with detergent solution, and add the rinsings to the sediment (Fig. 3).

Fig. 3. Washing the sides of the container with dilute detergent solution.

5. Remove the supernatant. If more than one centrifuge tube has been used in step 4, transfer all the sediments to one tube (remember to rinse thoroughly with detergent solution to ensure that no sediment is discarded), and recentrifuge at 1000 g for 15 min.

6. Suspend the pellet in an equal volume of acetoacetic buffer, pH 4.5 (i.e. if the volume of the pellet is 2 ml, add 2 ml of buffer) (see Note (3), p. 10). If the pellet is less than 2 ml, add buffer up to 4 ml to ensure that, after extraction with ethyl acetate (steps 7 and 8), there is sufficient volume of buffer above the pellet to allow the ethyl acetate layer to be poured off without resuspension of the pellet.

7. Add two volumes of ethyl acetate or ether (i.e. 4 ml in the above example) (Fig. 4), and mix the solution thoroughly in a vortex mixer. The sample can also be shaken by hand. This is quite acceptable if a mechanical mixer is not available (Fig. 5).

8. Centrifuge the sample at 1000 g for 15 min. The sample will now have separated into three distinct phases. All the non-fatty, heavier debris, including helminth eggs, larvae and protozoa, will be in the bottom layer. Above this will be the buffer, which should be clear. The fatty and other material moves into the ethyl acetate or ether and forms a thick dark plug at the top of the sample (Fig. 6).

Fig. 4. The pellet with 1 volume of buffer and 2 volumes of solvent.

Fig. 5a. Homogenization of the sample can be carried out with a vortex mixer.

Fig. 5b. The sample can also be homogenized by hand.

Fig. 6. Separation of the sample into three distinct phases after centrifugation.

Fig. 7. The supernatant is discarded, leaving only the pellet.

9. Record the volume of the pellet containing the eggs, and then pour off the rest of the

supernatant in one smooth action (Fig. 7). It may be necessary to loosen the fatty plug first by running a fine needle around the side of the centrifuge tube.

10. Resuspend the pellet in five volumes of zinc sulfate solution, (i.e. if the volume of the pellet is 1 ml, add 5 ml of $ZnSO_4$). Record the volume of the final product (*X* ml) (Fig. 8). Mix the sample thoroughly, preferably using a vortex mixer. Note that a minimum of 1.5 ml is required to fill a two-chambered McMaster slide.

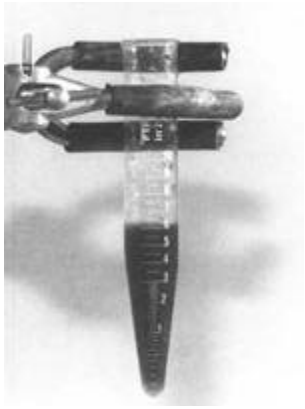

Fig. 8. The pellet, here 1 ml, is suspended in 5 volumes of zinc sulfate solution.

11. Quickly remove an aliquot with a Pasteur pipette and transfer to a McMaster slide (see p. 11) for final examination (Fig. 9).

12. Leave the full McMaster slide to stand on a flat surface for 5 min before examination. This allows all the eggs to float to the surface.

13. Place the McMaster slide on the microscope stage and examine under 10× or 40× magnification. Count all the eggs seen within the grid in both chambers of the McMaster slide (Fig. 10). For greater accuracy, the mean of two slides, or preferably three, should be recorded.

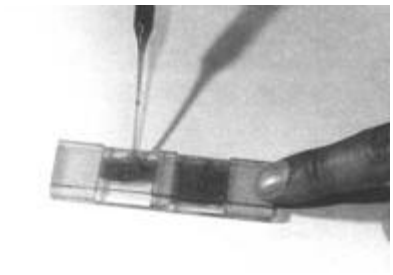

Fig. 9. Filling a McMaster slide: air bubbles must be avoided.

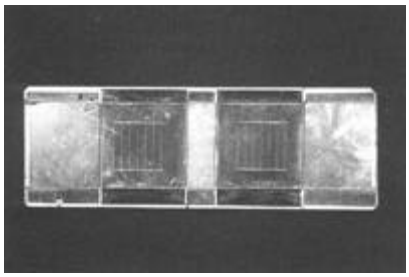

Fig. 10. Old-style McMaster slide: 0.15 ml is held under each grid.

14. Calculate the number of eggs per litre from the equation:

$$N = AX/PV$$

where:

N = number of eggs per litre of sample
A = number of eggs counted in the McMaster slide or the mean of counts from two or three slides
X = volume of the final product (ml)
P = volume of the McMaster slide (0.3 ml)
V = original sample volume (litres)

Remember that, if a single-chamber McMaster slide is being used, *P* = 0.15 ml (Fig. 11).

<<I>> p09c.jpg **Fig. 11.** New-style McMaster slide: 0.15 ml is held under the single grid.

Fig. 11. New style McMaster slide: 0.15 ml is held under the single grid

Note (1): sample size

It is assumed that eggs are uniformly distributed in the final processing stage. A multiplication step is therefore used to convert the number of eggs found to eggs per litre. If the only egg in the sample is detected, however, the final egg count may be greatly exaggerated. In addition, a small sample size plus the subsampling stage makes the detection of very small numbers of eggs improbable. The number of positive samples from treated wastewater is greatly increased by increasing the initial sample size to 10 litres (Ayres et al., 1991).

Note (2): sedimentation times

Stokes' law can be used to calculate the settling rates of nematode eggs in water. At 20°C the settling rates of the three most commonly found eggs are:

Ascaris lumbricoides	20 mm/min
Trichuris trichiura	16 mm/min
hookworms	6 mm/min

It is recommended that, to ensure the collection of all eggs, at least double the theoretical settling time for any container depth should be used.

Note (3): acetoacetic buffer

Extensive work by Bailenger (1979) showed that the removal of helminths from faecal samples was not just a matter of sedimentation or flotation based on relative density, but that the hydrophilic-lipophilic balance of the parasite eggs in relation to the extraction medium was also very important. By controlling the pH, the hydrophilic-lipophilic balance can be modified so as to optimize the concentration of parasite eggs. Acetoacetic buffer at pH 4.5 was found to be the most suitable for the concentration of a wide range of helminth eggs.

2.2 Basic laboratory skills

Microscope calibration

Materials

The following are required:

Stage micrometer: a microscope slide on which a 1 mm scale has been engraved, divided into 100 equal spaces. One space is equal to 10 µm.

Eyepiece micrometer, a special eyepiece on which a scale has been engraved. Not all eyepieces have the same size subdivisions (this depends on the manufacturer).

Calibration of the eyepiece micrometer

Each microscope and each eyepiece used must be individually calibrated, as follows:

1. Place the stage micrometer on the microscope stage and, using the lowest power dry objective, e.g. 4× or 10×, bring the scale into focus.

2. Insert the eyepiece micrometer and rotate it until the two scales overlap.

3. Move the mechanical microscope stage until the scales are aligned at the zero line.

4. Without moving the stage micrometer, find a point towards the extreme right where two other lines are exactly superimposed (Fig. 12).

5. Count the number of division lines on the eyepiece micrometer between the zero line and the point where the second set of lines is superimposed.

6. Repeat this process using each objective in turn, e.g. 4×, 10×, 40×, 100×. Note that the higher the magnification, the thicker the lines on the stage micrometer will appear. The line on the eyepiece scale which is aligned exactly with the centre (or right or left edge) of a line on the stage micrometer must be chosen.

7. Calculate the precise value of each division on the eyepiece scale with each objective as follows, using Fig. 12 as an example:

- with 10× objective: it can be seen that 33 divisions of the eyepiece micrometer are exactly superimposed over 22 divisions of the stage micrometer. For this objective each space of the eyepiece micrometer would correspond to 22 × 10/33 = 6.7 µm;

- with 40× objective: say, for example, 37 divisions of the eyepiece micrometer are exactly superimposed over 6 divisions of the stage micrometer. For this objective each space of the eyepiece micrometer would correspond to 6 × 10/37 = 1.6 µm.

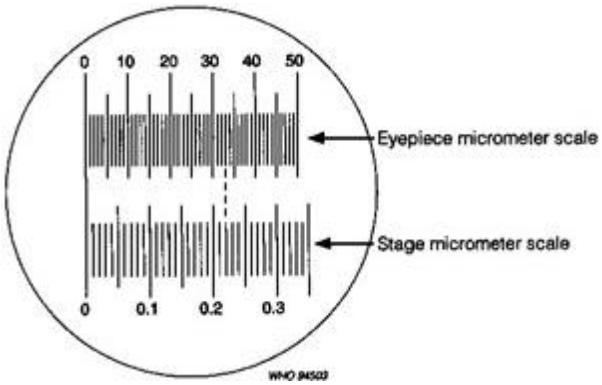

Fig. 12. Representation of part of an eyepiece micrometer superimposed over stage micrometer for microscope calibration

Use of the McMaster slide

A McMaster counting chamber is a specialized microscope slide which allows the number of helminth eggs or larvae to be counted in a known quantity of a flotation solution. Two models are now available through most commercial suppliers of laboratory equipment. In the older, more commonly used model (Fig. 10), the chamber is divided into two parts, each of which has a grid etched on to the glass of the upper surface. The precise volume contained under each of the two grids is 0.15 ml. In the new version (Fig. 11), there is only one chamber, but the volume held under the grid is still 0.15 ml.

The principle of the McMaster slide is that the eggs are lifted in the flotation solution and lie

immediately below the upper glass of the chamber, while the heavier debris settles to the floor. If the microscope is focused on the grid, the eggs will be clearly in focus while the debris is not. By searching up and down the grid systematically, the number of eggs in 0.15 ml of the suspension solution can be accurately counted.

To fill the two-chambered McMaster slide:

1. Mix the final flotation suspension thoroughly, preferably in a vortex mixer to ensure a homogeneous mix. Quickly fill a Pasteur pipette and carefully run the solution into one compartment of the McMaster slide. Fill the whole compartment completely even though it is only the section under the grid that is to be counted. Work quickly and smoothly at this stage so that eggs do not start to float in the test-tube or in the pipette. Ensure that there are no air bubbles under the grid.

2. Fill the other compartment of the McMaster slide, remembering to rehomogenize the solution first.

3. Leave the McMaster slide to stand for a few minutes before starting to count, thus ensuring that all the eggs have floated to the surface and that the debris has been allowed to settle.

4. Count the number of eggs under both grids. If there are large numbers of eggs and some are under the lines, it is usual to count those on two sides of the grid as “in” (e.g. on the top and left hand lines) and discard those under the other two lines (e.g. the bottom and right hand lines). This gives a good estimate of the number of eggs present in 0.3 ml.

5. Make at least two (preferably three) counts if there is enough flotation solution, and take a mean of the two (or three) counts. Calculate the number of eggs present in the original sample using the formula given on page 8 (remember that P = 0.15 if the new single-chamber counting slide is used).

McMaster counting chambers are usually made of glass and can be ordered from most major suppliers of scientific equipment. Some companies now produce less expensive (and less fragile) plastic chambers. If these are used, care must be taken to ensure that they do not become scratched.

Use of centrifuges

Most published methods that involve the use of centrifuges quote centrifuge speed in terms of relative centrifugal force. However, in some papers, speed is expressed in revolutions per minute (rpm). To convert rpm to force, the following formula is used:

$$\text{RCF} = r(\text{rpm})^2/k$$

where RCF = relative centrifugal force (g),

r = radius of the centrifuge from the spindle to the centre of the bucket (cm),

k = 89 456.

To convert force to rpm:

$$\text{rpm} = \sqrt{(k\text{RCF}/r)}$$

Identification of helminth eggs

Wastewater frequently contains the eggs of parasites of animals, e.g. rats, domestic animals such as pigs and dogs, and birds. Although it is not necessary to identify these positively, it is important to recognize that they are not of human origin. Plates I-XVII show a number of the eggs of the human parasitic helminths most frequently encountered in wastewater samples. Although these eggs are typical for each species, it must be remembered that not all eggs are absolutely uniform in size and shape. A number of books are available that will make identification easier (see Further reading, p. 31). It is sometimes almost impossible, however, to determine whether eggs are of human or animal origin, e.g. the eggs of *Ascaris suum* (from pigs) and *A. lumbricoides* (from humans) are morphologically indistinguishable. Similarly, the eggs of *Trichuris* spp. are all of similar colour and shape. Eggs of the human whipworm, *T. trichiura,* can only be separated from those of animal species by careful measurement. For a comparison of human and animal helminth eggs, the plates in Thienpont, Rochette & Vanparijs are excellent.

Human parasitic helminth eggs can be accurately identified using an eyepiece micrometer in a microscope calibrated using the method given on page 10.

For this purpose, the egg, e.g. a *Trichuris trichiura* egg, is moved under the eyepiece micrometer scale. If its length is found to cover 8 spaces of the scale using the standard 10× objective calibrated in the example on page 11, its real length is:

8 × 6.7 = 53.6 µm

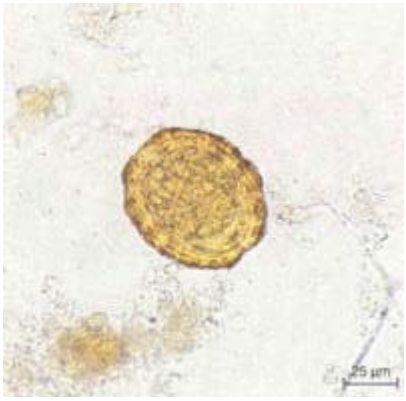

Plate I. *Ascaris lumbricoides*

Plate II. *Ascaris lumbricoides* (infertile)

Plate III. *Trichuris trichiura*

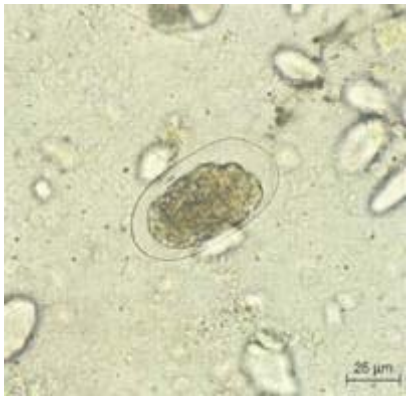

Plate IV. Hookworm

Plate V. *Enterobius vermicularis*

Plate VI. *Capillaria hepatica*

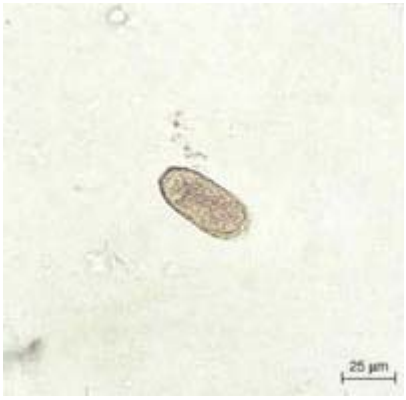

Plate VII. *Capillaria philippinensis*

Plate VIII. *Hymenolepis diminuta*

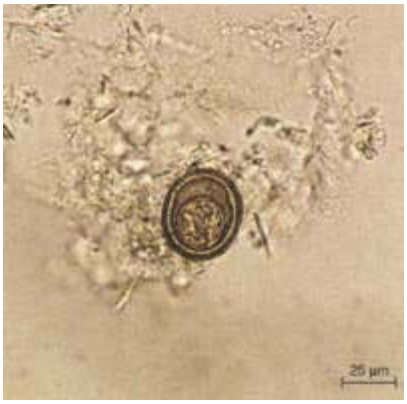

Plate IX. *Taenia* sp.

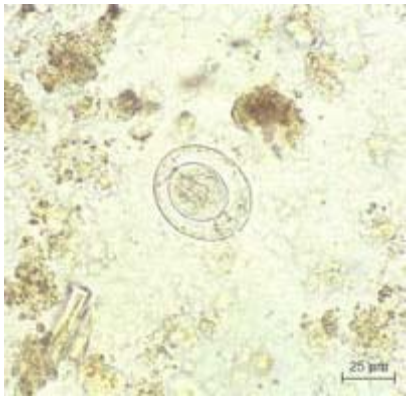

Plate X. *Hymenolepis nana*

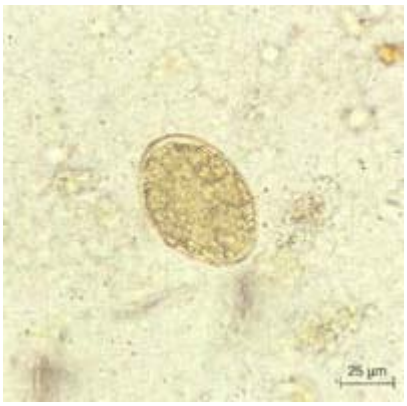

Plate XI. *Diphyllobothrium latum*

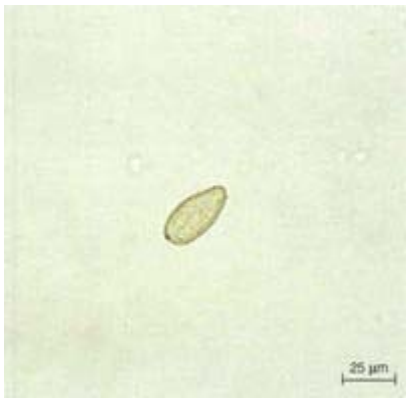

Plate XII. *Clonorchis sinensis*

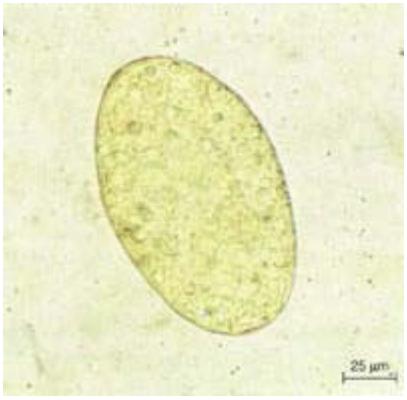

Plate XIII. *Fasciola hepatica*

Plate XIV. *Paragonimus westermani*

Plate XV. *Schistosoma haematobium*

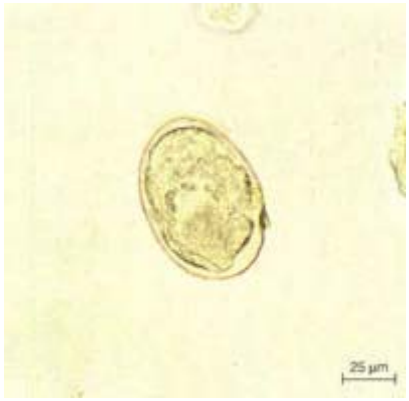

Plate XVI. *Schistosoma japonicum*

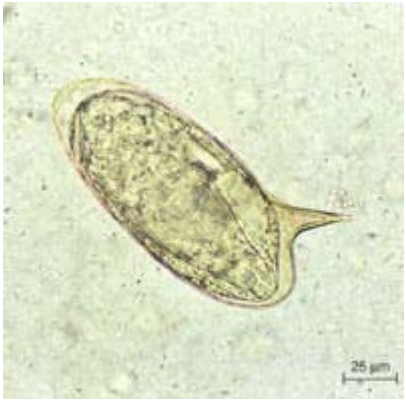

Plate XVII. *Schistosoma mansoni*

Similarly, if the same egg is measured again using the 40× objective calibrated in the example, and 33.5 spaces are covered by the length of the egg, its real length can be calculated as:

33.5 × 1.6 = 53.6 µm

The length thus obtained can be compared, for identification, with those of the typical specimens shown in the plates on pages 13-15.

Identification of free-living larvae and adults

Samples of raw wastewater and effluent from all types of wastewater-treatment plants frequently contain larval and adult stages of free-living nematodes which may be mistaken for parasitic ones, in particular, the rhabditiform or filariform stages of *Ancylostoma duodenale, Necator americanus* or *Strongyloides stercoralis.* These free-living nematodes feed on bacteria, algae or small organic particles. There are hundreds of different species and many have yet to be described.

In faecal samples, positive identification is quite straightforward, as free-living species are unlikely to be present. The rhabditiform larvae of hookworms and *Strongyloides stercoralis* can be identified from the comparative morphology of the buccal cavity. Likewise, close examination of the head and tail ends permits identification of the filariform larvae. However, in samples of wastewater, accurate identification of these stages is difficult because so many species of free-living nematodes are found in wastewater; it is therefore necessary to make detailed measurements of the relevant morphological features. To do this, individual nematodes must be mounted, stained and viewed under high-power magnification. Most methods for the examination of wastewater, including the modified Bailenger method, do not lend themselves to this procedure.

It is suggested that, unless it is possible to examine each individual nematode as described above, larval and adult stages should be ignored. Even if parasitic forms are present, not enough is known about their survival and viability in wastewater to make it possible to assess the risk to health that they pose.

3. Sanitary bacteriology

The numbers of faecal coliform bacteria in wastewater samples are usually counted by: (a) most probable number (MPN) methods; or (b) the membrane filtration method. Two MPN methods are described in section 3.1; membrane filtration is described in section 3.2.

3.1 MPN methods

MPN counts are statistical best estimates (hence the name, most probable number) obtained by culturing a number (usually five) of sample volumes and/or dilutions of such samples. These estimates are based on the principle of "dilution to extinction". For example, if a single 1-ml aliquot from each of a series of 1:10 dilutions (see p. 27) is examined and growth occurs at a dilution of 10^{-3} but not at 10^{-4}, the best estimate of the count is 10^{3} bacteria per ml. By increasing, usually to five, the number of 1-ml aliquots examined at each dilution, a better estimate of the count can be obtained.

In the first of the two MPN methods described here, five 1-ml aliquots of only one dilution are examined, and so only an approximate faecal coliform MPN count is obtained. This method, which is very simple and inexpensive, is suitable for the routine analysis of treated wastewaters that comply with the guideline value of no more than 1000 faecal coliforms per 100 ml (see Table 1).

In the second MPN method, five 1-ml aliquots of each of three dilutions are examined, so that a much better estimate of faecal coliform numbers is obtained. This method can be made suitable for the analysis of wastewater containing any number of faecal coliform bacteria by altering the dilutions examined (see method B, step 10, p. 20).

Equipment and consumables

Consumables

The chemicals listed below for medium A-1 will be needed, together with quarter-strength Ringer's solution (commercially available in tablet form) or sodium chloride solution (8.5 g NaCl per litre of distilled water). Non-absorbent cotton wool is also required.

Medium A-1 (American Public Health Association, 1995) is recommended, as it can be used for direct incubation at 44°C. It is not commercially available in dehydrated form and must be made up (see pp. 26-27) to the following formula:

lactose	5 g
tryptone	20 g
salicin	0.5 g
NaCl	5 g
Triton X-100	1 ml
distilled water	1 litre

It is dispensed in 5-ml quantities into test-tubes (or screw-capped bottles) each of which contains an inverted Durham tube (this is a very small test-tube). The test-tubes are closed with a plug of non-absorbent cotton wool and sterilized (see p. 27). During sterilization, the air in the Durham tube is expelled and it becomes completely full of medium.

Equipment

The following are required:

- 100-ml screw-capped bottles

- test-tubes (100 mm × 12 mm) or half-ounce (14-ml) screw-capped bottles

- 1-ml serological “blow-out” pipettes

- Bunsen burner

- test-tube rack

- incubator or water-bath

- autoclave or pressure cooker

- balance (± 0.01 g).

Illustrated step-by-step guide

Two MPN methods are described below. Method A is the simpler of the two and is suitable for the routine monitoring (see section 4) of treated wastewater effluents that contain around 1000 or fewer faecal coliforms per 100 ml.

Method B is more accurate and can also be used for samples containing 1000 or fewer faecal coliforms per 100 ml, or for those containing many more.

Method A

1. Collect a sample of wastewater effluent in a sterile 100-ml screw-capped bottle.

2. Shake the sample bottle thoroughly, and aseptically withdraw 1 ml using a sterile 1-ml “blow-out” pipette. Transfer this to a *sterile* test-tube or screw-capped bottle containing 9 ml of quarter-strength Ringer’s solution (or 8.5 g/l NaCl solution) (Fig. 13). **Do not pipette by mouth** - use a pipette suction pump.

3. Shake this 1:10 dilution of the sample thoroughly and, *using a single fresh sterile 1-ml pipette,* transfer 1 ml to each of five *sterile* test-tubes or screw-capped bottles containing an inverted Durham tube and 5 ml of medium A-1 (Fig. 14). Label each tube or bottle with a code for the sample, the date and 1:10.

4. Place these five test-tubes or bottles in an incubator or water-bath maintained at 44°C (± 0.25°C) (see p. 27).

5. After incubation for 24 h, examine each test-tube or bottle for gas production. (Faecal coliform bacteria produce gas from the lactose in medium A-1, and some of this gas is trapped in the inverted Durham tube.) Count the number of positive tubes or bottles (i.e. those with gas production) (Fig. 15) and determine the MPN from Table 2.

Fig. 13. Adding 1 ml of sample to 9 ml of diluent to make a 1:10 dilution of the sample.

Fig. 14. Adding 1 ml of the 1:10 dilution to a test-tube containing 5 ml of medium A-1 and an inverted Durham tube.

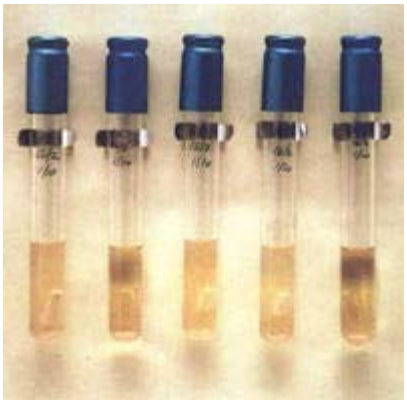

Fig. 15. After incubation at 44°C for 24 h, three of the tubes show gas production. The MPN is determined from Table 2 as 910 faecal coliforms per 100 ml of sample (treated wastewater).

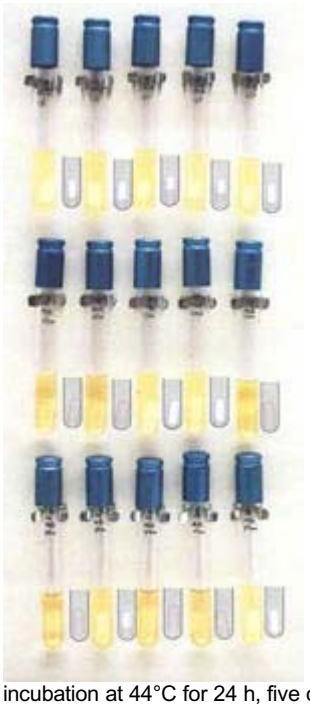

Fig. 16. After incubation at 44°C for 24 h, five of the tubes containing 1 ml of sample are positive (gas production), two of those containing 0.1 ml (1 ml of the 1:10 dilution), and two of those containing 0.01 ml (1 ml of the 1:100 dilution). For clarity, accompanying schematic drawings show positive and negative tubes. From Table 3, the MPN is 950 faecal coliforms per 100 ml of sample (treated wastewater).

Table 2.
Faecal coliform MPN per 100 ml of sample for one set of five tubes containing 0.1 ml of sample[a]

Number of positive tubes	MPN per 100 ml
0	240
1	350
2	540
3	910
4	1600
5	>1800

[a] Adapted from Department of the Environment (1994) by kind permission of Her Majesty's Stationery Office.

Method B

1. Collect a sample of wastewater as described in method A.

2. Make a 1:10 dilution (as described in method A) and a 1:100 dilution (this is simply a 1:10 dilution of the 1:10 dilution; see p. 27).

3. Label each of five sterile test tubes or screw-capped bottles containing an inverted Durham tube and 5 ml of medium A-1 with a code for the sample, the date and 1:100.

4. Repeat, labelling 1:10; repeat, labelling 1:1.

5. Using a fresh sterile 1-ml pipette, add 1 ml of the 1:100 dilution to each of the five sterile test-tubes or screw-capped bottles labelled 1:100.

6. Using the same pipette (but taking care not to lay it down on the bench or to touch anything with its tip), add 1 ml of the 1:10 dilution to each of the second set of five test-tubes or screw-capped bottles labelled 1:10.

7. Again using the same pipette, add 1 ml of the undiluted sample to each of the third set of five test-tubes or screw-capped bottles labelled 1:1.

8. Place the 15 tubes in a rack and transfer to an incubator or water-bath maintained at 44°C (± 0.25°C).

9. After incubation for 24 h, count the number of positive tubes (those with gas production) at each dilution (Fig. 16), and determine the faecal coliform MPN from Table 3.

10. If the faecal coliform count is much greater than 1800 per 100 ml, use smaller sample volumes (i.e. 1-ml quantities of higher dilutions). If the test-tubes contain 0.1 ml, 0.01 ml and 0.001 ml of sample, multiply the MPN given in Table 3 by 10. Similarly, if the test-tubes contain 0.01 ml, 0.001 ml and 0.0001 ml of sample, multiply the MPN given in Table 3 by 100.

Table 3.

Faecal coliform MPN per 100 ml of sample for three sets of five tubes containing 1 ml, 0.1 ml and 0.01 ml of sample respectively[a]

Number of positive tubes			MPN
1 ml	0.1 ml	0.01 ml	per 100 ml
0	0	0	0
0	0	0	20
0	1	0	20
1	0	0	20
1	0	1	40
1	1	0	40
1	2	0	50
2	0	0	40
2	0	1	50
2	1	0	50
2	1	1	70
2	2	0	70
2	3	0	110
3	0	0	70
3	0	1	90
3	1	0	90
3	1	1	130
3	2	0	130
3	2	1	160
3	3	0	160
4	0	0	110
4	0	1	140
4	1	0	160
4	1	1	200
4	2	0	200
4	2	1	250
4	3	0	250
4	3	1	310
4	4	0	320
4	4	1	380
5	0	0	220
5	0	1	290
5	0	2	410
5	1	0	310
5	1	1	430
5	1	2	600
5	1	3	850
5	2	0	500
5	2	1	700
5	2	2	950
5	2	3	1 200
5	3	0	750
5	3	1	1 100
5	3	2	1 400
5	3	3	1 750
5	3	4	2 100
5	4	0	1 300
5	4	1	1 700
5	4	2	2 200
5	4	3	2 800
5	4	4	3 450
5	5	0	2 400
5	5	1	3 500
5	5	2	5 400
5	5	3	9 100
5	5	4	16 000
5	5	5	>18 000

[a] Adapted from Department of the Environment (1994) by kind permission of Her Majesty's Stationery Office.

3.2 Membrane filtration method

Membrane filtration is a method of obtaining faecal coliform counts by filtering a known volume of a wastewater sample (or a dilution of it) though a membrane filter. This is a special filter-paper with a pore size of 0.45 µm, so that all faecal coliform bacteria are retained on it. The membrane filter is then placed on an absorbent pad saturated with a faecal coliform growth medium and incubated. During incubation, each faecal coliform bacterium develops into a visible yellow colony. After incubation, the yellow colonies are counted, and the count per 100 ml is calculated.

Equipment and consumables

Equipment

The following are required:

- membrane filter forceps
- Petri dishes (60-mm diameter glass or disposable plastic)
- membrane filtration units (glass or plastic)
- 5-ml or 10-ml and 1-ml serological "blow-out" pipettes
- pipette suction pump
- vacuum pump (electrical, manual or water Venturi pump)
- Bunsen burner
- incubator
- autoclave or pressure cooker
- balance (± 0.01 g).

Consumables

These comprise the following:

- membrane filters (0.45 µm pore size, 47 mm diameter)
- absorbent pads (47 mm diameter)
- membrane lauryl sulfate broth
- quarter-strength Ringer's solution
- ethanol.

Membrane lauryl sulfate broth is available commercially in dehydrated form. Alternatively, it may be made up in accordance with the following formula (Department of the Environment, 1994):

peptone	40 g
yeast extract	6 g
lactose	30 g
phenol red (4 g/l aqueous solution)	50 ml
sodium lauryl sulfate	1 g
distilled water (pH 7.6 before sterilization)	1 litre

Quarter-strength Ringer's solution is also available commercially in tablet form; alternatively, sodium chloride solution (8.5 g NaCl in 1 litre of distilled water) may be used.

Illustrated step-by-step guide

The following procedure is suitable for wastewater samples containing 200-2000 faecal coliforms per 100 ml. For samples containing more than 2000 per 100 ml, see step 11 below. Aseptic procedures should be used throughout (see chapter 3.3 Basic laboratory skills).

1. Collect a sample of wastewater in a sterile 100-ml screw-capped bottle.

2. Dip the membrane filter forceps in ethanol, and burn off in the flame of the Bunsen burner. Using the now sterile forceps, transfer a sterile absorbent pad to each of three sterile Petri dishes.

3. Using a sterile 5-ml or 10-ml pipette, aseptically add 1.8 ml of sterile membrane lauryl sulfate broth to each of the three Petri dishes, so as to just saturate (but not flood) each absorbent pad (Fig. 17).

Fig. 17. Adding 1.8 ml of sterile membrane lauryl sulfate broth to a Petri dish containing a sterile absorbent pad.

Fig. 18. Placing a sterile membrane filter on the membrane filtration unit.

4. Dip the membrane filter forceps in ethanol and burn off in the Bunsen flame. Aseptically place a sterile membrane filter in the membrane filtration unit (Fig. 18).

5. Pour in about 20 ml of sterile quarter-strength Ringer's solution (Fig. 19), and then add 5 ml of the wastewater sample to the membrane filtration unit using a sterile pipette (Fig. 20).

Fig. 19. Pouring about 20 ml of diluent into the membrane filtration unit.

Fig. 20. Adding 5 ml of sample to the membrane filtration unit.

6. Turn on the vacuum pump and, when all the liquid has been filtered through the membrane filter, switch off; a manual vacuum pump can also be used (Fig. 21).

Fig. 21. Using a manual vacuum pump to filter the sample through the membrane filter.

Fig. 22. With a rolling action, placing the membrane filter on an absorbent pad saturated with membrane lauryl sulfate broth.

7. Aseptically transfer the membrane filter to a sterile Petri dish containing an absorbent pad just saturated with sterile membrane lauryl sulfate broth. It is best to do this by a rolling action, so as to avoid air bubbles between the membrane filter and the absorbent pad (Fig. 22).

8. Repeat steps 4-7 twice.

9. Place all three Petri dishes upside down in an incubator maintained at 44°C (± 0.5°C).

10. After incubation for 24 h, count the number of yellow colonies, irrespective of size, on each of the three membrane filters (Fig. 23). (Faecal coliform bacteria produce acid from the lactose in membrane lauryl sulfate broth, and the acid changes the colour of the phenol red pH-indicator to yellow.) Calculate the mean of these three colony counts; since these counts are for 5 ml (the volume of sample filtered), multiply this figure by 20 to obtain the faecal coliform count per 100 ml.

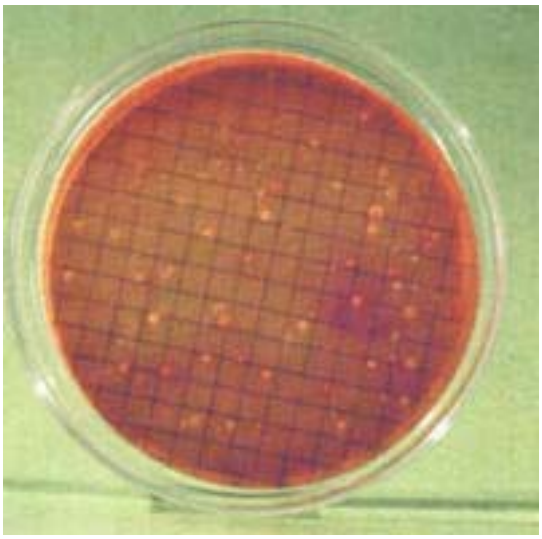

Fig. 23. After incubation at 44°C for 24 h, the yellow colonies on the membrane filter are counted. Here, the number of colonies was 40; this is the count per 5 ml (the volume filtered), so the corresponding faecal coliform count per 100 ml is 800.

11. Filter smaller volumes of the sample (or dilutions of it; see p. 27) if each membrane filter has more than 100 colonies growing on it, since it then becomes difficult to count them. Thus 1 ml of

the sample can be used for faecal coliform counts of up to 10 000 per 100 ml, 1 ml of a 1:10 dilution for counts up to 100 000 per 100 ml, and so on.

3.3 Basic laboratory skills

Asepsis

Special care has to be taken to ensure that the wastewater sample, or any dilution of it, does not become contaminated while it is being examined. In other words, it is necessary to ensure that only the faecal coliform bacteria in the sample are being counted, not those which may be on any glassware or on fingers. All glassware, media and diluent (quarter-strength Ringer's or salt solution), and membrane filters and absorbent pads are therefore sterilized before use (see below). However, sterilization is only a part of asepsis; in addition, the following standard aseptic procedures are necessary:

1. Wash hands thoroughly before starting work in the laboratory.

2. Ensure that the work is carried out in a dust-free and draught-free part of the laboratory and that the bench used is clean; swab it down with ethanol just before starting work.

3. Be careful not to touch the top of the bottles containing sterile medium or diluent, or of those used for sample collection. Similarly, do not touch the tip or bottom half of sterile pipettes.

4. When opening a bottle (or test-tube) containing sterile medium or diluent, or one containing the sample, quickly pass the open neck through a Bunsen flame while holding the cap of the bottle (or the cotton wool plug from the test-tube) in the other hand (using only the little finger for this - do so by curling it around the cap or plug). Similarly, when withdrawing a pipette from its sterile wrapping (or container), pass it quickly through the Bunsen flame, and be careful not to touch the pipette tip or allow it to touch anything else; if it does touch something, discard it and use another sterile pipette.

5. *Never* pipette by mouth. *Always* use a pipette suction pump.

If nobody in the laboratory has any bacteriological experience, it is best to contact a medical laboratory technician at a local hospital who will be able to demonstrate aseptic procedures.

Sterilization

Test-tubes containing MPN medium, bottles containing diluent and membrane filtration medium, and membrane filters and absorbent pads are all sterilized in an autoclave or pressure cooker (see p. 27).

Screw-capped bottles used for sample collection are sterilized in an oven at 160°C for 1 h. During sterilization, the cap should be slightly unscrewed (to allow air to escape, otherwise the bottle will explode); after sterilization and when the oven has cooled to room temperature, the bottles are removed, the caps screwed on tightly and a short length of adhesive tape (preferably marked STERILE) is placed over the top of each bottle.

Pipettes should also be sterilized in an oven at 160°C for 1 h. Before sterilization, the top of each pipette should be plugged with non-absorbent cotton wool. They are then wrapped either individually or in a group of up to five in aluminium foil; the tops should be marked (so that the other end is not opened and the tips contaminated).

Medium preparation and sterilization

This consists of the following steps:

1. Weigh out all the chemicals needed to make up the medium (see p. 17 and p. 22, respectively, for the formulas of medium A-1 and membrane lauryl sulfate broth), or weigh out the appropriate quantity of dehydrated membrane lauryl sulfate broth if this is being used. Add to the appropriate quantity of distilled water and allow to dissolve completely. (It may not always be advisable to make up 1 litre of medium as, once sterilized, it must be stored below 10°C in the dark and used within 3 months.)

2. Dispense the medium into bottles or test-tubes before sterilizing. Medium A-1 should be added in 5-ml quantities to screw-capped bottles or test-tubes each containing an inverted Durham tube; if test-tubes are used, these must be closed with a plug of non-absorbent cotton wool. Dispense membrane sodium lauryl sulfate broth in small quantities into screw-capped bottles (just under 6 ml are needed for testing each sample) (see p. 23, step 3). Make up diluent either by dissolving a tablet of quarter-strength Ringer's solution in 500 ml of distilled water, or by weighing out 8.5 g NaCl and dissolving it in 1 litre of distilled water (or *pro rata* for smaller quantities). Dispense the diluent in 9-ml quantities into screw-capped bottles (for making 1:10 dilutions) or in 100-ml quantities when it is to be used for membrane filtration (see p. 23, step 5).

3. Once the media and diluent have been dispensed, sterilize in an autoclave or pressure-cooker, as follows:

- medium A-1 and membrane lauryl sulfate broth: 115°C, 67 kPa (10 lb/in^2), for 10 min once the temperature and pressure are reached; and

- quarter-strength Ringer's solution or NaCl solution: 121°C, 101 kPa (15 lb/in^2), for 15 min once the temperature and pressure are reached.

4. Autoclave screw-capped bottles with their caps only loosely screwed down (to avoid explosions); after sterilization and when the bottles are at room temperature, screw the caps down tightly. Before use, store in a dust-free place and label as sterile.

Making serial 1:10 dilutions

Dilutions are used in both method A and method B for MPN counts (see pp. 18, 20) and may be used for membrane filter counts (see p. 25, step 11). The procedure is as follows:

1. With a sterile 1-ml pipette, aseptically add 1 ml of the wastewater sample to a sterile test-tube or bottle containing 9 ml of diluent. Mix thoroughly. This is a 1:10 dilution.

2. Using a fresh sterile 1-ml pipette, add 1 ml of the 1:10 dilution to a second sterile tube or bottle containing 9 ml of diluent. Mix thoroughly. This is a 1:100 dilution.

3. Step 2 is repeated as required, adding 1 ml of the last dilution made to another tube or bottle containing 9 ml of diluent. This gives higher dilutions in sequence: 1:1000, 1:10 000 and so on. (If these higher dilutions are to be used, remember that untreated wastewater usually contains 10^7-10^9 faecal coliforms per 100 ml.)

Incubation

Faecal coliform bacteria are incubated at 44°C (± 0.25°C). It is best to use a fan-assisted incubator (the fan ensures that all parts of the incubator are at the required temperature; without a fan this is difficult to achieve, and it is very important that incubation is carried out very close to

44°C), although a water-bath can be used for the test-tubes in the MPN procedures.

After incubation and examination of the samples, all the test-tubes or screw-capped bottles containing medium, and all Petri dishes containing membrane filters and absorbent pads, must be sterilized by autoclaving at 121°C for 15 min before disposal to ensure that the billions of bacteria that have grown during incubation are destroyed.

4. Routine monitoring programmes

The numbers of faecal coliform bacteria in the final effluent of a wastewater treatment plant vary much less with time (even over a 24-h period) than do the numbers of helminth eggs. The guideline values for faecal coliforms and helminth eggs given in Table 1 differ markedly (1000 faecal coliforms per 100 ml as compared with 1 egg per litre), so routine monitoring programmes for each are seeking to detect numerical changes of a different order of magnitude. A mean of 2 eggs per litre might be a result requiring action, but one of 1002 or 1020 faecal coliforms per 100 ml probably would not be (with bacteria the order of magnitude is much more important than the actual number). Because of this difference (1000 per 100 ml versus 1 per litre), and the different patterns of removal in wastewater-treatment works, routine monitoring programmes for faecal coliforms and helminth eggs are somewhat different.

4.1 Faecal coliforms

During the irrigation season, grab samples of the final effluent used for unrestricted crop irrigation should be taken at least once, and preferably twice, a week and faecal coliform numbers determined. The time of day that such samples are collected should be established by conducting a few diurnal studies, as follows:

1. Over a 24-h period, take samples every 3 h (eight samples in all), and determine the faecal coliform numbers N_1, N_2, N_3, etc. in each.

2. Calculate the geometric mean count for the 24-h period from the formula:

Geometric mean count = $(N_1 \times N_2 \times N_3 \times N_4 \times N_5 \times N_6 \times N_7 \times N_8)^{1/8}$

3. Determine which of the individual counts is closest to the mean count, and then take the routine samples at this time (usually between 08:00 and 10:00). As each sample is taken during the irrigation season, calculate the running geometric mean count; it is this value that should not exceed 1000 faecal coliforms per 100 ml.

Running geometric mean = $(N_1 \times N_2 \times N_3 \times \ldots \times N_n)^{1/n}$

4.2 Helminth eggs

Time of day

The concentration of human parasitic helminth eggs in raw wastewater varies considerably over 24-h periods. If raw wastewater is being monitored, it is important to take 24-h composite samples, or to take a representative grab sample as described in section 4.1 (the arithmetic, rather than geometric, mean egg count should be used).

Running arithmetic mean = $(N_1 + N_2 + N_3 + \ldots + N_n)/n$

The diurnal variation in the numbers of helminth eggs in effluents from treatment plants (especially waste-stabilization ponds) is less marked.

Frequency of sampling

Frequency of sampling depends on the objectives of the monitoring regime and the method of wastewater treatment. A diurnal study and an initial period of intense sampling (once or twice a week) for several weeks, should be carried out at every treatment plant.

Waste-stabilization ponds remove helminth eggs at a fairly constant rate, despite periodic

overloading and changes in flow; this preliminary sampling should therefore give a good indication of the long-term effluent quality. Egg-removal rates from conventional treatment plants tend to be more variable, and intense monitoring should therefore cover a range of operating conditions.

After this preliminary sampling, the frequency of sampling will depend on whether the effluent is being used for restricted or unrestricted irrigation. If the wastewater is being treated in waste-stabilization ponds and used for unrestricted irrigation (Category A in Table 1), samples need only be taken once or twice a month, since the retention time in ponds required to achieve the faecal coliform guideline value vastly exceeds that for the egg guideline. However, if treatment is by another process, or the wastewater is going to be used for restricted irrigation (Category B, Table 1), samples need to be taken at least once a week. Regular monitoring should always be carried out while the effluent is being used for irrigation.

Number of samples

If differences over time in the number of eggs per litre are to be determined, several samples should be taken on each occasion. A minimum of three is suggested. The actual number of samples required will depend on the smallest true difference to be detected and the level of significance desired. This is described in most statistical textbooks (e.g. Sokal & Rohlf, 1981).

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Wastewater reuse

Mara DD, Cairncross S (1989) *Guidelines for the safe use of wastewater and excreta in agriculture and aquaculture: measures for public health protection.* Geneva, World Health Organization.

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Appropriate technology for the treatment of wastewaters for small rural communities. Report on a WHO meeting.
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