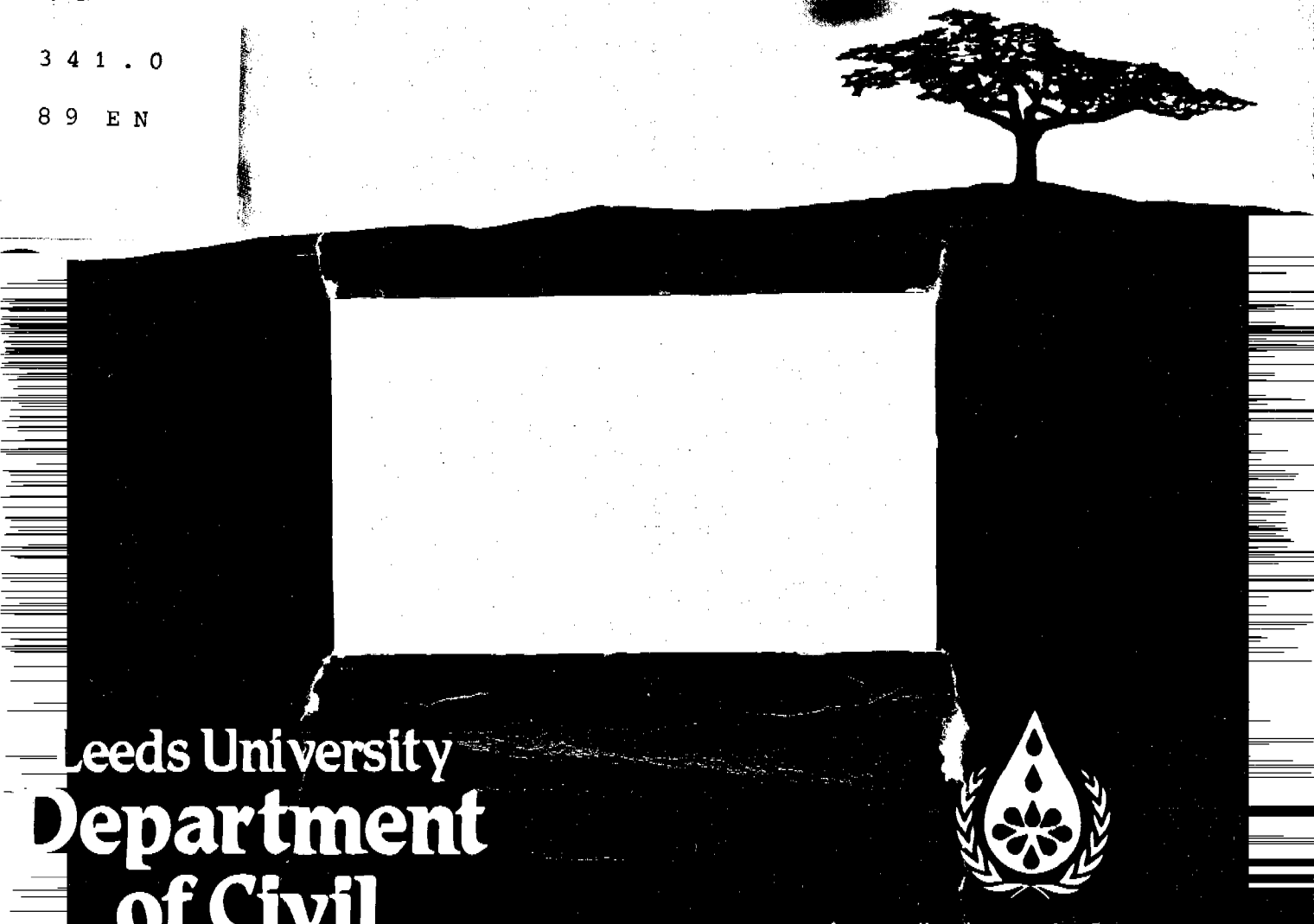
Tropical Public Health Engineering



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Final Report

THE ENUMERATION OF HUMAN INTESTINAL NEMATODE EGGS IN RAW AND TREATED WASTEWATERS

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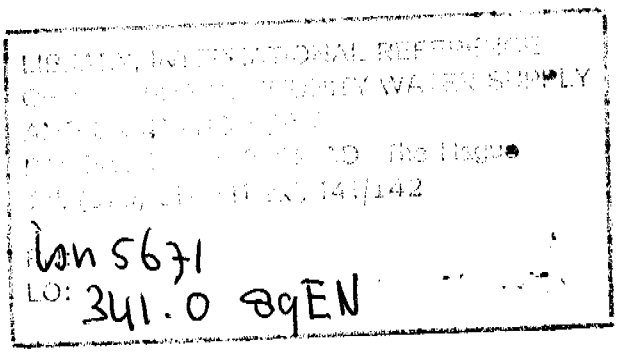
March 1989



February 1989

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SUMMARY

The recently proposed World Health Organisation guidelines for the microbiological quality of treated wastewaters used for crop irrigation 1/ , which are based on the 1985 Engelberg guidelines2/ , recommend that the concentration of human intestinal nematode eggs be no more than one per litre. This guideline value is applicable to treated wastewaters used for both restricted and unrestricted irrigation, where the latter refers to the irrigation of crops for direct human consumption including those eaten raw.

The Engelberg Report recognised the need for the development of a simple and reliable technique to determine such low numbers of eggs in wastewater and this research has developed the Leeds II technique for samples of waste stabilisation pond effluent. The Leeds I technique was also developed as a simple robust technique for the evaluation of raw wastewater where the numbers of helminth eggs are high and there is a minimum of equipment available.

Laboratory experiments done in Leeds using wastewater seeded with animal intestinal nematode eggs, and in field studies conducted at EXTRABES, the Federal University of Paraiba's experimental pond research station in Campina Grande, northeast Brazil, using samples of raw wastewater and pond effluent showed that both of these techniques were superior to that described by Teichmann 3/ and that in routine use at EXTRABES 4/ . The Leeds I and II techniques are given on pages iv and vi of this summary.

The Leeds II Technique

The probability of recovering a single helminth egg in a one litre sample of waste stabilisation pond effluent using the Leeds II technique was found to vary with the concentration of suspended solids as shown in Table 1. It was also found that the distribution of helminth eggs in pond effluent followed a Poisson distribution. Using this distribution the minimum number of 1-litre samples needed to detect an underlying (or "true") mean of 1-2 eggs per litre are given in Table 2. It is recommended that at least four 1-litre samples are taken on each sampling occasion, but the total number of 1-litre samples given on Table 2 may be collected over a period of several weeks (even months), providing that the samples are taken at the same time of day on each occasion.

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1. Prost,A. (1988). Revision of the 1973 WHO guidelines: a WHO Scientific Group proposes revised health guidelines for the use of wastewater. IRCWD News No.24/25, p.11. Duebendorf, Switzerland: International Reference Centre for Wastes Disposal.
2. IRCWD. (1985). Health Aspects of Wastewater and Excreta Use in Agriculture and Aquaculture: The Engelberg Report. IRCWD News No.23, pp.11-18. Duebendorf,Switzerland: International Reference Centre for Wastes Disposal.
3. Teichmann, A. (1986). Quantitative determination of helminth eggs in wastewater. Angewandte Parasitologie, 27, pp. 145-150.
4. Mara, D.D. & Silva, S.A. (1986). Removal of intestinal nematode eggs in tropical waste stabilisation ponds. Journal of Tropical Medicine and Hygiene, 89, pp.71-74.

Table 1. The probability of recovering 1 helminth egg per litre over a range of suspended solids concentrations.

SS concentration (mg/l)	Probability
0 - 30	0.8
30 - 70	0.7
70 - 115	0.6
>115	0.5

Table 2. The minimum number of 1 litre samples needed to detect an underlying mean concentration of 1 or 2 helminth eggs per litre using the Leeds II technique.

Underlying mean concentration	Minimum number of one litre samples required for 80% confidence for xp value of:				for 90% confidence for xp value of:			
	0.8	0.7	0.6	0.5	0.8	0.7	0.6	0.5
1	32	36	42	50	125	143	167	200
2	16	18	21	25	63	72	83	100

The Leeds I Techique

This technique was used to enumerate intestinal helminth eggs in raw wastewater from Campina Grande arriving at the waste stabilisations ponds at EXTRABES and 24 hour sampling showed that samples taken at 0800 h were representive of the mean daily load of helminth eggs entering the pond system, and that the diurnal variation of Ascaris lumbricoides reflected human defecation patterns in the catchment population. For example numbers of eggs were highest in samples taken at 1100 h corresponding to defecation early in the morning prior to leaving the home for work or school, whilst very few eggs were recovered from samples taken during the night.

Pond performance

Monitoring of the effluent from maturation ponds using the Leeds II technique showed that the series comprising of an anaerobic, a facultative and three maturation ponds produced effluent of a quality well within the Engelberg guideline of < 1 helminth egg per litre. A mean of 0.11 eggs per litre was recorded over a period of 7 weeks. Similarly, samples from a single facultative pond with a mean hydraulic retention time of 15 days showed an overall mean helminth egg concentration of 0.82 eggs/litre, also within the WHO standard.

Hookworm larvae in raw and treated wastewater

Samples of raw and treated wastewater were examined for hookworm larvae and it was concluded that it is extremely difficult to differentiate hookworm larvae from the many species of free-living nematodes which are normally present in wastewater. This can only be successfully done by experienced nematologists for research purposes rather than as a routine laboratory process.

THE LEEDS II TECHNIQUE, FOR THE ENUMERATION OF INTESTINAL HELMINTHS IN THE EFFLUENT OF WASTE STABILISATION PONDS

The Leeds II technique as finally developed during the course of this study comprises of the following steps:

1. Collect four 1 litre grab samples of final effluent in clean plastic containers. The containers should have smooth internal walls and be prewashed with a detergent to remove any grease and thus decrease the chances of helminth eggs adhering. Add 10 ml formalin to the sample.

2. Leave the samples to stand for at least 1 hour. This is the minimum amount of time with a generous safety margin required for the lightest of the helminth eggs to sediment in a standard 1 litre beaker. The sedimentation time for any particular container can be calculated using Stokes equation (Appendix VIII) It is suggested that the calculated time required is always doubled for safety.

3. Remove the supernatant carefully using a siphon or a suction pump leaving 60-70 ml sediment. The supernatant must <u>not</u> be poured off.

4. Transfer the sediment of each sample into a 100 ml centrifuge tube. The sides of the sedimentation vessel should be rinsed thoroughly with a solution of 0.01% Triton X-100 and the rinsings added to the appropriate centrifuge tube.

5. Centrifuge the sample at 600 g for 10 min.

6. Remove the supernatant using a vacuum pump leaving a small pellet of about 2-3 ml at the bottom of the tube. It is very important that the supernatant is removed carefully at this stage as it is very easy to suck up eggs with other debris in the pellet. A glass pipette or plastic pipette tip may be inserted into the siphon tube for greater control.

7. Add a solution of NaCl with a specific gravity of 1.04 to a depth of 5 cm and leave to stand for 1 hour. To ensure a homogenous mix of pellet and salt solution it may be necessary to add a few ml of flotation solution, and agitate before adding the rest.

8. Remove the supernatant and discard leaving a pellet of 2-3 ml in the bottom of the tube.

9. Pour the final pellet into a Doncaster counting dish and examine under a high power microscope for eggs and larvae of parasitic helminths. If a Doncaster counting dish cannot be obtained the pellet can be examined in several batches in a slightly smaller counting cell such as a Sedgewick-Rafter slide. The 100 ml tube should be rinsed with the Triton X-100 solution and the rinsings examined as well.

Notes:

(a) In a salt solution of specific gravity 1.04 many of the algae which are normally found in effluent from waste stabilisation ponds will remain in suspension, whilst the helminth eggs and larvae will sediment out thus leaving a cleaner pellet for final examination. One hour has been determined as the optimum time for maximum helminth sedimentation and minimum algal sedimentation.

(b) A Doncaster counting dish is a counting chamber which is used directly under a microscope. It is a round dish (diameter 75 mm) with concentric circular divisions which keep eggs and larvae in defined areas. A total volume of 4 to 5 ml can easily and efficiently be examined under a magnification of x40.

Doncaster counting dishes can be obtained from:

Cox Thermoforming Ltd
London Road
Tring
Hertfordshire HP23 6HB
UK

Telex: 825389
Telephone: 044-282-4222

Advantages of this method

There is no subsampling step in this method. All the solid debris of a specific gravity 1.04 is examined for any given sample.

Freeliving forms of parasitic nematodes are extremely difficult to identify when encountered in wastewater as there are likely to be so many free-living species present. Suspect nematodes need to be examined under high power in a clean microscope slide preparation. Use of a Doncaster dish enables individual nematodes to be lifted out using a mounted needle and examined on a separate microscope slide.

LEEDS I TECHNIQUE FOR THE ENUMERATION OF HELMINTH EGGS IN RAW WASTEWATER

The Leeds I technique, as finally developed during the course of the study, comprises the following steps:

1. Take 1 litre grab sample of raw sewage.

2. Pour the sample into a series of 100 ml centrifuge tubes, and centrifuge at 600 g for 10 min.

3. Carefully remove the supernatant using a simple filter pump attached to a water tap. It is important that the supernatant is not poured off as eggs can be lost in this way. If the whole of the original sample is not centrifuged the first time, top up the tubes with the remaining sample and centrifuge again for 10 min at 600 g and remove the supernatant as above.

4. Rinse the sediments from each tube into a single centrifuge tube using a solution of 0.01% Triton X-100 dispensed from a wash bottle. Each centrifuge tube should be rinsed twice and the rinsings added to the final tube.

5. Centrifuge the combined sediments and rinsings for 10 min at 600 g.

6. Remove the supernatant as described above leaving about 5 ml (pellet and supernatant) in the bottom of the tube.

7. Using a glass rod stir the pellet thoroughly and divide between six 15 ml centrifuge tubes. Make sure that both the glass rod and the original centrifuge tube are rinsed well with Triton X-100 and that the rinsings are added to the 15 ml tubes.

8. Centrifuge the sample at 600 g for 10 min as described above.

9. Remove the supernatant carefully using a vacuum suction pump.

10. Add 3-4 ml of saturated $MgSO_4$ to each tube and agitate thoroughly (the pellet must be loosened and mixed with the salt solution). An automatic vortex mixer or a simple mounted needle may be used.

11. Top each centrifuge tube up to just below the rim with the salt solution and leave to stand for 5 min.

12. Centrifuge the tubes for 1 min at 100-150 g.

13. Put the tubes in a test tube rack and top up with salt solution using a glass pipette until a positive miniscus is formed. Rest a coverslip on the top of the tube and leave to stand for 30 mins. A glass or plastic coverslip may be used. During this time helminth eggs will float to the surface.

14. Remove the coverslip using a firm upward movement and put on a glass slide for examination under a high power microscope. The eggs collect under the coverslip and are removed in the meniscus of salt solution as the coverslip is lifted off.

15. After the first coverslip has been removed for examination it is immediately replaced with a second. A total of 4 coverslips should be examined for each tube of sediment, topping up with flotation solution if necessary. The sediment from a 1 litre sample is usually divided between six 15 ml centrifuge tubes and thus a total of 24 coverslips are examined for each sample.

    Although further eggs may be recovered by examining more coverslips, the percentage added to the total is very small and, given the time involved in coverslip examination, the test would be prohibitively long if the samples were examined exhaustively. The data collected from trial runs with seeded samples of raw sewage showed that the examination of 4 coverslips would be the most practical and most efficient number to examine.

A C K N O W L E D G E M E N T S

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The fieldwork for this project was conducted in Campina Grande, north east Brazil, at the Universidade Federal da Paraiba's wastewater treatment experimental station, EXTRABES, and we particularly wish to thank Professor Salomao A Silva, Director of Research, for providing us with facilities for the work. We are also grateful to the technical staff at EXTRABES, and to our colleagues, Tom Curtis and Martin Gambrill, for their unstinting support.

We are also grateful to the following organisations for providing research facilities at EXTRABES: Universidade Federal da Paraiba, Companhia de Aguas e Esgotos da Paraiba, Superintendencia para o Desenvolvimento do Nordeste, Banco Nacional de Desenvolvimento Economico, Conselho National de Desenvolvimento Cientifico e Tecnologico, Financiadora de Estudos e Projetos, the Canadian International Development Agency, and the UK Science and Engineering Research Council.

I. INTRODUCTION

The use of treated wastewater for irrigation is now becoming more common, especially in arid and semi-arid areas. Guidelines for the microbiological quality of such waters for the irrigation of agricultural crops were developed at a meeting of specialists convened by the International Reference Centre for Wastes Disposal, the World Bank and the World Health Organisation and held in Engelberg, Switzerland in June 1985. These guidelines (IRCWD, 1985), which were based on an appraisal of the best available epidemiological evidence for disease transmission caused by wastewater irrigation (Shuval et al., 1986), state that treated wastewater used for irrigation, either restricted or unrestricted (the latter referring to the irrigation of crops for direct human consumption inc.salad crops), should not contain more than one viable human intestinal nematode egg per litre - the intestinal nematodes being (Ascaris lumbricoides), the human whipworm (Trichuris trichiura) and the human hookworms (Necator americanus and Ancylostoma duodenale). For restricted irrigation there is the additional guideline quality of less than 1000 faecal coliform bacteria per 100 ml.

These 'Engelberg' guidelines were accepted, with minor modification, at the meeting in Geneva in November 1987 of the World Health Organisation Scientific Group on Health Aspects of Use of Treated Wastewater in Agriculture and Aquaculture (Prost, 1988). The minor modification was to drop the word 'viable' from the nematode guideline.

At the Engelberg meeting it was recognised that such low levels of nematode eggs could be simply and reliably achieved by a properly designed series of waste stabilisation ponds, for example by a series comprising a 1-day anaerobic pond followed by a 5-day facultative and a 5-day maturation pond (Mara and Silva, 1986). However it was also recognised that there were no simple techniques to determine, both reliably and at low cost, low egg numbers in treated wastewaters on a routine basis. This research project was therefore initiated with the objective of developing such a technique for both raw and treated wastewaters.

Preliminary research work was done at the University of Leeds using samples of wastewater seeded with animal nematode eggs (Ascaris suum, Trichuris muris, T.ovis and Nippostrongylus brasiliensis). This led to the development of the 'Leeds I' technique for helminth enumeration in samples of raw wastewater and the 'Leeds II' technique for samples of effluent from waste stablization ponds.

Following this work in Leeds, fieldwork was done in Campina Grande, northeast Brazil at the Federal University of Paraiba's experimental wastewater treatment station, EXTRABES 1/. Human intestinal nematode eggs were counted in samples of raw wastewater and waste stabilization pond effluents using the Leeds I and Leeds II techniques, respectively. Comparisons were made with Teichmann's (1986) technique and that which was in routine use at EXTRABES (Mara and Silva, 1986) (see Sections 3 and

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1. Estacao Experimental de Tratamentos Biologicos de Esgotos Sanitarios (address: Caixa Postal 306, 58.100 Campina Grande - PB, Brazil).

4) 2/. Studies were also made on the diurnal variation in egg concentration in both raw and treated wastewater at EXTRABES (Sections 8 and 9).

The experimental pilot-scale ponds at EXTRABES (Figure 1) that were used in this study were:

a) three maturation ponds, coded M12, M13 and M14; These ponds were the third to the fifth in a series and were preceded by an anaerobic and a secondary facultative pond. They were all equally sized, with dimensions 10.00 x 3.35 x 2.20 m and with an individual mean hydraulic retention time of 5 days; and

b) a primary facultative pond, coded F18, which received raw wastewater; its dimensions were 25.40 x 7.15 x 2.20 m, and its mean hydraulic retention time was 15 days.

Full details of the experimental facilities at EXTRABES are given in Silva (1982). Throughout the period of study reported herein the mean daily mid-depth pond temperatures were 24-26 °C.

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2. A very limited amount of comparative work has since been done on the technique developed at the Universite de Nancy, France, by Stien and Schwartzbrod (1988). This technique although excellent as a research tool, was not found suitable for routine quality control work in developing countries. Our work with this technique which was done in Amman, Jordan, will be presented elsewhere.

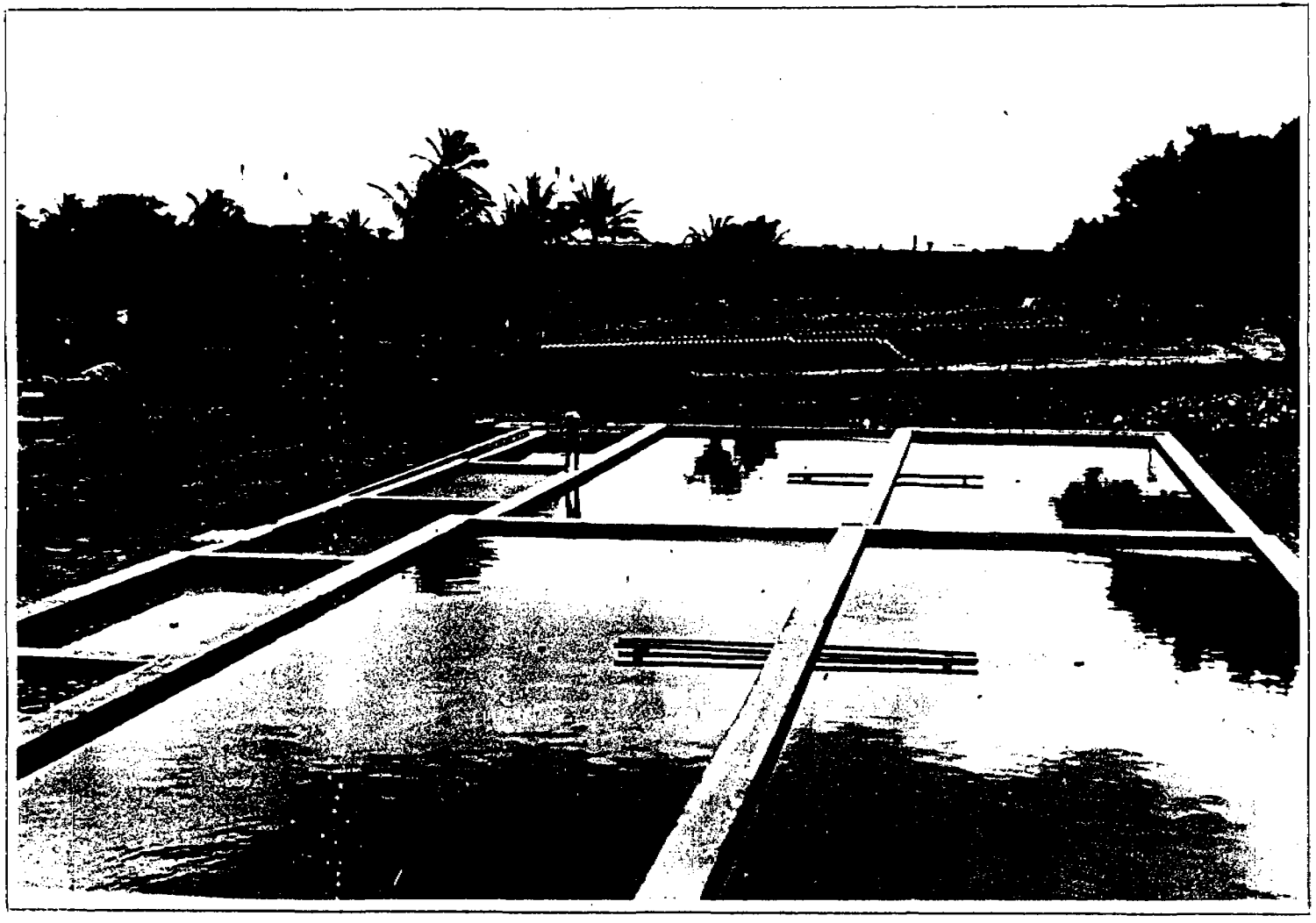

Fig. 1. The pilot-scale waste stabilisation ponds at EXTRABES showing the series and the four single facultative ponds.

2. DEVELOPMENT OF A TECHNIQUE FOR ENUMERATING INTESTINAL NEMATODE EGGS IN RAW SEWAGE

2.1 Introduction

A wide range of published techniques exists for the enumeration of helminth eggs in faeces or sewage sludge but relatively few have been developed specifically for the examination of wastewater (Teichmann, 1986; Schwartzbrod 1987; Crewe, pers. comm., 1988). The enumeration of helminth eggs in wastewater is not straightforward: they are not normally present in such large numbers that they can be found by making a simple direct smear, and thus it is necessary to use a technique which harvests them from a relatively large sample of material. Different helminth eggs and samples of wastewater from different treatment processes vary in their properties and therefore it is not possible to recommend one 'perfect' multipurpose technique. In the search for the most appropriate method for their enumeration in raw wastewater, a wide range of clinical and vetinerary techniques was investigated along with the published and unpublished techniques, and a method referred to as the Leeds I technique was evolved.

A useful summary of the range of techniques available for the enumeration of helminth eggs in faeces, sludge and wastewater has been compiled by Dr S Crewe (Liverpool School of Tropical Medicine) and is shown in Table I. The removal of helminth eggs from wastewater or faeces can be divided into the six stages described in the Table:

(i) **Primary cleaning** removes or breaks up larger particles which may be present in the sample and may interfere with egg recovery; coarse sieves or homogenisers are normally used.

(ii) **Secondary cleaning** removes water soluble material and smaller particles which may be in suspension by a series of washing steps.

(iii) **Primary treatment** aims to separate eggs from other particles by sieving to break physical bonds or chemically using an anionic detergent.

(iv) **Secondary treatment** separates eggs from unwanted material by flotation away from the particulate material or by sedimentation leaving unwanted material in suspension.

(v) **Harvesting** is the collection of eggs from the final sample. If eggs have been separated from unwanted material by flotation they can then be collected on the surface of a coverslip. Other methods include the use of membrane filters or sedimentation in clean water.

(vi) **Examination for final counting of eggs** is either carried out by mounting the coverslip or membrane filter directly on a microscope slide and examining them under a compound microscope.

During experimentation in the laboratories at Leeds University the most appropriate method was selected from each section and a technique developed, the 'Leeds I' technique, which was then compared for its efficiency with the Teichmann technique (Teichmann, 1986; see Appendix IV) and the technique currently in use at EXTRABES (Mara and Silva, 1986; see Appendix III).

A summary of the methods investigated and the results obtained are given below; full experimental details are given in Appendix IX.

PRIMARY CLEANING To remove or break up larger particles	SECONDARY CLEANING To remove smaller particles which will float, and water-soluble material	PRIMARY TREATMENT To break the attachment between eggs and other particles, and possibly remove some of the organic material	SECONDARY TREATMENT To separate eggs from unwanted material	HARVESTING Collection of eggs	EXAMINATION For identification of eggs
Coarse sieve (tea-strainer) Graduated sieves a) 10, 20 and 40-mesh sieves b) 250um, 120um, 30um c) 150um, 30um wanted material is back-washed from final sieve in methods (b) and (c) Homogenisation (for dried sludge) a) In blender b) In a shaker with glass beads	Repeated washing in water, and sedimentation The "Liverpool method" used 24-hour intervals, but other workers use as little as 15 minutes. The washing may be speeded by centrifuging	Chemical a) Sodium hydroxide 0.1N Sodium hydroxide 0.4N b) Sodium hypochlorite 2.5% for most eggs * For Taenia eggs, solutions must not contain more than 5% available chlorine. 0.5% Chloros is suitable. c) Anionic detergents 0.05% Tween 20 1.0% Tween 80 1.0% 7X 10.0% 7X 0.01% Triton 100 * commercial sodium hypochlorite (BDH) diluted 1:4 has been used by some workers. Physical a) Force material through a fine mesh b) Homogenisation	Flotation Zinc chloride 1.55 1.89 Magnesium sulphate 1.14 1.275 1.3 (Sat.) Zinc sulphate 1.18 1.275 1.4 (Sat.) Sodium chloride 1.2 (Sat.) Sodium nitrate 1.375 (Sat.) Sugar solutions 1.4 or as density gradients Glycerine solutions various strengths Sodium dichromate 1.2 1.375 Zinc chloride + sodium chloride 1.53 Magnesium sulphate + 5% potassium iodide 1.33 Magnesium sulphate + sodium chloride Potassium mercury iodide 1.44 (dangerous and expensive) Sodium salicylate Caesium chloride Potassium dichromate Sedimentation 10% formol-ether 10% formol saline-ether 5% acetic acid-ether 10-15% hydrochloric acid-ether 10% formol-ethyl acetate 0.8% sodium chloride (for Taenia) Special 'Zonal centrifugation', i.e., continuous-flow centrifugation combined with sedimentation in density gradient. (not suitable for Taenia eggs)	Bacteriological loop Coverslip floating in beaker (picked up by tweezers) Coverslip on centrifuge tube Sedimentation in water a) in cylinder (gravity) b) in tube (centrifugation) (for small quantities) Filter membranes a) Millipore 14um in Swinnex 25 attachment to syringe b) Millipore 14um in funnel c) Millipore 8um in funnel d) Millipore 0.45 um in funnel	Coverslip preparation on microscope slide Filter membrane mounted on microscope slide Material on membrane back-washed a) into Petri dish and examined under microscope b) into centrifuge tube and examined on microscope slide with coverslip

Table.1. Summary of the range of techniques which have been used for the enumeration of helminth eggs in faeces, sludge and wastewater. (from Dr.S.Crewe,Liverpool School of Tropical Medicin).

2.2 Primary Cleaning

Several techniques use a series of coarse or graduated sieves to remove or break up larger particles or organic matter (for example, Satchwell, 1986; Allen and Ridley, 1970). However, our own laboratory observations showed that, when samples of raw wastewater seeded with helminth eggs were passed through sieves, large numbers of the eggs became trapped in the organic debris retained on the sieves and this significantly reduced the efficiency of recovery. It was decided that too many eggs might be lost and relatively little solid matter removed if a primary cleaning step were included in the technique for wastewater. This is in agreement with Meyer et al. (1978) who suggested that a positive advantage of their method is that 'sieving' is eliminated from the initial steps in the handling of sludge since, if large particulates are removed by the sieving process, eggs may be eliminated with them.

2.3 Secondary Cleaning

The 'Liverpool' method (Crewe, pers. comm. 1988; see Appendix V) involves washing samples by letting the solid matter, including the eggs, sediment for 24 h, removing the supernatant and then adding more water; this is repeated several times with 24 h settling periods and thus removes most of the smaller particles and water-soluble material found in sewage sludge, for which it was developed. The method was tried with samples of wastewater collected from Pudsey sewage treatment works, West Yorkshire, and seeded with *Ascaris suum* eggs. The rate of recovery of eggs was found to be very low (mean 5.2%). During each washing stage it was observed that a large amount of material remained in suspension. These 'flocs', which may well have had eggs attached to them, were discarded with the supernatant, and this will have been the main cause of the very low recoveries recorded. It was concluded that secondary cleaning by repeated washing was not required for the examination of raw sewage samples as it was the cause of unacceptably high egg losses.

2.4 Primary Treatment

Crewe describes the function of primary treatment as being to 'break the attachment between eggs and other particles and possibly remove some of the organic matter' (unpublished, 1985). The limiting factor in the recovery of helminth eggs from faeces, sludge or raw wastewater is likely to be the amount of organic matter to which eggs can become attached. This is particularly true of *Ascaris* spp, whose eggs have a mucilaginous outer coat which causes them to adhere to almost all surfaces. In faeces or sludge samples where there are likely to be very high numbers of helminth eggs, physical homogenisation or sieving of the material is very effective, since the loss of eggs during these processes makes relatively little difference to the final results. However this would not be appropriate in wastewater where numbers are much smaller. A wide range of other techniques for breaking down the attachment of eggs to particles or breaking the particles themselves was tried and these are outlined below. It was eventually decided that the use of a solution of Triton X-100 for cleaning all containers at each stage of the process was the most effective way of preventing eggs from sticking to the sides of containers and tubes.

2.4.1 Floc Dispersal

Flocs of material found floating in sewage samples are clumps of bacteria and organic debris bound together by their respective electrical charges.

Calgon (sodium hexametaphosphate), a widely used dispersant, was used to try and disperse flocs by interfering with the electric charges. It appeared to have no effect on the structure of the flocs.

2.4.2 Destruction of solids using cellulase

One of the main organic solids found in domestic sewage is cellulose. Microscopic examination of Leeds sewage revealed many long filamentous particles which were most probably incompletely digested vegetable material or toilet paper. Sewage samples examined in Campina Grande showed far less of this material; this may have been because it is not customary in Brazil to dispose of toilet paper in the toilet in order to avoid blocking the small pipes which connect the toilet to the sewer.

Several experiments were carried out to see if cellulase would break down the long chain cellulose molecules and thus reduce the size of the organic particles to which eggs might become attached. It was found that the quantity of settleable solids was decreased from about 1.5 ml to 0.25-0.5 ml in 400 ml raw sewage when 0.03 g of Sigma cellulase was used. The percentage recovery of Ascaris suum eggs was tested on four 1 litre samples using the technique described in Appendix I. However despite the use of cellulase there was no significant improvement in the rate of recovery of eggs, which was 26% (Appendix IX) as compared to 24 and 22% with the Leeds I and Teichmann techniques respectively (Appendix VI). Cellulase is an expensive material for routine laboratory use, and it is only available from a limited range of sources. It was therefore concluded that it would not be worthwhile to include cellulase treatment in a technique for the enumeration of helminth eggs in raw wastewater from the points of view of both cost and performance.

2.4.3 Destruction of cellulose using Cuprammonium solution.

The pulp and textile industries traditionally use Cuprammonium solution (Schweizer's reagent, 0.3% $Cu(OH)_2$ in 20% NH_4OH) to dissolve cellulose and it is available at relatively low cost. Observations were carried out on the effect of various concentrations of the reagent on the destruction of organic debris from samples of raw sewage. The amount of organic matter was decreased by treatment with the reagent from 1.5 ml to 0.25-0.5 ml per 400 ml sample. Microscopic examination showed that the particle size was much smaller and very few long cellulose filaments were present. However Schwiezer's reagent has the serious disadvantage that it is extremely unpleasant to work with due to its strong odour and it should only be used in a fume cupboard. Work with this reagent was not therefore continued.

2.4.4 Use of a coating material on glassware and plasticware

Helminth eggs, particularly those of Ascaris spp, may get lost during any enumeration technique by becoming stuck to the sides of glass or plastic containers. Dimethyl-dichlorosilane (DD) is a strong water repellant and may be used to coat the surfaces of scientific equipment and glassware. It was decided to investigate the ability of DD to repel helminth eggs and thus improve the rates of egg recovery. Samples of sewage seeded with Ascaris suum eggs were processed using the Leeds I technique (Appendix I). Despite treating all the glassware with DD a recovery rate of only 12% was achieved compared with the mean recovery rate of 24% for the Leeds I technique.

2.5 Secondary Treatment

2.5.1 Sedimentation

Unwanted organic matter, particularly light solids and fats, can be removed in organic solvents in a non-water-miscible layer above the helminth eggs which sediment. Formol-ether is used in several techniques mainly for the extraction of eggs from faecal samples (Allen and Ridley, 1970; Schwartzbrod, 1987; Thienpont et al., 1986). Satchwell (1986) examined a modification of the Allen and Ridley technique for the determination of helminth eggs in sewage sludge: much of the unwanted debris was removed quite efficiently but the overall percentage recovery remained very low (mean 7.74%). Satchwell established that eggs were lost at all stages of the process but that most of them were lost at the formol-ether stage. However, as the reproducibility of the results was fairly good (standard deviation 1.36), and as there was no correlation between egg recovery and total solids concentration, Satchwell adopted the technique and used it for routine monitoring of sludge prior to its application to land.

Schwartzbrod et al. (1987) developed a technique for the examination of wastewater, based on the Janeckso and Urbanyi (1931) technique. The Janeckso-Urbanyi technique was modified by the addition of n-butanol for viability determination (Stien and Schwartzbrod, 1988). The technique has an efficiency of about 50% and varies little with egg load. It is suitable for the examination of wastewater where there are few parasites as the sample size is large, 25 l. One disadvantage of the technique is the use of ether, which is unpleasant to work with, having a very pungent smell and is also dangerous as it has a very low flash point and for these reasons should only be used in a fume cupboard. Another disadvantage is that the mercuric iodide in the Janeckso-Urbanyi reagent, which is used for final flotation, is both expensive and toxic. The authors are currently working on an alternative cheaper and less hazardous flotation solution (pers. comm.).

This technique would be suitable for research purposes or in well equipped laboratories where availability of chemicals and equipment such as fume cupboards are not limiting factors. However it was felt that sedimentation using any of the ether techniques was not appropriate for a simple field technique.

2.5.2 Flotation

Because wastewater contains large quantities of heavy material, it is usually easier to separate any eggs by floating them away from the heavier debris than by sedimenting them away from lighter material. Most techniques therefore use a centrifugation step to remove the water and concentrate the solids and then use a strong salt solution for flotation (Teichmann, 1986; MAFF, 1986; Satchwell, 1986; Caceres, 1987; Steer et al., 1974; Kazacos, 1983; Story and Philips, 1982). A wide range of salt solutions is suggested in the literature (Table I), and the efficiency of recovery seems to depend on the species of helminth and the specific gravity of the salt solution, which must be greater than that of the helminth eggs. Parfitt (1969) found that the highest rate of recovery of Gongylonema pulchrum eggs from goat faeces was achieved using saturated $MgSO_4$, whilst Story and Phillips (1982) found saturated $ZnSO_4$ to be the most effective for the recovery of Taenia saginata from soil drainage water, river water and vegetation. Parfitt (1969) notes that 'saturated solutions of NaCl, $ZnSO_4$ and $MgSO_4$ are widely used and satisfactory for many helminths'.

It was decided to investigate the use of saturated $MgSO_4$ (specific gravity 1.28-1.30) and NaCl (specific gravity 1.198) for helminth egg extraction, as all the human parasitic helminths which may be found in raw sewage have a specific gravity of less than 1.18, although there is variation in the calculated specific gravity of some eggs, in particular Taenia spp (see Table 2). Initial qualitative observations showed that eggs of Ascaris suum, Trichuris ovis and Hymenolepis diminuta could be readily separated from sewage sample using either of these salts and relatively little debris floated. Laboratory trials were carried out using $MgSO_4$, as its higher specific gravity was expected to give better results and is recommended in the final version of the Leeds I technique.

Table 2. Specific Gravities of Helminth Eggs

Helminth	Specific Gravity (range)	Reference
Ancylostoma caninum	1.0559 (1.1283-1.1310)	David and Lindquist, (1982)
Ascaris suum	1.1299 (1.0549-1.0573)	
Trichuris suis	1.1299 (1.1283-1.1310)	
Taenia spp	1.2251 (1.2244-1.2257)	
Ascaris lumbricoides	1.11	Shuval et al. (1987)
Hookworm	1.055	
Trichuris trichiura	1.15	
Schistosoma spp	1.18	
Taenia saginata	1.1	

2.5.3 Zonal centrifugation

This involves the separation of helminths from unwanted debris along a density gradient. Tests using a sucrose gradient (MAFF, 1987) showed that there were several practical limitations to the use of the technique. Firstly, it was found to be quite difficult to actually construct the sucrose gradient, although this presumably would become easier with practice. Secondly, organic debris also spread along the gradient, such that the band in which the eggs might be expected to be found was full of organic matter and it was consequently difficult to remove and examine. This technique was not therefore investigated further.

2.6 Harvesting

This is the collection of eggs separated during secondary treatment. The two most commonly used techniques, which are most appropriate for the examination of wastewater, are coverslip collection and sedimentation in water.

Coverslip collection involves placing a glass or plastic coverslip on a positive meniscus of flotation solution in a centrifuge tube. The helminth eggs which have floated to the surface become adhered to the coverslip which can then be removed after a certain length of time and examined under a microscope. The usual procedure is that the combined sediments from a sewage sample are divided between several small centrifuge tubes, the flotation solution added and the mixture agitated to suspend the solid matter. After leaving for 5-10 minutes to allow the eggs to start floating upwards and the sediment to sink, the tubes are usually centrifuged for a short period at a low velocity. More flotation solution is added to form the positive meniscus and the coverslip placed on the top. Several centrifugation times for this final stage have been suggested in the literature and these are summarised in Table 3.

Table 3. Range of times and velocities suggested for centrifugation of samples prior to coverslip harvesting

Reference	Time	Velocity
Teichmann (1986)	3 min	2500 rpm
Crewe (unpub)	1 min	300 rpm
Satchwell (1986)	1 min	683 g
Parfit (1969)	2 min	1000 rpm
Meyer et al.(1978)	2 min	800 g

The Teichmann technique uses sedimentation as the final method for harvesting. Helminth eggs separated by salt flotation are removed using a pipette and dropped into 1 litre of clean water in which the eggs sediment and can be collected by centrifugation after the removal of the supernatant. It was found when using this technique that much more floating debris was collected with the pipette than with the straight coverslip recovery, thus making the final examination on microscope slides much more difficult and time consuming, although the percentage recovery rate using the Teichmann technique was only slightly less than that obtained with the Leeds I technique (24 and 26% respectively).

For the Leeds I technique it was decided to use the coverslip recovery method and samples were centrifuged at 150 g for 1 min.

2.7 Final Examination

After sedimentation the eggs can be quantified in any counting chamber or the debris can be examined drop by drop on a microscope slide. Due to the amount of debris collected with the eggs during the sedimentation procedure with the Teichmann technique, McMaster slides and Sedgewick-Rafter counting chambers were found to be less useful and counts were always made on microscope slides.

Membrane filters mounted on a microscope slide were tried, but this was found to be suitable only where helminth eggs were being extracted from clean water and there was no debris to obscure the eggs on the filter.

Examination for the identification and enumeration of helminth eggs after coverslip recovery is done on a microscope slide, at x40 magnification with a higher power being used for confirmation if necessary. This method was adopted for the final stage of the Leeds I technique.

2.8 The Leeds I Technique

The following technique was devised on the basis of the observations and experiments described above.

1. Take a 1 litre grab sample of raw sewage.

2. Pour the sample into a series of 100 ml centrifuge tubes, and centrifuge at 600 g for 10 min.

3. Carefully remove the supernatant using a simple filter pump attached to a water tap. It is important that the supernatant is not poured off as eggs can be lost in this way. If the original sample is not all centrifuged the first time, top up the tubes with the remaining sample and centrifuge again for 10 min at 600 g and remove the supernatant as above.

4. Rinse the sediments from each tube into a single centrifuge tube using a solution of 0.01% Triton X-100 dispensed from a wash bottle. Each centrifuge tube should be rinsed twice and the rinsings added to the final tube.

5. Centrifuge the combined sediments and rinsings for 10 min at 600 g.

6. Remove the supernatant as described above leaving the pellet plus a few ml of supernatant in the bottom of the tube.

7. Using a glass rod, stir the pellet thoroughly and divide between 6 x 15 ml centrifuge tubes or less if there is very little sediment. For an effective flotation no more than 3 ml of sediment should be added to each tube. Make sure that both the glass rod and the original centrifuge tubes are rinsed well with Triton X-100 and that the rinsings are added to the 15 ml tubes.

8. Centrifuge the sample at 600 g for 10 min as described above.

9. Remove the supernatant carefully using a vacuum suction pump.

10. Add 3-4 ml of saturated $MgSO_4$ to each tube and agitate thoroughly (the pellet must be loosened and mixed with the salt solution). An automatic vortex mixer or a simple mounted needle may be used.

11. Top each centrifuge tube up to just below the rim with the salt solution and leave to stand for 5 min.

12. Centrifuge the tubes for 1 min at 150 g.

13. Put the glass tubes in a test tube rack and top up with salt solution using a glass pipette until a positive miniscus is formed. Rest a coverslip on the top of the tube and leave to stand for 30 min. A glass or plastic coverslip may be used. During this time helminth eggs will float to the surface.

14. Remove the coverslip using a firm upward movement and put on a glass slide for examination under a high power microscope. The eggs collect under the coverslip and are removed in the meniscus of salt solution as the coverslip is lifted off.

15. Four coverslips should be examined from each tube. (Only an insignificant number of eggs are recovered if more coverslips are examined; see Appendix I.)

3.0 COMPARISON OF THREE TECHNIQUES FOR THE ENUMERATION OF HELMINTH EGGS IN RAW SEWAGE

3.1 Introduction

Three methods for the recovery of eggs from raw wastewater were evaluated in laboratory trials at Leeds University and in field trials at EXTRABES. The Leeds I and Teichmann methods were compared with the method described by Mara and Silva (1986), which is currently used at EXTRABES to evaluate the number of parasitic helminths in samples of raw and treated wastewater.

3.2 Laboratory comparison of the Leeds I, EXTRABES and Teichmann techniques

3.2.1 Methods

Samples of raw sewage taken from Pudsey sewage treatment works were seeded with a range of number of Ascaris suum eggs,1/.

Eggs were then recovered and counted using the Leeds I, EXTRABES and Teichmann techniques described in Appendices I, III and IV respectively.

The percentage recovery was calculated for each sample, and a regression analysis was carried out to investigate if there was any relationship between the original egg number and percentage recovery.

3.2.2 Results and Discussion

Table 4 shows the percentage recoveries of Ascaris suum from 1 litre samples of raw sewage using the Leeds I, Teichmann and EXTRABES methods. It should be noted that 1 litre samples were used to evaluate the EXTRABES technique, although 500 ml samples are actually taken in the field. The percentage recovery is clearly most variable in the EXTRABES technique, and there was a very large variation even within replicates from the same samples (see Appendix VI). This can be explained by the fact that the technique utilizes the examination of very small subsamples which inherently builds in a large error since it is unlikely that helminth eggs remain evenly distributed within a sample after centrifugation and remixing. In the technique a subsample volume of only 0.2 ml is taken for microscopic examination from a final reference volume of 5 or 10 ml, and the results are then calculated to give a final count of helminth eggs per litre. Both the Leeds I and the Teichmann methods produced mean percentage recoveries that were higher than the EXTRABES technique but nonetheless these were still

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1. The eggs or larvae used for seeding experimental samples were always counted individually in a Doncaster counting dish or watch glass before being washed into the sample container using a solution of 0.01% Triton X-100 dispensed from a wash bottle. The use of Triton ensured that all eggs were rinsed into the sample container without becoming stuck to the counting dish. This is different to most published techniques where a suspension of eggs is made up (x/ml) and an aliquot is removed and added to the sample. Where very large numbers of eggs are being used this is the only practical method, but for small numbers it can be quite inaccurate, particularly with Ascaris suum eggs which tend to clump together unless kept well homogenised.

relatively low. However, as subsamples are not used in these techniques the variation of their results is greatly reduced.

Table 4. Percentage recovery of Ascaris suum eggs obtained from 1 litre samples of raw sewage using the Leeds I, Teichmann and EXTRABES techniques

Technique	No. of Samples	Range of numbers of eggs seeded per litre	Percentage recovery Mean	Range	95% confidence limits
Leeds I	14	99 - 840	24	11 - 44	4%
Teichmann	17	98 - 828	22	13 - 34	3%
EXTRABES	8	349 - 674	11	0 - 60	6%

The regression analysis of the original number of eggs seeded against percentage recovery (Table 5) shows no correlation between the number of eggs present and the percentage recovery obtained with any of the methods used. It is concluded that the percentage recovery is more likely to be affected by the quantity and quality of organic matter in the sample rather than by the absolute number of helminth eggs present.

Table 5. Regression of numbers of eggs seeded against the percentage recovery

Technique	Correlation coefficient	f ratio	Probability
Leeds I	0.055	0.0456 (N.S.)	0.828
Teichmann	0.2034	0.518 (N.S.)	0.507
EXTRABES	0.018	0.1089 (N.S.)	0.743

N.S. - non significant

3.3 Field comparison of the Leeds I, Teichmann and EXTRABES methods

3.3.1 Method

Five hundred ml grab samples of raw wastewater are taken twice a week at 0800 h by the technical staff at EXTRABES and analysed for parasitic helminth eggs and larvae using the EXTRABES technique. During April to June, two 1 litre grab samples were taken simultaneously from the same sump and analysed using the Leeds I and Teichmann methods. Samples were taken twice weekly wherever possible.

The EXTRABES method is also used at EXTRABES for the examination of protozoa in raw wastewater. Random samples were examined after extraction using the Leeds I and Teichmann techniques for protozoa by the EXTRABES technician concerned.

3.3.2 Results and discussion

Helminth Eggs

Table 6 shows the number of parasitic helminth eggs per litre of raw wastewater at EXTRABES determined by the Leeds I, Teichmann and EXTRABES techniques.

Table 6. Number of helminth eggs per litre of raw wastewater in samples taken twice weekly during April - June 1988

Date	A.lumbricoides			T.trichiura			H.diminuta			H.nana			Hookworm eggs		
	L	T	E	L	T	E	L	T	E	L	T	E	L	T	E
20.4	406	-	1000	14	-	0	-	-	-	3	-	0	2	-	0
25.5	390	654	700	10	05	100	-	-	0	6	6	0	0	0	0
27.4	429	422	1300	11						9	2	0	1	4	0
02.5	269	390	500	08	04	0	-	-	0	1	3	0	3	0	0
05.4	370	392	400	07	10	0	-	-	0	3	4	0	8	0	0
09.5	-	-	-	-	-	-	-	-	0	-	-	0	-	-	0
11.5	197	514	450	0	10	100	-	-	0	7	4	0	3	1	0
17.5	242	460	300	08	03	0	-	-	0	2	3	0	2	0	50
18.5	379	455	300	09	06	0	-	-	-	3	2	0	7	0	50
23.5	215	314	250	03	01	50	-	-	0	1	2	0	3	1	50
25.5	223	244	300	0	02	0	02	03	0	1	0	0	0	0	100
30.5	474	595	600	10	10	50	05	07	0	3	1	0	5	3	50
01.6	595	442	700	19	07	0	08	0	0	4	1	0	6	1	100
06.6	588	666	1100	16	11	0	05	10	0	7	10	0	6	4	100
08.6	389	476	200	11	13	100	10	01	0	4	2	100	2	0	0
13.6	702	445	300	15	05	0	07	03	0	2	3	0	5	2	300
15.6	348	415	200	08	05	0	04	02	0	1	0	0	3	0	100

L Leeds I technique
T Teichmann technique
E EXTRABES technique
- not determined

As in the laboratory trials in Leeds, the results obtained with the EXTRABES method were much more variable than those from both the Leeds I and the Teichmann methods. Of course the absolute number of eggs will vary considerably from day to day as environmental conditions (primarily rainfall) change, and also over a longer period as incidence and prevalence vary. However, these differences were greatly exaggerated by the EXTRABES technique, particularly when very low numbers of eggs were present.

The Teichmann technique was found to be very time consuming as the sample has to sediment overnight or for at least 8 hours (this allows a very generous margin of safety and consideration of the eggs terminal settling

velocities (Appendix VIII) indicates that the time could be considerably reduced). Furthermore, the final sediment requires the examination of 20-30 coverslips and, although this is only a few more than the number examined in the Leeds I method, it tends to be much more time consuming as there is usually much more debris through which to look and this large amount of debris can easily mask some of the eggs, so reducing the percentage recovery.

The Leeds I technique was found to be the most suitable of the three techniques: it was much less time-consuming than the Teichmann technique and just as accurate, and considerably more consistent than the EXTRABES technique.

Protozoa

No protozoa were detected in any of the random samples examined using either the Leeds I or Teichmann techniques, although they were detected by the EXTRABES technique but it is not known with what effectiveness. Thus it seems that, if it is of interest to count the number of excreted protozoa, the effectiveness of the EXTRABES technique should be evaluated and, if necessary, an improved specific technique developed.

4.0 DEVELOPMENT OF A TECHNIQUE FOR ENUMERATING INTESTINAL NEMATODE EGGS IN THE EFFLUENT OF WASTE STABILISATION PONDS.

4.1 Introduction

A techique is required to detect very low numbers of parasitic helminths, particularly Ascaris lumbricoides, in the final effluent of waste stabilisation ponds. The new World Health Organisation guidelines for the microbiological quality of treated wastewater used for crop irrigation is <1 intestinal nematode egg per litre, based on the Engelberg guideline value of <1 viable intestinal nematode egg per litre (IRCWD, 1985; Prost, 1988).

Such low levels of intestinal nematode eggs are readily achievable by a well designed series of waste stabilisation ponds, but the problem is that there is no simple reliable method for counting such low numbers of eggs. Most of the techniques described in the literature have evolved from the medical field (faeces examination), or from looking at highly contaminated raw sewage or sludge. These methods generally rely on a subsampling step which, as explained in Section 3.2, can lead to very large errors when looking for very low numbers of eggs, particularly if small samples of effluent are taken. These subsamples are taken so that 0.3 or 1.0 ml of the final sample can be examined in a McMaster or Sedgewick-Rafter counting chamber. It was decided to try to evolve a technique which does not rely on a subsampling step but which examines all the contents of a single grab sample using the larger 'Doncaster' counting dish which holds 4-5 ml.

The principal constituent of the suspended solids fraction of waste stabilisation ponds is single celled algae (particularly in the case of facultative ponds), some of which can be of similar size and shape to parasitic nematode eggs. This leads to problems when attempts are made to count helminth eggs using any of the available techniques as most use either a flotation step using concentrated salt solutions in which eggs, larvae and algae will all float, or a sedimentation stage during which all three will settle together. Thus one of the first aims of the work was to devise a simple method for the separation of parasitic helminths and algae in pond effluent samples leaving a small amount of cleaner water which could be examined for helminths in a Doncaster dish under low power magnification.

4.2 Removal of algae

4.2.1 Introduction

Several methods were tried to destroy or break up single celled algae and to separate them from helminths. It was hoped that small fragments of algal cells could be separated from helminths by sieving or floating away the fragments on a salt solution of low specific gravity in which the helminths would not float. Other methods to separate whole algae from helminths were tried and are described below.

4.2.2 Methods

One litre samples of water containing cultures of Euglena and Chlorella were concentrated by centrifugation, the supernatant removed and various treatments applied to the algal concentrate. Methods tried for breaking down the algal were destruction using high osmotic pressure (saturated solutions of sodium chloride and sodium nitrate) and cell wall disintegration (Triton X-100, sodium hypochlorite, heat treatment and sonication). Methods for separating whole algae from helminths were filtration, charge separation, flotation in concentrated salt, sucrose gradient separation and flotation in weak salt solutions.

Algae were kept in petri dishes and observed at regular intervals for changes in their structure. Where a technique looked promising observations were also made on parasitic helminths (eggs of Ascaris suum, Hymenolepis diminuta, Trichuris ovis and eggs and larvae of Nippostrongylus brasiliensis).

4.2.3 Results and discussion

The results of experiments to obtain the separation of helminth eggs and larvae from algae with and without destruction of the algae are shown in Table 7, together with notes on the efficacy and practicality of the methods.

Table 7a. Separation of helminth eggs and larvae without algal destruction.

METHOD	REMARKS
Filtration	Sieves of small enough mesh size to trap the smallest of the helminth eggs (hookworm) would also trap most of the algae. Sieving was abandoned at an early stage as previous work had already shown that large numbers of helminth eggs are lost during this procedure (Section 2.2).
Charge separation	The charges on A. suum eggs were measured using a Rank Brothers Electrophoresis Mobility Apparatus with a view to possibly separating eggs from algae along an electrical gradient. It was found that they had a weak negative charge as do the majority of algae. It was considered unlikely that this method would yield any useful results.
Flotation on concentrated salt solutions	Algae and helminth eggs and larvae were found to float in concentrated salt solutions.
Sucrose gradient separation	This technique is frequently used for 'cleaning' suspension of adult or larval nematodes (MAFF, 1986a). It was difficult to obtain a clear interphase containing the eggs, and the helminth eggs and larvae tended to become distorted in the concentrated sucrose.
Flotation in weak salt solutions	It was found that most of the algae in waste stabilisation pond will remain in suspension in relatively weak solutions of salt (NaCl and $MgSO_4$, specififc gravity 1.04 and 1.07) for 1-1.5 hours. The helminth eggs of interest in this project have mean specific gravities 1.0559 (Ancylostoma) - 1.1299 (Trichuris) (David & Lindquist, 1982). Trials were carried out in 50 ml centrifuge tubes filled to a depth of 5 cm with flotation solution. Although not all of the algae remained in suspension a significant amount could be separated in this way from the helminths which, being heavier, sedimented more rapidly.

Table 7b. Separation of helminth eggs and larvae with algal destruction.

METHOD		REMARKS
Heat treatment		Suspensions of algae were boiled for 0-20 min with regular subsampling for microscopic examination. No change in structure was observed although chlorophyll was lost. This treatment would certainly kill all nematodes making viability assessments impossible.
High osmotic pressure		Algae were suspended in solutions of saturated salt (NaCl and $NaNO_3$) for 60 min. Samples were removed and examined regularly. No change in structure was observed so the algae would continue to be a problem in this separation method.
Sonication		Algae were treated using a MSE Ultrasonicator at 6 microns for 1 min. The treatment separated clumps of algae and clumps of eggs but no change in the structure of either was observed. Sonication would probably destroy cell contents making viability assessments impossible.
Cell wall destruction	a)	Algae were left in a solution of 1% Triton X-100 for 1 hour. Samples were taken every 10 min for microscope examination. No change in structure was observed.
	b)	Algae were left in a solution of 50% sodium hypochlorite. Within minutes certain algae from pond water in West Yorkshire started to disintegrate. Although initially promising, this result could not be reproduced with algae from waste stabilisation ponds in the field and the technique was abandoned.

4.2.4 Conclusions

Flotation of algae in a weak salt solution showed the most promise as a technique for separating algae from helminth eggs and larvae. It was found with this method that a proportion of algae will stay suspended in a salt solution sedimenting slowly over 1-1.5 hours. In order to allow the lightest of the helminth eggs and larvae to sediment out (Ancylostoma spp., specific gravity 1.0559) a solution of specific gravity 1.04 should be used. It was observed that the rate of algal sedimentation was only slightly slower in the denser solution (specific gravity 1.07).

4.3 Optimum sedimentation times for eggs and larvae

4.3.1 Introduction

Results from the algal removal experiments (section 4.2) suggested that the simplest method to separate helminth eggs and larvae from algae may be in a weak solution of salt in which the algae may float or at least sediment more slowly than the helminth eggs or larvae. A series of observations was made to determine the optimum time for complete sedimentation of eggs and larvae and the maximum removal of algae.

4.3.2 Method

Sedimentation times for eggs of Ascaris suum, Trichuris muris and Hymenolepis diminuta and eggs and infective stage larvae of Nippostrongylus brasiliensis were measured in a solution of magnesium sulphate of specific gravity 1.04.

Known numbers of eggs or larvae (range 100-200) were washed from a counting dish into 50 ml centrifuge tubes using a solution of 0.01% Triton X-100. The tubes were centrifuged at 1500 rpm for 3-4 min. The supernatant was removed using a simple tap-attachment vacuum pump, leaving 2-3 ml of sediment. Then 30 ml (5 cm depth) of $MgSO_4$ solution, specific gravity 1.04, was added to each tube and allowed to stand. The supernatant was removed (using the same pump) at various time intervals and the number of eggs in the sediment counted. Sedimentation in 30 ml tap water was used as a control.

4.3.3 Results and discussion

Optimum recovery rates for all the helminth eggs were obtained within 1 hour (Table 8), and up to 87% of the infective stage larvae could be recovered if the samples were left to stand for 2 hours. However because earlier observations had shown that the majority of algae found in pond effluent will also settle out in 2 hours, so leaving a sediment which is difficult to examine under the microscope, a sedimentation time of 1 hour was chosen for the technique, even though this meant that the recovery rate for hookworm larvae might be rather poor. This may not be a serious problem as, for reasons outlined in Section 10, hookworm larvae are unlikely to be found in large numbers in the final effluent of waste stabilisation ponds.

Table 8. Percentage recovery of helminth eggs after sedimentation in $MgSO_4$ (specific gravity 1.04) and regression against time

Helminth	15 min	30 min	1 hr	2 hr	1 hr control	Regress. coeff.	Probability
A.suum	-	86±46 (3)	87 (10)	-	89±18 (3)	0.3833	0.13967
H.dim.	80±6 (10)	88±5 (10)	91±4	-	95±3 (10)	0.543	2.0×10^{-3}
T.muris	71±6 (10)	91±3 (10)	98±5 (10)	-	92±4 (10)	0.5753	9.68×10^{-4}
N.bras eggs	-	30±18 (10)	85±13 (10)	-	87±10 (10)	0.9495	4.62×10^{-4}
N.bras larvae	26±12 (4)	21±19 (5)	55±12 (5)	87±13 (4)	80±18 (4)	0.9336	4.34×10^{-8}

N.bras = N.brasiliensis
H.dim = H.diminuta
(No. of replicates in brackets.)
95% confidence intervals calculated using $x \pm t(s/\sqrt{n})$ for small sample sizes (Sokal and Rohlf, 1981).

Following these experiments a final technique was evolved which is described for the time being as the Leeds II technique. It is hoped that other alternative final examination chambers may ultimately be used for the technique, as Doncaster counting dishes are currently only available from one source (Appendix II). The technique is described in detail below.

4.4 The Leeds II technique

For the enumeration of intestinal helminths in the effluent of waste stabilisation ponds:

1. Collect four 1 litre grab samples of final effluent in clean plastic containers. The containers should have smooth internal walls and be prewashed with a detergent to remove any grease and thus decrease the chances of helminth eggs adhering. Add 10 ml formalin to the sample.

2. Leave the samples to stand for at least 1 hour. This is the minimum amount of time with a generous safety margin required for the lightest of the helminth eggs to sediment in a standard 1 litre beaker. The sedimentation time for any particular container can be calculated using the Stokes equation (Appendix VIII). It is suggested that the calculated time required is always doubled for safety.

3. Remove the supernatant carefully using a siphon or a suction pump leaving 60-70 ml sediment. The supernatant must not be poured off.

4. Transfer the sediment of each sample into a 50 or 100 ml centrifuge tube. The sides of the sedimentation vessel should be rinsed thoroughly with a solution of 0.01% Triton X-100 and the rinsings added to the appropriate centrifuge tube.

5. Centrifuge the sample at 600 g for 10 min.

6. Remove the supernatant using a vacuum pump leaving a small pellet of about 2-3 ml at the bottom of the tube. It is very important that the supernatant is removed carefully at this stage as it is very easy to suck up eggs with other debris in the pellet. A glass pipette or plastic pipette tip may be inserted into the siphon tube for greater control.

7. Add a solution of NaCl with a specific gravity of 1.04 to a depth of 5 cm and leave to stand for 1 hour. In order to ensure a homogenous mix of pellet and salt solution it may be necessary to first add a few ml of flotation solution, and agitate before adding the rest.

8. Remove the supernatant and discard leaving a pellet of 2-3 ml in the bottom of the tube.

9. Pour the final pellet into a Doncaster counting dish and examine under a high power microscope for eggs and larvae of parasitic helminths. If a Doncaster counting dish cannot be obtained the pellet can be examined in several batches in a slightly smaller counting cell such as a Sedgewick-Rafter slide. The centrifuge tube should be rinsed with the Triton X-100 solution and the rinsings examined as well.

Notes:

(a) In a salt solution of specific gravity 1.04 many of the algae which are normally found in effluent from waste stabilisation ponds will remain in suspension whilst the helminth eggs and larvae will sediment out, thus leaving a cleaner pellet for final examination. One hour has been determined as the optimum time for maximum helminth sedimentation and minimum algal sedimentation.

(b) A Doncaster counting dish is a counting chamber which is used directly under a microscope. It is a round dish (diameter 75 mm) with concentric circular divisions which keep eggs and larvae in defined areas. A total volume of 4 to 5 ml can easily and efficiently be examined under a magnification of x40.

4.5 Rate of recovery of helminths and total suspended solids

4.5.1 Introduction

The single factor most likely to affect the rate of recovery of helminth eggs and larvae from the final effluent of waste stabilisation ponds using the Leeds II technique is the amount of total suspended solids present in the form of algae which may be of a similar shape and size to the helminth eggs. Eggs may adhere to algae during the flotation stage of the counting techique, or may be obscured by algae when the samples are finally examined microscopically in a counting dish.

Historic data from the pond series at EXTRABES were examined to assess the range of total suspended solids found in the effluents of the three maturation ponds (M7, M8, M9) over a one year period and are shown in Table 9.

Table 9. Total suspended solids in the effleunt of maturation ponds M7, M8 and M9 from 2 July 1985 - 1 July 1986.

TSS concentration (mg/l)	M7	M8	M9
Mean TSS	44	30	28
Maximum	144	97	79
Minimum	21	6	1
95% confidence limits	± 7.6	± 5.4	± 7.8

Since total suspended solids can readily be measured (APHA, 1985), a series of experiments was carried out to investigate how the rate of recovery of single eggs in 1 litre samples of final effluent varied with the total suspended solid concentration.

Experiments were designed to test the rate of recovery over the full range of concentrations of total suspended solids which were found during this 1 year period.

4.5.2 Method

Water containing algae was taken when required from a disused primary sedimentation tank at EXTRABES. Examination of a sample taken at 0800 h on 26 June 1988 showed that the following species of algae were present:

Ankistrodesmus
Chlorella (*)
Ciclotella
Diatomocea (*)
Euglena (*)
Oscillatoria
Scenedesmus
Tetraedron

Species usually found in the final effluent of waste stabilisation ponds at EXTRABES are marked with an asterisk.

Three samples of pond water, each of 1 litre, were examined for helminth eggs. The samples were centrifuged for 10 min at 600 g and all the sediment examined in several batches in a Doncaster counting dish. No helminth eggs were found. Suspended solids were measured using the method described in Standard Methods (APHA, 1985).

Single eggs of Ascaris lumbricoides, Trichuris ovis, and Hymenolepis diminuta, were pipetted onto a watch glass together and then rinsed (using 0.01% Triton X-100 to ensure that the eggs did not adhere to the watchglass) into 1 litre volumes of pond water containing a range of quantities of algae, and then recovered using the Leeds II

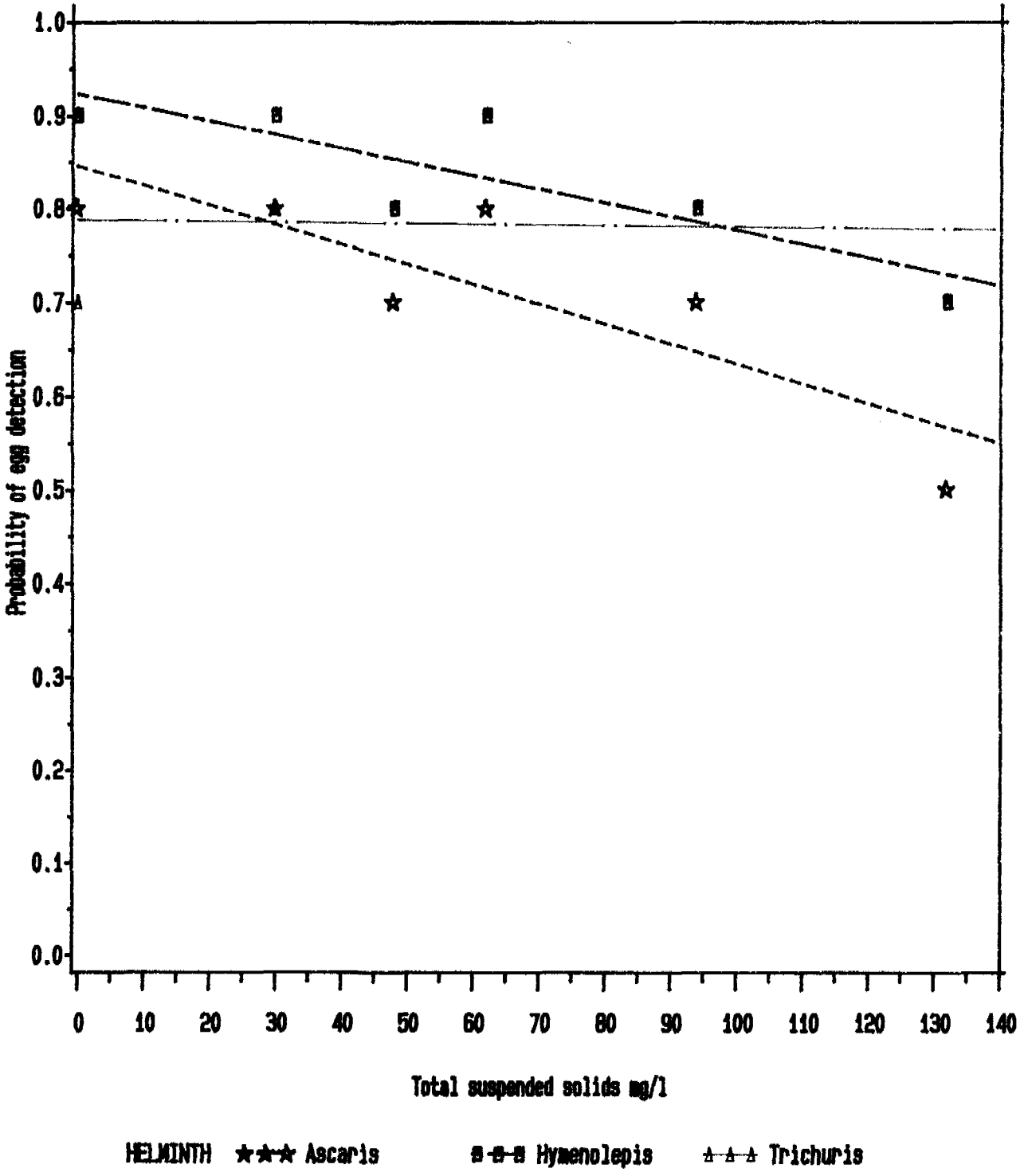

Fig. 2: Probability (regression analysis) of detecting 1 egg per litre over a range of total suspended solids concentrations

technique described in Appendix II. To reduce the concentration of suspended solids the pond water was diluted with tap water. To increase the total suspended solids concentration, 1 litre samples were centrifuged, the supernatant poured off and the sediment combined until the required concentration was reached. Concentrations of 0, 30, 48, 62, 94 and 132 mg/l were used. Ten replicates were processed for each concentration of total suspended solids and ten controls were carried out using 1 litre samples of tap water.

4.5.3 Results and discussion

There was a small but significant correlation between the total suspended solids and the probability of recovery of single eggs of Ascaris lumbricoides and Hymenolepis diminuta (Table 10). As the amount of suspended solids increased, so the probability of egg recovery decreased (Figure 2). When the quantity of suspended solids was low the technique was very effective: there was an 80 percent chance of recovering a single A.lumbricoides egg from an effluent containing suspended solids in the range 0-30 mg/l.

Table 10. Regression analysis of the probability of recovering a single helminth egg against total suspended solids concentration

Suspended solids concentration (mg/l)	Probability of egg recovery (mean of 10 reps) for Ascaris lumbricoides	Trichuris ovis	Hymenolepis diminuta
0	0.8	0.7	0.9
30	0.8	0.8	0.9
48	0.7	0.8	0.8
62	0.8	0.8	0.9
94	0.7	0.6	0.8
132	0.5	0.5	0.7
f ratio	10.13 S	4.713 NS	10.463 S
p	3.419×10^{-2}	9.513×10^{-2}	3.262×10^{-2}

S = significant at the 0.5% level, NS = not significant

As the amount of algae increased it became more difficult to remove them by flotation, and thus the eggs may have been more obscured by debris in the counting dish. There was no correlation between the recovery of Trichuris ovis eggs and suspended solids, possibly because the egg, although a little smaller than those of Ascaris lumbricoides and Hymnolepis diminuta, is very much easier to see, being bright orange-tan in colour with a distinct "elongated lemon" shape.

5.0 COMPARISON OF THE EXTRABES AND LEEDS II TECHNIQUES

5.1 Introduction

Samples of effluent of all the waste stabilisation ponds at EXTRABES are taken routinely once a week by the technicians at EXTRABES and analysed for parasitic helminths eggs and larvae using the technique described in Appendix III. Over a period of 7 weeks samples were also analysed using the Leeds II technique in order to compare the relative effectiveness of the two techniques.

5.2 Evaluation of maturation pond effluent

5.2.1 Methods

Samples of effluent from maturation ponds M12, M13 and M14 were taken at 0800 h every Tuesday over 5 weeks from 2 August - 4 October 1988. 500 ml was taken for analysis using the EXTRBES technique (Appendix III) and four 1-litre samples for analysis using the Leeds II technique (Appendix II). Total suspended solids were also measured on each occasion (APHA, 1985). Samples for both techniques were taken at the same time in order to minimise intrasample variation.

5.2.2 Results and discussion

No eggs were detected in the effluent of any of the maturation ponds using the EXTRABES technique, whereas mean egg counts of 0.3, 0.15 and 0.11 eggs/litre were obtained over the period for effluents of M12, M13 and M14 respectively using the Leeds II technique (Table 11).

The numbers of helminth eggs were extremely low in all samples. However the larger sample size of 4 litres used in the Leeds II technique was more effective in detecting those eggs present. There was no correlation between the amount of total suspended solids and the egg count per litre, suggesting that helminth eggs do not move up and down the pond profile with the algae (Mara,1976). However it is known that the amount of total suspended solids does affect the accuracy of the Leeds II technique slightly (Section 4.5).

Table 11. Helminth eggs per litre recovered from 0800 h samples of effluent from M12, M13 and M14 and regression of egg count per litre versus total suspended solids concentration.

Date	Mean egg count per litre in effluent from pond								
	M12			M13			M14		
	EXT	LII	TSS	EXT	LII	TSS	EXT	LII	TSS
02:08:88	0	0.75	32	0	0	17	0	0.5	12
30:08:88	0	0.25	80	0	0.25	21	0	0	8
06:09:88	0	0.25	75	0	0.25	52	0	0.25	33
13:09:88	0	0.25	66	0	0.25	96	0	0	18
20:09:88	0	0	52	0	0	96	0	0	25
27:09:88	-	-	-	-	-	-	0	0	16
04:10:88	-	-	-	-	-	-	0	0	43
mean	0	0.30		0	0.15		0	0.11	
correlation coefficient		-0.577			-0.002			-0.161	

EXT = EXTRABES technique
LII = Leeds II technique
TSS = Total Suspended Solids (mg/l)

The data suggest that the pond series is working very effectively in terms of helminth removal, with the majority being removed in the anaerobic and facultative ponds. The effluent of the first maturation pond in the series, M12 (likely to be the most contaminated), was shown to be of a much cleaner quality than that recommended by the Engelberg Report (IRCWD, 1985) for effluent reuse. The effluent quality did not appear to vary greatly over the sampling period.

In order to confirm the results of these observations it was decided to sample an effluent which was more likely to be contamined with helminth eggs. The unbaffled facultative pond, F18, was selected for this purpose.

5.3 Evaluation of facultative pond effluent

5.3.1 Introduction

Routine weekly sampling of the final effluent of maturation ponds M12, M13, and M14, showed that more eggs were consistently recovered using the Leeds II techique than the EXTRABES technique (Section 5.2). However, egg numbers were consistently very low (mean <0.3 per litre) and in order to make further comparisons between the techniques it was decided to use the effluent of one of the facultative ponds F18. This pond operates as a single facultative reactor, receiving raw

wastewater and having a retention time of 15 days. The pond was unbaffled and recent tracer work at EXTRABES showed that there was some short circuiting occuring with tracers appearing within 2-3 h in the effluent (Silva, pers. comm.). It was considered therefore that there was a greater chance of finding eggs in the effluent of this pond.

5.3.2 Methods

Samples of the effluent from pond F18 were taken at 0800 h on seven occasions between 21 and 29 October 1988 and the number of helminth eggs analysed using the EXTRABES and Leeds II techniques (sample size: 500 ml and 4 x 1 litre, respectively). The suspended solids were also measured on each occasion (APHA, 1985).

5.3.3 Results and discussion

Table 12 shows that on every occasion more eggs were found using the Leeds II technique than the EXTRABES technique.

Table 12. Helminth egg count per litre recovered by the EXTRABES and Leeds II techniques from samples of effluent taken at 0800 hr on seven occasions from pond F18.

	Helminth eggs per litre		
Date	EXTRABES technique	Leeds II technique (*)	Total suspended solids (mg/l)
21:09:88	0	0.75	18
22:09:88	0	0.25	27
23:09:88	0	0.75	35
26:09:88	0	3.50	40
27:09:88	0	1.50	49
28:09:88	0	0.75	93
29:09:88	0	0.25	149
mean	0	1.107	59

* = all eggs recovered were Ascaris lumbricoides

As before (Section 5.2), there was no correlation between the number of eggs per litre in the effluent and the amount of total suspended solids (correlation coefficient = -0.2777), confirming that there is no direct relationship between the movement of algae up and down the water column and the appearance of helminth eggs in the final effluent.

6 DISTRIBUTION OF HELMINTH EGGS IN THE FINAL EFFLUENT OF FACULTATIVE PONDS

6.1 Introduction

In order to attach confidence levels to any particular sampling regime, the distribution pattern of helminth eggs in the effluent that is being sampled must be known. The most likely distribution for eggs in the final effluent of waste stabilisation ponds is a Poisson distribution, where events occur randomly and independently from one another (Sokal and Rolfe, 1981). Data collected from daily sampling, 24 hour sampling and multiple samples at a single point in time were analysed to determine whether or not egg numbers did in fact follow a Poisson distribution.

6.2 Method

In order to test the distribution of eggs in the final effluent of waste stabilisation ponds, twenty 1-litre samples were taken in rapid succession from the facultative pond F18 and analysed using the Leeds II technique (Appendix II). These data, those obtained from 24-hour sampling periods (Section 9) and those from routine daily and weekly monitoring (Section 5) were tested for Poisson distribution using the Kolmogorov-Smirnov one-sample test (Siegel, 1956). This is a test of goodness of fit which is concerned with the degree of agreement between the distribution of a set of sample values and a theoretical distribution. The test involves specifying the cumulative frequency distribution which would occur under the theoretical distribution and comparing that with the observed frequency distribution. The Kolmogorov-Smirnov test treats individual observations separately and thus, unlike the Chi squared test, does not lose information through the combination of categories.

The Null Hypothesis (Ho) which was set states that the samples have been drawn from the specified theoretical Poisson distribution, and that the variation in the data can be explained by this.

6.3 Results and discussion

The three sets of data described above were analysed separately and together to test the null hypothesis. For calculation the following parameters were used:

(a) Fo(X): a completely specified theoretical cumulative frequency under a Poisson distribution, where the mean of the sample is used.

(b) Sn(X): observed cumulative frequency of a random sample of N observations. X is any possible number of eggs/litre. Sn(X) = K/N where K = the number of observations equal or less than X.

(c) D max.: maximum value for Fo(X) - Sn(X)

A critical value for D is found from Kolmogorov-Smirnov tables (Rohlf and Sokal, 1981) for n degrees of freedom where n = number of categories. If D max. < D critical, the Ho must be rejected.

The results are shown in Tables 13, 14, 15 and 16. In each of the analyses the value for D max. was well below the critical value for D. This means that the null hypothesis that the distribution follows a Poisson distribution must be accepted, and that in each case this can be accepted with a maximum degree of confidence of 95 percent (p = 0.05)

Table 13. Twenty 1 litre samples of effluent from F18 taken sequentially at 12.30 on 30 September 1988.

No. eggs per litre	Freq. of observation	Fo (X) Mean = 0.55 ± 0.47	Sn (X)	D max
0	13	0.5769	0.65	0.0731
1	5	0.8944	0.90	
2	1	0.9817	0.95	
3	0	0.9977	0.95	
4	1	1.0000	1.00	

D max. = 0.07. D critical (for n = 5) = 0.56, p = 0.05
Ho cannot be rejected.

Table 14. Daily sampling of effluent from pond F18 (four x 1 litre samples taken at 0800 h -Section 9.

No. eggs per litre	Freq. of observed	Fo (X) Mean = 1.107 ± 0.47	Sn (X)	D max
0	9	0.3306	0.3200	
1	13	0.6965	0.7857	0.0892
2	3	0.8990	0.8926	
3	0	0.9737	0.8926	
4	3	1.9944	1.000	

D max. = 0.089. D critical (for n = 5) = 0.56, p = 0.05
Ho cannot be rejected.

Table 15. 24 hour period sampling of effluent from F18 (samples taken every three hours;data from 28 September and 4 October combined Section 9).

No. eggs per litre	Freq. observed	Fo (X) Mean = 0.75 ± 0.44	Sn (X)	D max
0	21	0.4724	0.5833	0.1109
1	11	0.8267	0.8888	
2	0	0.9596	0.8888	
3	2	0.9928	0.9444	
4	1	0.9990	0.9722	
5	0	0.9999	0.9722	
6	1	1.000	1.0000	

D max = 0.11 D critical (for n = 7) = 0.52, p = 0.05
Ho cannot be rejected

Table 16. Combination of all distribution data from, Tables 13, 14 and 15.

No. eggs per litre	Freq. observed	Fo (X) Mean = 0.82 ± 0.26	Sn (X)	D max
0	43	0.4398	0.5119	0.0721
1	29	0.8004	0.8571	
2	4	0.9483	0.9048	
3	2	0.9887	0.9286	
4	5	0.997	0.9881	
5	0	0.9984	0.9881	
6	1	1.0000	1.0000	

D max = 0.072 D critical (for n = 7) = 0.52, p = 0.05
Ho cannot be rejected

7 EFFICIENCY OF THE LEEDS II TECHNIQUE AND SAMPLING SIZES

7.1 Introduction

Knowing that the distribution of helminth eggs in the final effluent of the facultative waste stabilisation pond is randomly distributed following a Poisson distribution, and assuming that this is likely to be the pattern of egg distribution in most pond effluents (Section 6), it is possible to calculate the probability of sampling one or more helminth eggs over a range of theoretical mean egg concentrations. It is also possible to calculate the minimum number of samples needed to be confident that the sample mean is a good estimate of the underlying mean and to attach confidence levels to the data obtained.

7.2 Probability of recovering one helminth egg per litre

Table 17 shows the efficiency of the technique for the recovery of one Ascaris lumbricoides egg per litre over a range of suspended solids concentrations. It has been extrapolated from the regression analysis shown on fig.7.

Table 17. Probability of recovering 1 egg/litre over a range of suspended solids (SS) concentrations.

SS (mg/l)	Probability of recovering 1 egg/litre
0-30	0.8
30-70	0.7
70-115	0.6
>115	0.5

7.3 Probability of sampling ≥ 1 helminth egg

Assuming a mean concentration (x) of 1 egg/litre, then the probability of sampling no eggs, one egg, two eggs etc (r = 0,1..) is computed using the Poisson distribution, as follows:

$$Pr(r) = \exp(-x)x^r/r!$$

For example, the probability of sampling no eggs when x = 1, is:

$$Pr(r=0) = \exp(-1)$$
$$= 0.368$$

and the probability of sampling one or more eggs when x = 1, is:

$$Pr(r\geq 1) = 1 - Pr(r=0)$$
$$= 1 - 0.368$$
$$= 0.632$$

7.4 Probability of detecting ≥ 1 helminth egg

Detecting an egg involves both processes of sampling and recovery, and the probability of detecting one or more eggs per litre also follows a Poisson distribution of the form:

$$Pr(r) = \exp(-xp)xp^r/r!$$

where x = the underlying mean concentration of eggs per litre

p = the probability of recovery

Thus the probability of detecting no eggs in a one litre sample of an effluent with a mean egg concentration of one per litre (x=1), and a suspended solids concentration of 0-30 mg/l,is calculated as:

$$Pr(r=0) = \exp[-1(1)(0.8)]$$
$$= 0.449$$

and the probability of detecting one or more eggs will be:

$$Pr(r\geq1) = 1- Pr(0)$$
$$= 1- 0.449$$
$$= 0.551$$

Table 18 shows the probabilities of detecting one or more eggs over a range of recovery efficiencies and with a varying number of one litre samples, assuming a mean concentration of one egg per litre (x=1).

Table 18. Probability of detecting ≥ 1 egg using the Leeds II technique assuming a mean egg concentration of one per litre.

No. of one litre samples	Probability of detecting ≥ 1 egg/l for values of xp of:			
	0.8	0.7	0.6	0.5
1	0.551	0.503	0.451	0.393
2	0.800	0.750	0.700	0.632
3	0.910	0.877	0.835	0.766
4	0.960	0.940	0.910	0.865

The probability of detecting one or more eggs using four 1 litre samples, assuming a mean egg concentration of 1/litre will vary from 0.96 to 0.87 depending on the amount of suspended solids in the samples. Thus there is a minimum probability of 0.87 of detecting at least one egg when four 1 litre samples are taken of an effluent from waste stabilisation ponds. If there is a higher concentration of eggs in the effluent, then the probability will be even higher.

7.5 Minimum number of samples required

To find the minimum number (n) of samples needed to be sure that the sample mean provides a good estimate of the underlying mean the following equation can be used (Elliot, 1983):

$$D = 1/\sqrt{(nx)}$$

where D, the index of precision, is the tolerable error. For example, if a confidence of 80% is required, then the error is 20% and D is 0.2.

This equation can be used to calculate the minimum number of samples required to detect an underlying mean of 1 egg/litre, but it needs modification to incorporate the probability of egg recovery (p), as follows:

$$D = 1/\sqrt{(nxp)}$$

Thus n is given by:

$$n = 1/(D^2xp)$$

The minimum number of 1 litre samples needed to be 80% (D=0.2) or 90% (D=0.1) sure that the sample mean is a good estimate of the underlying mean is shown in Table 19 for a range of detection rates.

Table 19. Number of samples needed for given levels of confidence where the underlying mean is predicted to be 1 or 2 eggs/litre.

Underlying mean concentration	Minimum number of one litre samples required for 90% confidence for xp value of:				for 90% confidence for xp value of:			
	0.8	0.7	0.6	0.5	0.8	0.7	0.6	0.5
1	32	36	42	50	125	143	167	200
2	16	18	21	25	63	72	83	100

Once a number (n) of 1 litre samples has been taken and analysed the data can be used to estimate the underlying mean number of helminth eggs in the effluent. Following this, 95% confidence limits for the underlying mean are computed in two stages. Firstly 95% confidence limits are determined for the mean modified by the rate of recovery:

$$xp \pm t(s/\sqrt{n})$$

where xp = mean number of helminth eggs per litre from n samples
t : value of student t for 95% level of confidence with n-1 degrees of freedom
s : standard deviation
n : total number of 1 litre samples taken

Secondly these 95% confidence limits (y and z) must be divided by the probability of recovery (p) to arrive at the 95% confidence limits for the underlying mean. Thus:

$$y/p \leq x \leq x/p$$

For example, using the data in Table 16 for the single primary facultative pond:

$n = 84$
$xp = 0.82$
$s = 1.21$
$t = 1.99$ for 83 d.f and 95% confidence
$p = 0.7$ (for SS of 30-70 mg/l)

then (1): $xp \pm t(s/\sqrt{n})$
$0.82 \pm 1.99\ (1.21/\sqrt{84})$
0.82 ± 0.26

so that $y = 0.56$ and $z = 1.08$

and (2): $y/p \leq x \leq z/p$
$0.56/0.7 \leq x \leq 1.08/0.7$
$0.8 \leq x \leq 1.54$

In this case it can therefore be said with 95% confidence that the underlying mean lies between 0.8 and 1.54.

To be 80% sure that the observed mean approximates the underlying mean using the Leeds II technique and assuming that a minimum of 1 egg per litre must be detected, then between 32 and 50 1-litre samples, depending on the suspended solids concentration (Table 18), must be analysed. Providing that the samples are routinely taken at the same time of day, the suspended solids should not vary too greatly and it is suggested that the average suspended solids concentrations over the sampling period be used for final evaluation of the underlying mean. If the suspended solids are not, or cannot be, measured as part of the routine effluent analylsis, it may be necessary to assume a worst case situation and allow 50 samples to be taken before a decision is made, and to use a probability of recovery of 0.5 throughout all the above calculations.

It is not necessary to take all the samples at the same time, sampling may be spread over several weeks. However, it is recommended that four 1-litre samples be taken on each sampling occasion in order to have a 0.96-0.87 probability of egg detection in effluents containing a minimum of one egg per litre. Thus the 32 - 50 samples referred to above would be taken on 8 - 13 sampling occasions, which may be spread over 8 - 13 weeks or even months. The justification for this is that pond performance is unlikely to vary greatly over these periods with respect to helminth egg removal, and any decision regarding the acceptability of the effluent for irrigation would not be made on a weekly or monthly basis but as part of the long term evaluation of pond performance. If a pond is found to produce an effluent of consistently poor quality, the effluent should not be reused until modifications to the pond design have been made. For example the addition of one or more maturation ponds would improve the removal of helminths and produce a consistently clean effluent.

8 DIURNAL VARIATION OF HELMINTH EGG NUMBERS IN RAW SEWAGE

8.1 Introduction

Samples of raw wastewater are routinely taken twice a week by the technicians at EXTRABES at 0800 h and analysed using the EXTRABES technique (Appendix III) to assess the number of helminth eggs entering the waste stabilisation ponds. It was not known whether there were marked diurnal fluctuations in the numbers of helminth eggs in the raw sewage or if the samples were representative of the number of eggs entering the ponds. The flow and strength of raw sewage varies throughout the day, the magnitude of the peaks depending on the size of the contributing population. Work at EXTRABES in 1978 and 1979 (Silva, 1982) showed that there is a well marked diurnal variation in the quantity of suspended solids, biochemical oxygen demand and faecal coliforms, with lower values being detected in the early mornings and higher values around midday. These parameters follow practically the same diurnal variation in both the dry and the rainy seasons.

Raw sewage was sampled at EXTRABES over three 24-hour periods at approximately one month intervals (May-July 1988) to determine whether there was any diurnal variation in helminth egg numbers and to find out if a single sample taken at 0800 h was representative of the 24 h period.

8.2 Method

Samples of raw sewage were taken over a 24 hour period on 3 May, 7 June and 14 July 1988 (all weekdays). Samples were taken every three hours starting at 0800 h. On 3 May, four 500 ml samples were collected on each occasion but, due to the amount of time required for subsequent sample examination, the number of replicates was reduced to three on 7 June and 14 July. Samples were collected from a raw wastewater sump, and stored with 5 ml of formalin in a glass screw-top bottle. Helminth eggs were counted and identified using the Leeds I technique (Appendix I) which has an average recovery rate of 24%.

The raw data were analysed using a statistics package ("Statspack") developed for the BBC Microcomputer by the Department of Pure and Applied Biology, University of Leeds, using analysis of variance to look at intrasample differences and the t-test to test for intersample differences.

8.3 Results and discussion

The diurnal variations of egg numbers per litre of raw wastewater determined on 3 May, 7 June and 14 July are given in Tables 20, 21 and 22 respectively. The amounts of rainfall which occurred on these dates were 5.1, 1.0 and 52.2 mm respectively. The tables show that significant diurnal variation in the numbers of *Ascaris lumbricoides* eggs on each of the three occasions (see also Figure 3,) with the high rainfall on the third occasion having little effect on either the egg count or its pattern of diurnal variation. The recorded range of *A.lumbricoides* egg numbers was 38-670 eggs/litre.

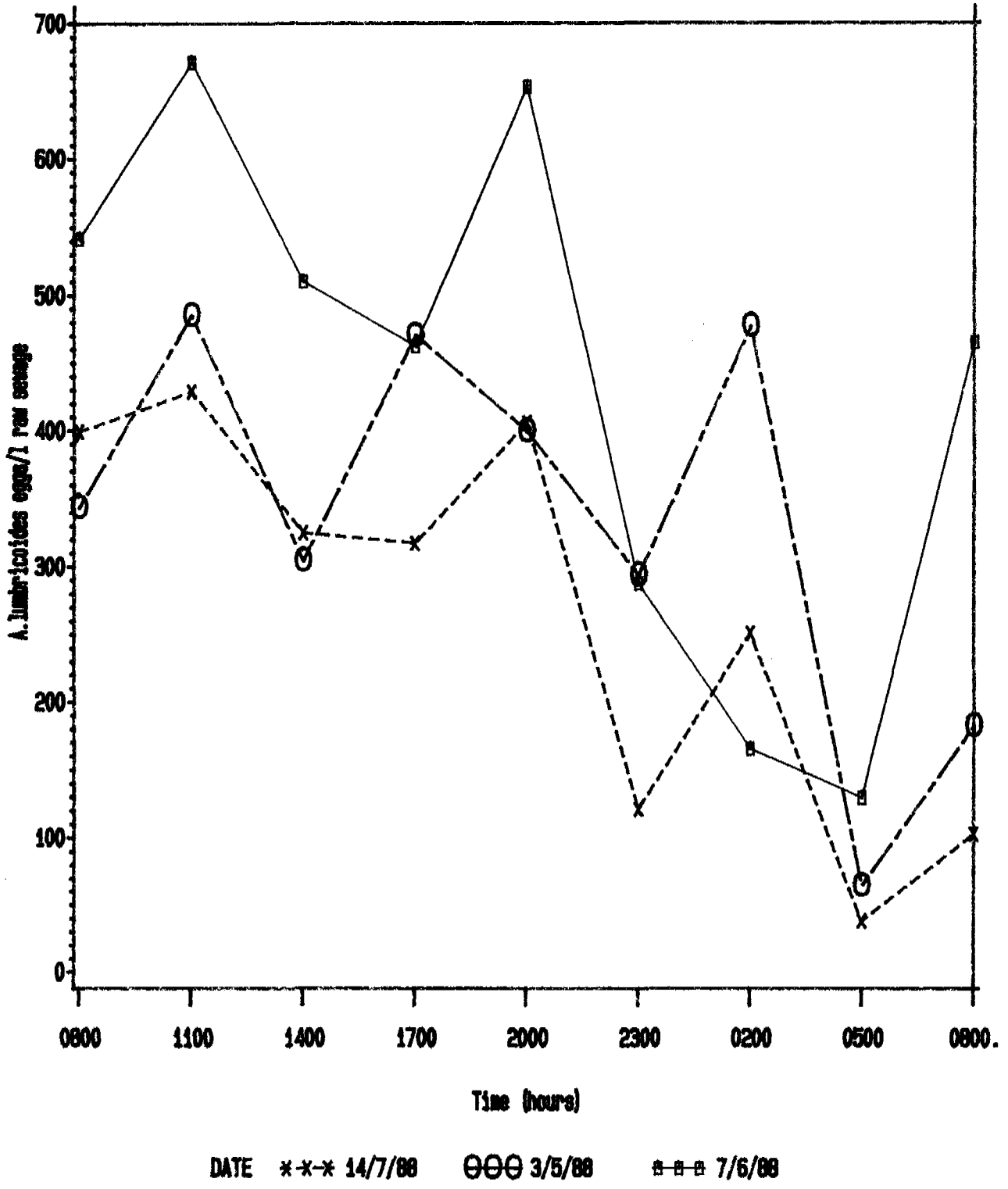

Fig. 3. Diurnal variations in A.lumbricoides eggs in raw sewage

Table 20. Mean egg counts per litre over 24 hour period, (May 1988), with analysis of variance and comparison of means

Time	A.lumbricoides	T.trichuria	Hookworm	Hymenolepis sp
08.00	343	7	2	2
11.00	485	9	3	5
14.00	304	3	0	3
17.00	470	5	3	4
20.00	399	5	1	4
23.00	293	3	4	4
02.00	477	4	6	3
05.00	64	1	4	3
08.00	182	1	1	1
	Analysis of Variance			
f ratio	31.31 ***	4.24 *	1.716 NS	0.7002 NS
CV	11.66	55.25	84.82	108.1
	Detection of significant differences in means			
5%	49.7	2.48	-	-
1%	67.1	3.35	-	-
0.1%	88.9	4.46	-	-

CV = coefficient of variation
*** = significant at 0.01% level
** = significant at 0.1% level
* = significant at 0.5% level
NS = not significant

Table 21. Mean egg counts per litre over 24 hour period, (7 June 1988) with analysis of variance and comparison of means.

Time	A.lumbricoides	T.trichiura	Hookworm	H.total	H.dimin	H.nana
08.00	541	4	4	12	8	4
11.00	671	9	3	14	7	7
14.00	510	7	1	6	3	3
17.00	462	11	3	7	4	3
20.00	653	8	5	11	5	6
23.00	287	3	3	11	7	3
02.00	165	1	1	3	3	1
05.00	129	3	1	5	3	1
08.00	465	6	1	9	5	4
	Analysis of variance					
f ratio	21.94 ***	2.565 *	1.337 NS	1.866 NS	1.066 NS	3.388 *
CV	12.53	65.72	89.91	77.59	97.85	55.68
	Detection of significant differences in means					
5%	72.1	3.78	-	-	-	1.97
1%	98.7	5.18	-	-	-	2.70
0.1%	134.5	7.06	-	-	-	3.68

Hookworm = Ancylostoma duodenale or Necator americanus
H.total = Hymenolepis nana and H. diminuta

CV = coefficient of variation
*** = significant at 0.01% level
** = significant at 0.1% level
* = significant at 0.5% level
NS = not significant

Table 22. Mean helminth egg counts per litre over a 24 hour period, (14 July 1988) with analysis of Variance and comparison of means.

Time	A.lumbricoides	T.trichiura	Hookworm	H.total	H.dimin	H.nana
08.00	399	11	2	11	8	3
11.00	429	8	1	9	3	6
14.00	325	6	1	3	2	1
17.00	317	11	1	1	1	0
20.00	406	11	2	2	1	1
23.00	121	12	1	3	2	1
02.00	251	1	0	0	0	0
05.00	38	0	0	1	1	1
08.00	103	0	1	1	0	0
	Analysis of variance					
f ratio	21.51 ***	8.552 ***	0.8214 NS	6.821 ***	4.482 **	2.3 NS
CV	12.76	28.43	106.3	34.01	46.28	69.77
	Detection of significant differences in means					
5%	53.9	2.9	-	2.6	2.0	-
1%	73.8	4.0	-	3.5	2.8	-
0.1%	100.5	5.4	-	4.8	3.8	-

Hookworm = Ancylostoma duodenale or Necator americanus
H.total = Hymenolepis nana and H. diminuta

CV = coefficient of variation
*** = significant at 0.01% level
** = significant at 0.1% level
* = significant at 0.5% level
NS = not significant

T-tests on the differences between the means show that on 3 May there were significant differences in the numbers of eggs recovered on each sequential occasion (Figure 4a). These differences were less marked between 1400 h and 1700 h. On all three occasions peak egg numbers occurred in the 1100 h and either the 1700 h or 2000 h sample, whilst the smaller numbers were found in the 0500 h sample (Figures 4a, 5a and 6a). These diurnal patterns clearly reflect the patterns of human activity in the catchment area. The average time between the sewage leaving the resdential areas and arriving at EXTRABES is about 3 h, and thus it can be deduced that the peak of egg numbers found in the 1100 h sample reflects defecation early in the morning prior to leaving the house for work or school, whilst the peak found in the 1700 h samples corresponds to defecation during the lunch-time period (most people in Campina Grande return home for lunch between 1200 h and 1400 h).

The diurnal variation in numbers of Trichuris trichiura eggs generally followed a similar pattern to that of Ascaris lumbricoides, although the absolute numbers of eggs found per litre were very much smaller (range 0-12 over the three days). The analysis of variance for each date is significant at the 0.1% level.

Hookworm eggs were found in very low numbers on all three occasions, but there was too much variation between replicates to permit a meaningful analysis of variance (a much larger sample size would have been necessary to detect significant differences with time). Despite the lack of statistical significance, Figures 4a and 5a for 3 May and 7 June respectively, indicate a diurnal variation which matches that found for A.lumbricoides and T.trichiura. Although there is known to be a high prevalence of hookworm infection in northeast Brazil, it is probably more prevalent in the rural sector and those harboring hookworm in Campina Grande are most likely to live in unsewered areas.

The numbers of Hymenopis spp eggs showed a significant variation over 24 h in the 14 July sample only (Figure 6b), but the pattern did not match that found for A. lumbricoides or T. trichiura in that peak egg counts were found in the 0800 h and 1100 h samples with a steady decline throughout the day, with no eggs being found in either the 0200 h or 0500 h samples. There was no significant variation in Hymenolepis spp on 3 May or 7 June (Figures 4b and 5b respectively).

Table 23 shows that samples taken on a regular basis at 0800 h do seem to be fairly representative of the mean numbers of Ascaris lumbricoides entering the waste stabilisation ponds over 24 hour periods. Given that the absolute numbers of eggs will vary from day to day and season to season depending on human behavioral and environmental patterns, a sample taken at 0800 h on a regular basis will give a reasonably good prediction of the loading of eggs entering the ponds over 24 hour periods. The estimate of other helminth eggs is less likely to be representative given the very low number of eggs recovered.

Table 23. Mean numbers of eggs per litre over a 24 hour period compared with the routine 0800 h sample

Date	Helminth parasite			
	A. lumbricoides mean 0800 h	*T. trichiura* mean 0800 h	Hookworm mean 0800 h	*Hymnolepis spp* mean 0800 h
3 May	335 (343)	4 (7)	3 (2)	3 (2)
7 June	431 (541)	5 (4)	2 (4)	9 (12)
14 July	265 (399)	7 (11)	1 (2)	3 (11)

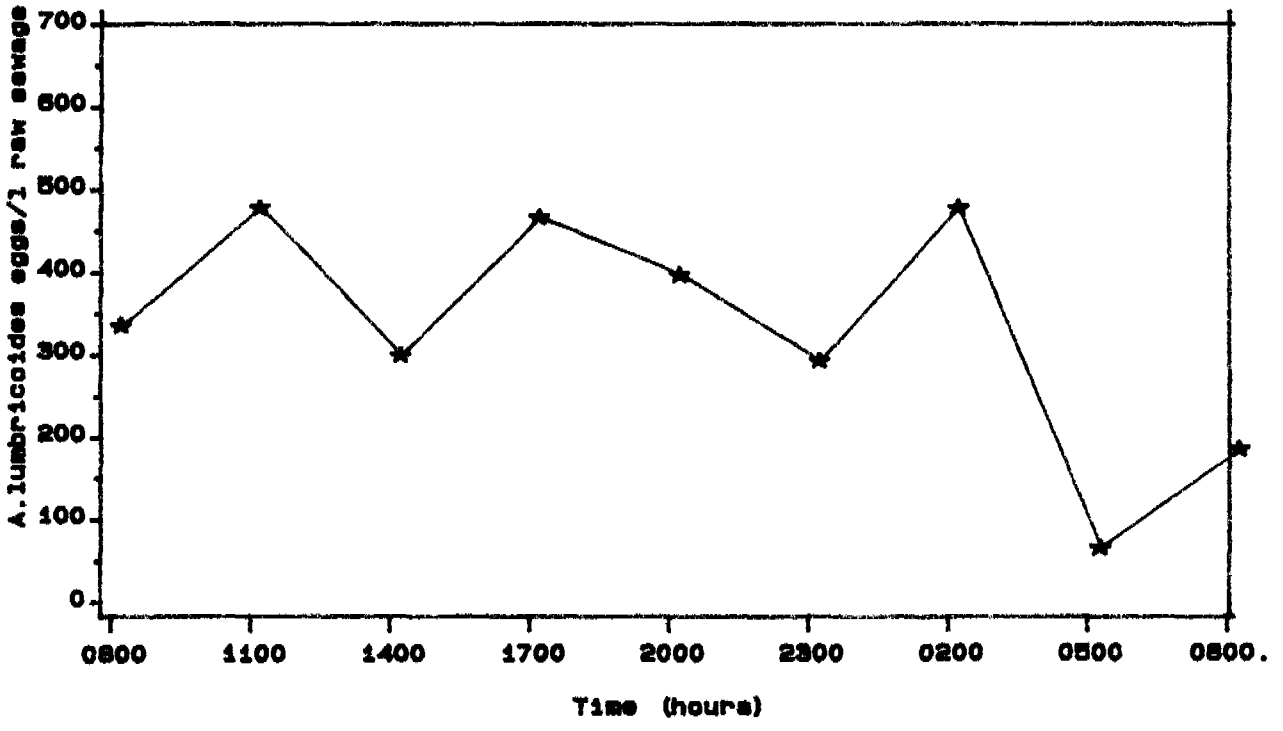

Fig. 4a: Diurnal variation in Ascaris lumbricoides eggs in raw sewage on 3/5/88

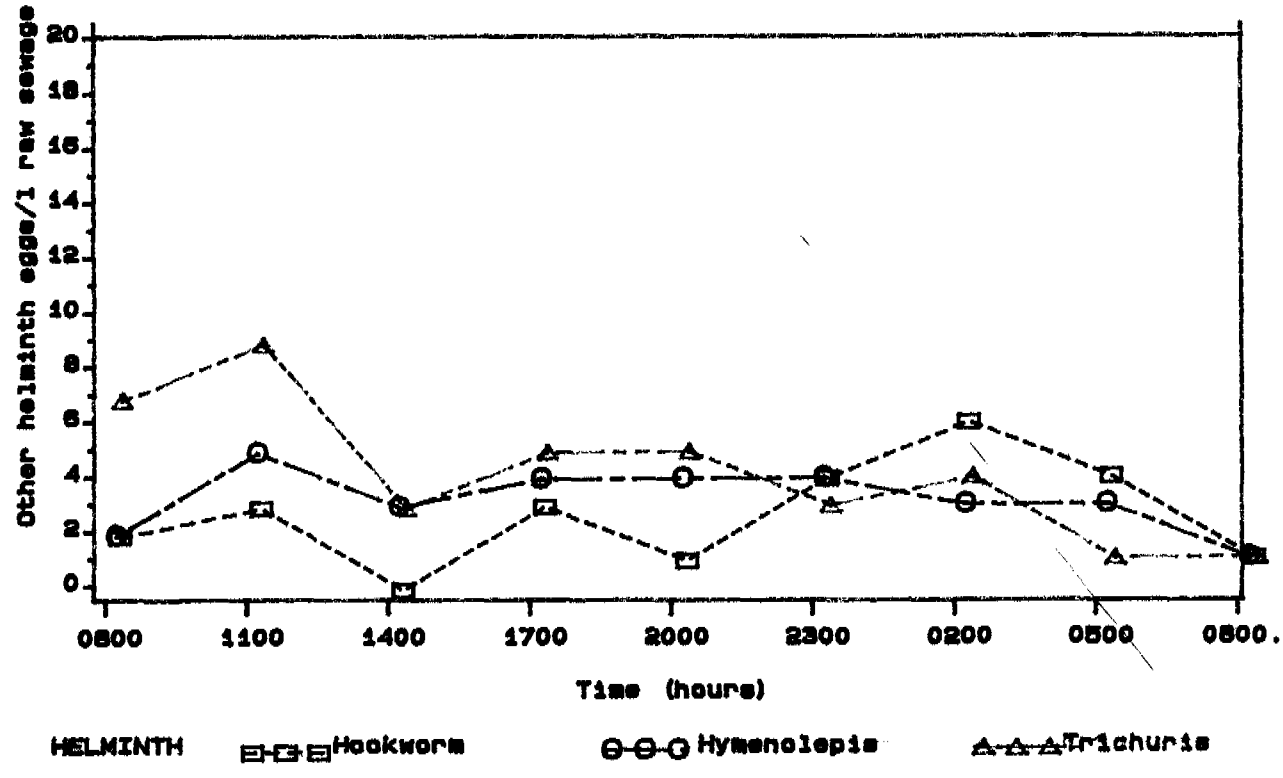

Fig. 4b: Diurnal variation in other helminth eggs in raw sewage on 3/5/88

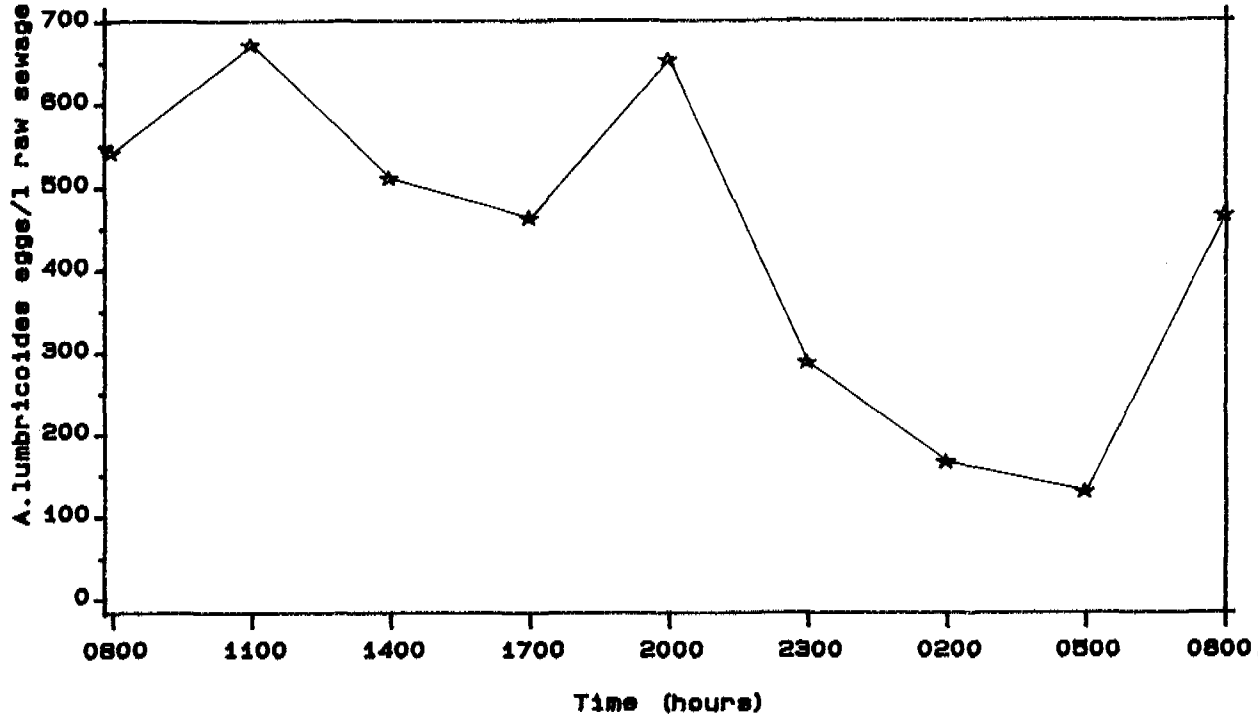

Fig. 5a: Diurnal variation in Ascaris lumbricoides eggs in raw sewage on 7/6/88

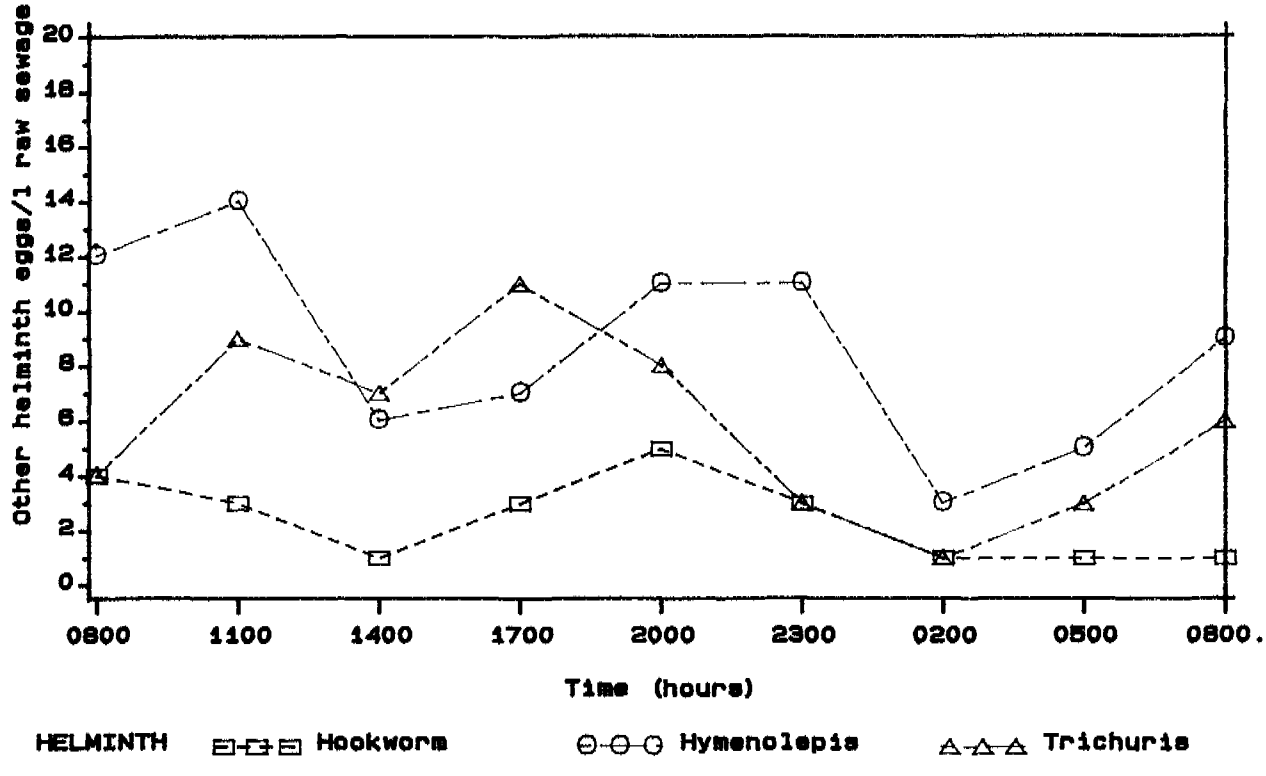

Fig. 5b: Diurnal variation in other helminth eggs in raw sewage on 7/6/88

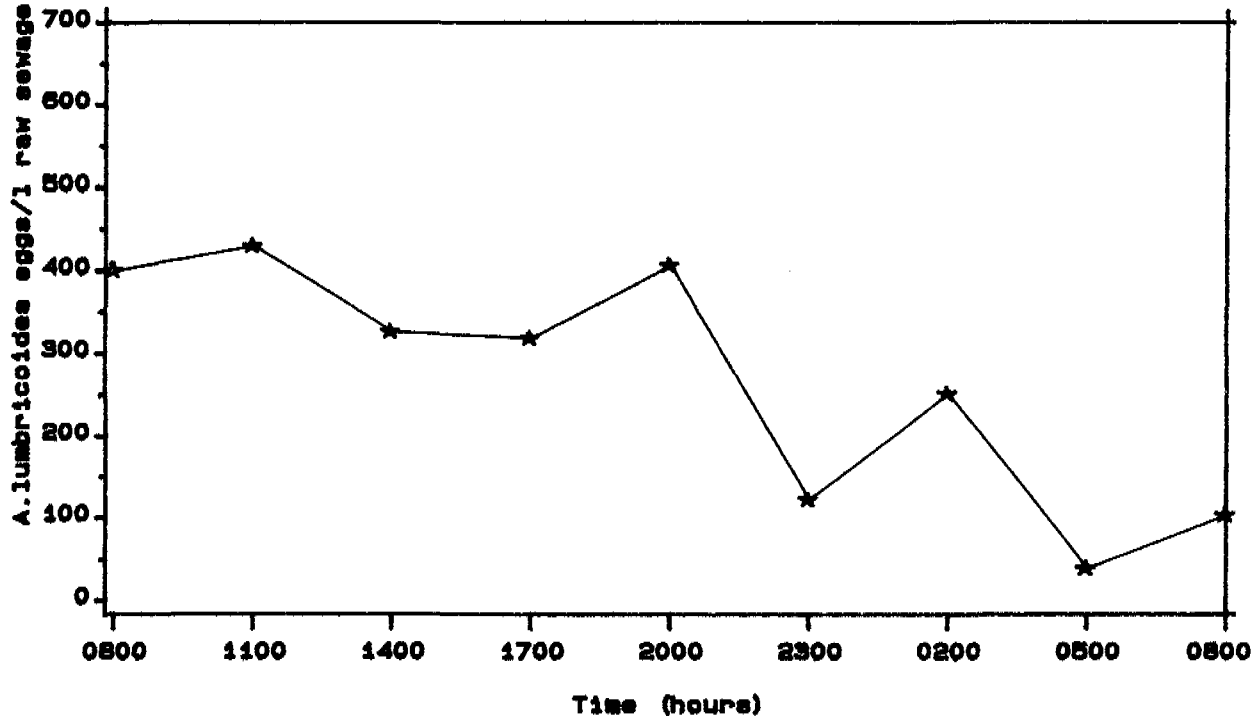

Fig. 6a: Diurnal variation in Ascaris lumbricoides eggs in raw sewage on 14/7/88

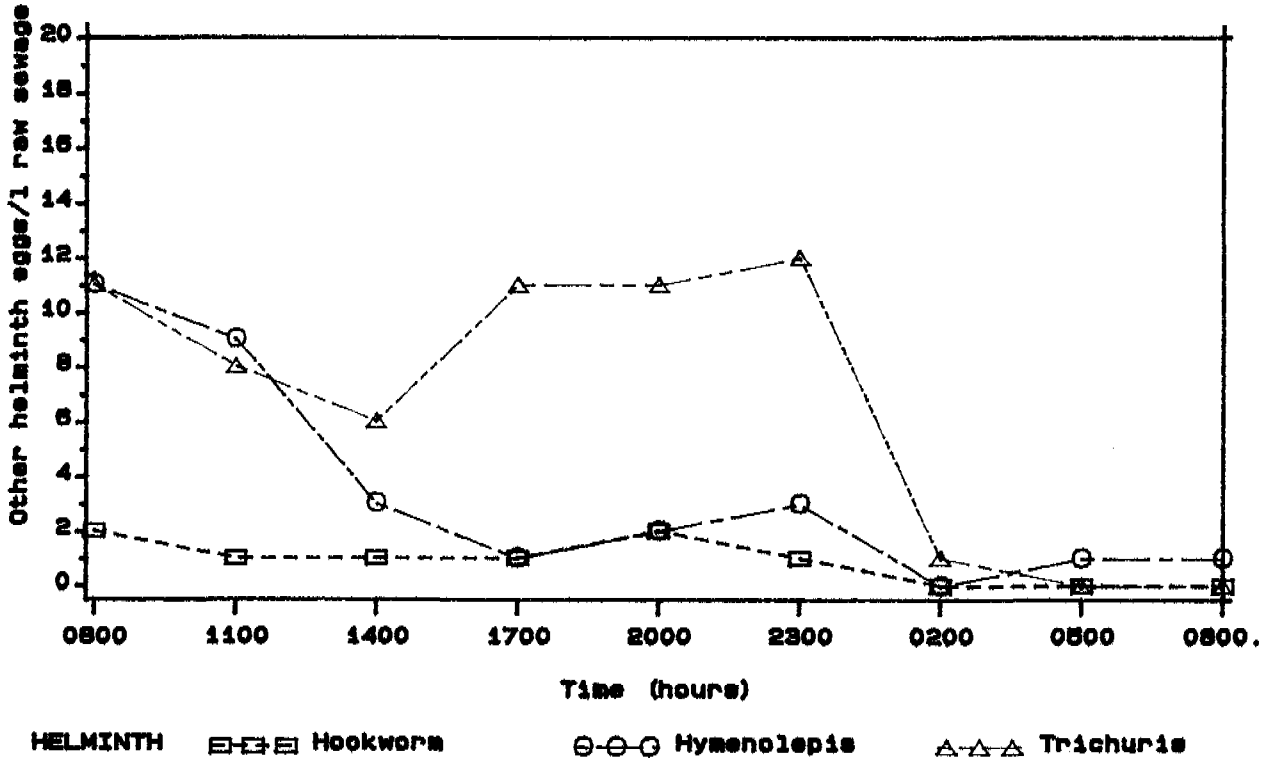

Fig. 6b: Diurnal variation in other helminth eggs in raw sewage on 14/7/88

9 DIURNAL VARIATION OF EGG NUMBERS IN FINAL EFFLUENT

9.1 Introduction

If the final effluent of waste stabilisation ponds is to be assessed for the quantity of intestinal nematode eggs prior to use for irrigation, it is important to know whether the quantity is likely to vary over a 24 hour period and at what time of day the most representive sample might be taken. The loading of helminths in raw sewage varies significantly with human behaviour over 24 hours (section 8), but it is not known if there is a similar variation in egg numbers in pond effluents.

If there is a diurnal variation of helminths in the effluent, it may be possible to take a composite sample once a day which is representative. It has already been shown that total water column samples provide rapid and accurate daily mean values for effluent quality in terms of algal quantity, chemical oxygen demand, chlorophyll a and faecal coliform bacteria (Pearson et al., 1987). This method is used routinely at EXTRABES to obtain mean effluent quality data for algae. The vertical distribution of algae within a water column of a waste stabilisation pond varies with organic loading and also with the time of day. These diurnal fluctuations in vertical algal distribution effect the quantity of algae leaving the pond in the effluent. The variation ranges from the algae being almost homogneously distributed with respect to depth, to being concentrated into a narrow and motile layer which moves through the water column in response to environmental factors such as light and temperature. It is possible that helminth eggs could be transported up and down the water column profile during the day by this algal band and an experiment was designed to compare a single water column sample with a series of effluent samples taken over a 24 hour period to elucidate this point.

The effluent from maturation pond M14 was monitored over two 24 hour periods. Only one helminth egg was found during this period and column sampling for helminth eggs was found not to be not very successful due to the amount of algae in the sample interfering with the recovery technique. The observations were then repeated on the effluent of facultative pond F18.

9.2 Method

Four 1 litre samples of effluent were collected over a 24 hour period from pond M14 on 14 and 28 September, and from pond F18 on 28 September and 4 October. Samples were taken every three hours starting at 0800 h and analysed for parasitic helminth eggs and larvae using the Leeds II technique. (Appendix II). A column sample was taken at 1100 h from M14 at a point close to the outlet pipe using a simple plastic tubular water column sampler (Pearson et al., 1987). A piece of wood was secured to the end of the sampler to ensure that its base did not touch the floor of the lagoon, to avoid any collection of sludge. The results were compared with the mean of the 24h effluent grab samples. The column sampling was not carried out during the monitoring of F18. The suspended solids were also measured on each occasion.

9.3 Results and Discussion

Only one egg of Ascaris lumbricoides was recovered from the effluent of M14 (Table 24) over the two 24 h periods. This supports much of the data in the literature which claims that a well designed series of waste stabilisation ponds can achieve almost 100% removal of helminth eggs (Feachem et al., 1983). No eggs were found in either of the two column samples taken, but examination of the samples was extremely difficult due to the large amount of algae present which were not satisfactorily removed during sample processing. It was decided to divide the final samples between 3-4 Doncaster dishes for examination in order to improve the rate of recovery.

Significant differences were found in the numbers of eggs in the effluent of pond F18 over the 24 hours on both sampling occasions (Table 24). All the eggs recovered were those of Ascaris lumbricoides.

During each 24 hour period the highest number of eggs in the effluent of F18 was recovered from the 1100 h sample, corresponding with peak egg numbers found in the raw sewage samples taken earlier. A very poor correlation was again found between the total suspended solids and helminth eggs per litre.

Table 24 Mean helminth egg counts over 24 hours in the effluent of pond M14

Time	14 September		28 September	
	Eggs/litre	TSS	Eggs/litre	TSS
08.00	0	6	0	6
11.00	0	10	0	3
14.00	0	77	0	3
17.00	0	6	0.25	3
20.00	0	6	0	10
23.00	0	8	0	4
02.00	0	9	0	3
05.00	0	6	0	7
08.00	0	7	0	5
mean 24hrs	0		0.027	
column sample	0		0	

TSS = Total suspended solids.

Table 25. Mean helminth egg counts per litre over 24 hours in the effluent of pond F18.

Time	14 September Eggs/litre	TSS	28 September Eggs/litre	TSS
08.00	0.75	93	0.25	43
11.00	4.00	29	0.75	37
14.00	0.25	17	0	52
17.00	0.5	130	0.25	43
20.00	0	59	0	27
23.00	0	44	0	64
02.00	0	84	0	48
05.00	0	50	0.5	39
08.00	0.25	149	0	121
f ratio	*** 18.79		* 2.481	
Detection of significant differences in means				
5%	0.566		0.337	
1%	0.765		0.451	
0.1%	1.018		0.605	
regression coefficients	-0.304		-0.388	

*** - significant at 0.01% level
* - significant at 0.5% level

10 HOOKWORM LARVAE IN RAW AND TREATED WASTEWATER

10.1 Introduction

Hookworm larvae have been reported in samples of fresh wastewater on several occasions. They have been recorded in experimental work from EXTRABES (Mara and Silva, 1986) and in samples taken from open wastewater drains in Ibadan, Nigeria (Striker et al., 1981). This situation needs investigation for epidemiological and biological reasons which are outlined below. Hookworm eggs are liberated into the environment through faeces at the 2-8 cell stage of cleavage. They then require 24-48 hours to develop and hatch into the first stage rhabtidid larval form (Levethal and Cheadle, 1979; Desowitz, 1980; Schmidt and Roberts, 1981). The sewage takes only 2-3 hours to reach EXTRABES from its catchment area in Campina Grande, thus it would seem most unlikely that first stage rhabditid nematodes would be found in the samples. It is not known how old the samples examined from Ibadan were, but the wastewater would be unlikely to be more than 10-12 hours old.

Samples of final effluent from waste stabilisation ponds have also been reported as being contaminated with hookworm larvae (Mara and Silva, 1986; Lakshminarayana and Abdulappa, 1969). Schmidt and Roberts (1981) report that one of the factors necessary for hookworm survival is the presence of oxygen as hookworm larvae have an aerobic metabolism. It seems improbable that eggs or larvae would survive in anaerobic ponds, although they might survive in facultative ponds providing the total retention time was less than a month. Banwell and Schad (1979) report that less than 1% of hookworm larvae can survive more than a month under optimum conditions, and most texts quote an average of three weeks survival (Schmidt and Roberts, 1981; Desowitz, 1980).

10.2 Identification and Occurrance of Hookworm Larvae in Wastewater and Pond Effluent

During normal routine monitoring of wastewater, numerous free-living nematodes are found. Accurate identification is difficult as the final examination of samples of wastewater for parasites is carried out on a microscope slide or a McMaster counting cell in most techniques (Teichmann 1986; Schwartzbrod, 1987; Mara and Silva 1986; Crewe, unpublished). In this situation nematodes can be partially obscured by detritus and it is impossible to remove individuals to clean microscope slides for detailed examination.

General guides to identification can be obtained from a variety of textbooks and papers (Jeffrey and Leach, 1975; Thienpont et al., 1986; Chitwood and Chitwood, 1959; Goodey, 1963; Svensson, 1926; Leventhal and Cheadle 1979; Desowitz, 1980). However most of these are medical texts and concentrate on the features which differentiate the various parasitic larvae which may be found in human faeces, or demonstrate the difference between Ancylostoma duodenale and Necator americanus larvae (the eggs which are recovered from the faeces are inseparable and larvae must be cultured to distinguish the two). These texts are not very suitable for separating possible hookworm larvae from the free-living larvae which are found in wastewater, many of which belong to the Rhabditidae, and which resemble first and second stage hookworm larvae. Attempts were made to identify hookworm larvae from wastewater and from the final effluent of the waste stabilisation ponds at EXTRABES; any suspect specimens were sent to the British Museum (Natural History) in London for further identification.

Free-living nematodes were extracted from 1 litre samples of wastewater by means of a modified Baermann apparatus (MAFF, 1986a; MAFF, 1986b). The nematodes were examined in a Doncaster counting dish under low power magnification and those of appropriate size and shape were mounted on microscope slides in a solution of TAF fixative 1/. (MAFF, 1986a) for further examination under high power. In cases of doubt, mounted specimens were sent to the British Museum (National History) for identification. Samples of final effluent collected routinely were examined for eggs and larvae of human parasites using both the Leeds II and EXTRABES methods (Appendices II and III).

No hookworm larvae were found in any of the samples examined. Most free-living larvae observed had very elongated tails, which differentiates them immediately from hookworms. Of 20 specimens sent to the British Museum (National History) none could be confirmed as hookworm. These results support the theoretical reasons why hookworm larvae would be unlikely to be found in wastewater arriving at a sewage treatment works within 24-48 hours.

No hookworm larvae and very few free-living larvae were ever recovered from the final effluent of the small pond series or the facultative pond F14.

10.3 Hookworm Survival in Waste Stabilisation Ponds

10.3.1 Introduction

There is a variety of evidence for and against the survival of hookworm larvae in the waste stabilisation pond environment. In a series of laboratory tests Lakshminarayana (1969) found that a model anaerobic/aerobic pond system achieved complete removal of Ancylostoma duodenale eggs. Three experiments were run for 16 days; A. duodenale eggs were innoculated at day 1 and no eggs were ever found in the effluent. All the eggs recovered from the sludge were found to be non-viable. In a second aerobic system into which eggs were seeded at day 1, rhabditid and filariform larvae were found in comparison with the numbers seeded. This suggests that, although hookworm eggs may develop and hatch in aerobic or facultative ponds, if the retention time is long enough then the numbers of infective larvae in the effluent should be minimal. Hookworm larvae have also been recorded as being present in the effluent of full scale aerobic sewage treatment plants (Cram, 1943; Leibamnn, 1964) and in the effluent of single facultative ponds at EXTRABES (Mara and Silva, 1986). Hookworm larvae were also reported in the final effluent of the series of ponds at EXTRABES which includes an anaerobic pond.

It is known that the optimum conditions for egg development and hatching include "adequate but not excessive" oxygen and moisture and a pH of near neutrality (Schmidt and Roberts, 1981). Lakshminarayana (1969) suggested that it is principally the lack of dissolved oxygen which causes the rapid death of hookworm eggs in anaerobic ponds.

If hookworm eggs can survive and hatch in the saturated, oxygenated, environment of facultative and maturation ponds, the reasons for the die off

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1. TAF - formalin (40% formaldehyde) 7 ml
triethanolamine 2 ml
distilled water 91 ml

as suggested by the work of Lakshminarayana are largely unknown. The possibilities are: that the saturated environment is unfavourable to the larvae, that larvae are removed by sedimentation as are helminth eggs, or that high pH found during the diurnal cycle in most facultative and maturation ponds (Mara, 1976) is not tolerated. The saturated environment is unlikely to be limiting in itself over a short period of time as hookworm larvae can be kept alive for several weeks in well aerated tap water. Larvae will sediment in water as they can only show negative geotropism when there is a firm substrate against which they can move: for example, they can move up and down soil profiles (Wallace, 1963). Little is known about the effect of pH on hookworm larvae. The algae-induced diurnal changes in pH ponds can result in pH maxima which may be as high as pH 10-11. An experiment was therefore carried out to see if the high pH found in ponds would have any effect on the survival of hookworm larvae. Infective stage, filariform larvae of Nippostrongylus brasiliensis (a parasite of rats) were used as a laboratory model for human hookworms.

10.3.2 Method

Infective stage larvae of Nippostrongylus brasiliensis were assessed for viability by counting 4 x 1 ml aliquots of nematodes in a suspension of water in a Sedgewick-Rafter counting chamber. 200 ml solutions of sodium hydroxide (NaOH) and calcium hydroxide ($Ca(OH)_2$) were made up to the required pH as shown in Table 26. The latter was included as there was a lime sedimentation plant being evaluated at EXTRABES at the time, and the pH of this plant is 11.0-11.5. One ml of larval suspension containing (approximately 11,600 larvae) was added to each test solution at time 0. At intervals of 30 min, 1, 2, 4 and 6 hours, a 1 ml aliquot was removed and the nematodes were assessed for viability in a Sedgewick-Rafter counting chamber under a magnification of x 40. The larval suspensions were agitated on a magnetic stirrer for a few seconds prior to examination to ensure that a random sample was taken.

Table 26. pH values tested (pH at time 0)

$Ca(OH)_2$	NaOH	Distilled Water
8.6	8.5	7.1
10.0	10.5	
11.5	11.5	

Viability was assessed by using the following reasoning and criteria to assess "dead" larvae. Live larvae of N.brasiliensis move vigorously in water under light and in warm conditions. As conditions become adverse they tend to slow down and become motionless, remaining quiescent in a 'C' or 'S' shape (this happens for example when larvae are kept in the refrigerator at 4°C). Nematode larvae tend to die in a characteristic shape and those of N.brasiliensis were observed to become completely straight when dead. It was therefore assumed that larvae which remained straight and motionless for several seconds under the microscope were dead.

10.3.3 Results and discussion

Table 27 shows that there was a mean of 4.2% mortality in the stock solution at time 0, and results were transformed accordingly to allow for this.

Table 28 shows that the only treatment which appeared to have any effect on the survival of filariform hookworm larvae was $Ca(OH)_2$ at pH 11.5, where only 12% of the larvae were alive after 6 hours. As this effect was not found with the NaOH at pH 11.5 it can be assumed that it is not the pH itself which is causing the mortality. Clearly there was some slight inaccuracy in the assessment of mortality as the percentage survival marginally increased on two occasions (distilled water control and NaOH at pH 10.0).

It can be concluded therefore that it is unlikely that the daily change in pH itself would cause the mortality of hookworm larvae in facultative and maturation ponds.

Table 27. Assessment of percentage mortaility in original stock solution of N.brasiliensis larvae

Aliquot no.	No. of live larvae /ml	No. of dead larvae /ml	Percentage mortality
1	182	8	4.2
2	147	7	4.5
3	130	5	3.7
4	180	11	5.8
		mean:	4.2

Table 27 Variation of percentage survival of filariform larvae of N.brasiliensis with time in solutions of various pH.

Time	$Ca(OH)_2$ pH 8.6	$Ca(OH)_2$ pH 10.0	$Ca(OH)_2$ pH 11.5	NaOH pH 8.5	NaOH pH 10.0	NaOH pH 11.5	Dist. Water
30 min	95	96	89	97	93	98	100
1 hr	97	91	64	100	103	98	95
2 hr	100	101	55	90	98	99	101
4 hr	100	98	25	100	101	95	101
6 hr	100	100	12	98	103	97	101
Final pH	9.3	9.7	11.4	7.7	9.7	11.4	7.5

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12. APPENDICES

I Leeds I technique for the enumeration of helminth eggs in raw wastewater

II Leeds II technique for the enumeration of helminths in the effluent of waste stabilisation ponds

III EXTRABES technique for the enumeration of helminth eggs in wastewater

IV Teichman technique for the enumeration of helminth eggs in wastewater

V Liverpool technique for the enumeration of helminth eggs in sewage sludge

VI Evaluation of the rate of recovery of Ascaris suum eggs from raw sewage using three techniques, (Leeds I, Teichmann and EXTRABES techniques raw data)

VII Percentage survival of filariform Nippostrongylus brasillensis over a range of pH raw data

VIII Stokes'law for the calculation of settling velocities

IX Development of the Leeds I technique for enumerating eggs of intestinal nematodes in raw sewage: details of methods from section 2.

APPENDIX I

LEEDS I TECHNIQUE FOR THE ENUMERATION OF HELMINTH EGGS IN RAW WASTEWATER

The Leeds I technique, as finally developed during the course of the study, comprises the following steps:

1. Take 1 litre grab sample of raw sewage.

2. Pour the sample into a series of 100 ml centrifuge tubes, and centrifuge at 600 g for 10 min.

3. Carefully remove the supernatant using a simple filter pump attached to a water tap. It is important that the supernatant is not poured off as eggs can be lost in this way. If the whole of the original sample is not centrifuged the first time, top up the tubes with the remaining sample and centrifuge again for 10 min at 600 g and remove the supernatant as above.

4. Rinse the sediments from each tube into a single centrifuge tube using a solution of 0.01% Triton X-100 dispensed from a wash bottle. Each centrifuge tube should be rinsed twice and the rinsings added to the final tube.

5. Centrifuge the combined sediments and rinsings for 10 min at 600 g.

6. Remove the supernatant as described above leaving about 5 ml (pellet and supernatant) in the bottom of the tube.

7. Using a glass rod stir the pellet thoroughly and divide between six 15 ml centrifuge tubes. Make sure that both the glass rod and the original centrifuge tube are rinsed well with Triton X-100 and that the rinsings are added to the 15 ml tubes.

8. Centrifuge the sample at 600 g for 10 min as described above.

9. Remove the supernatant carefully using a vacuum suction pump.

10. Add 3-4 ml of saturated $MgSO_4$ to each tube and agitate thoroughly (the pellet must be loosened and mixed with the salt solution). An automatic vortex mixer or a simple mounted needle may be used.

11. Top each centrifuge tube up to just below the rim with the salt solution and leave to stand for 5 min.

12. Centrifuge the tubes for 1 min at 100-150 g.

13. Put the tubes in a test tube rack and top up with salt solution using a glass pipette until a positive miniscus is formed. Rest a coverslip on the top of the tube and leave to stand for 30 mins. A glass or plastic coverslip may be used. During this time helminth eggs will float to the surface.

14. Remove the coverslip using a firm upward movement and put on a glass slide for examination under a high power microscope. The eggs collect under the coverslip and are removed in the meniscus of salt solution as the coverslip is lifted off.

15. After the first coverslip has been removed for examination it is immediately replaced with a second. A total of 4 coverslips should be examined for each tube of sediment, topping up with flotation solution if necessary. The sediment from a 1 litre sample is usually divided between six 15 ml centrifuge tubes and thus a total of 24 coverslips are examined for each sample.

    Although further eggs may be recovered by examining more coverslips, the percentage added to the total is very small and, given the time involved in coverslip examination, the test would be prohibitively long if the samples were examined exhaustively.

    The data collected from trial runs with seeded samples of raw sewage showed that the examination of 4 coverslips would be the most practical and most efficient number to examine (Table 28).

Table 28. Percentage recovery of Ascaris suum eggs by coverslip examination from 15 ml test tubes of raw sewage sediment.

Coverslip No.	% Recovery (3 replicates) 1	2	3
1	42	29	22
2	22	46	21
3	12	8.5	16
4	19	15	12
5	5	0.5	2
6		0	6
7		1	4
8			5
9			7
10			4
11			0
12			0
13			0
14			0
15			1

APPENDIX II

THE LEEDS II TECHNIQUE, FOR THE ENUMERATION OF INTESTINAL HELMINTHS IN THE EFFLUENT OF WASTE STABILISATION PONDS

The Leeds II technique as finally developed during the course of this study comprises of the following steps:

1. Collect four 1 litre grab samples of final effluent in clean plastic containers. The containers should have smooth internal walls and be prewashed with a detergent to remove any grease and thus decrease the chances of helminth eggs adhering. Add 10 ml formalin to the sample.

2. Leave the samples to stand for at least 1 hour. This is the minimum amount of time with a generous safety margin required for the lightest of the helminth eggs to sediment in a standard 1 litre beaker. The sedimentation time for any particular container can be calculated using Stokes equation (Appendix VIII) It is suggested that the calculated time required is always doubled for safety.

3. Remove the supernatant carefully using a siphon or a suction pump leaving 60-70 ml sediment. The supernatant must <u>not</u> be poured off.

4. Transfer the sediment of each sample into a 100 ml centrifuge tube. The sides of the sedimentation vessel should be rinsed thoroughly with a solution of 0.01% Triton X-100 and the rinsings added to the appropriate centrifuge tube.

5. Centrifuge the sample at 600 g for 10 min.

6. Remove the supernatant using a vacuum pump leaving a small pellet of about 2-3 ml at the bottom of the tube. It is very important that the supernatant is removed carefully at this stage as it is very easy to suck up eggs with other debris in the pellet. A glass pipette or plastic pipette tip may be inserted into the siphon tube for greater control.

7. Add a solution of NaCl with a specific gravity of 1.04 to a depth of 5 cm and leave to stand for 1 hour. To ensure a homogenous mix of pellet and salt solution it may be necessary to add a few ml of flotation solution, and agitate before adding the rest.

8. Remove the supernatant and discard leaving a pellet of 2-3 ml in the bottom of the tube.

9. Pour the final pellet into a Doncaster counting dish and examine under a high power microscope for eggs and larvae of parasitic helminths. If a Doncaster counting dish cannot be obtained the pellet can be examined in several batches in a slightly smaller counting cell such as a Sedgewick-Rafter slide. The 100 ml tube should be rinsed with the Triton X-100 solution and the rinsings examined as well.

Notes:

(a) In a salt solution of specific gravity 1.04 many of the algae which are normally found in effluent from waste stabilisation ponds will remain in suspension, whilst the helminth eggs and larvae will sediment out thus leaving a cleaner pellet for final examination. One hour has been determined as the optimum time for maximum helminth sedimentation and minimum algal sedimentation.

(b) A Doncaster counting dish is a counting chamber which is used directly under a microscope. It is a round dish (diameter 75 mm) with concentric circular divisions which keep eggs and larvae in defined areas. A total volume of 4 to 5 ml can easily and efficiently be examined under a magnification of x40.

Doncaster counting dishes can be obtained from:

Cox Thermoforming Ltd
London Road
Tring
Hertfordshire HP23 6HB
UK

Telex: 825389
Telephone: 044-282-4222

Advantages of this method

There is no subsampling step in this method. All the solid debris of a specific gravity 1.04 is examined for any given sample.

Free-living forms of parasitic nematodes are extremely difficult to identify when encountered in wastewater as there are likely to be so many free-living species present. Suspect nematodes need to be examined under high power in a clean microscope slide preparation. The use of a Doncaster dish enables individual nematodes to be lifted out using a mounted needle and examined on a separate microscope slide.

APPENDIX III

THE EXTRABES TECHNIQUE FOR THE ENUMERATION OF HELMINTH EGGS IN WASTEWATER

The technique for counting helminth eggs at EXTRABES comprises the following steps (Mara and Silva, 1986):

1. Take a 500 ml grab sample of wastewater.
2. Centrifuge in 100 ml alliquots at 2500 rpm for 10 min.
3. Remove the supernatant by a vacuum pump.
4. Rinse the sediment into 50 ml tubes and centrifuge again at 2500 rpm for 10 min.
5. Remove the supernatant and combine all the sediments in one 50 ml centrifuge tube.
6. Make up to 50 ml with water and centrifuge at 2500 rpm for a further 10 min.
7. Remove either 45 or 49 ml of supernatant depending on the amount of solid material in the sample.
8. Take a 0.2 ml aliquot of well mixed sample and examine under a microscope at x100 magnification. The eggs are recorded and the number of eggs per litre calculated.

APPENDIX IV

THE TEICHMANN TECHNIQUE FOR THE ENUMERATION OF HELMINTH EGGS IN WASTEWATER

Teichmann's (1986) technique comprises the following steps:

1. Take 1 litre of raw wastewater and leave to sediment overnight in a 1 litre container.

2. Remove the supernatant using a vacuum suction pump.

3. Transfer the sediment to a series of 15 ml centrifuge tubes, with a maximum of 3 ml per tube. Rinse the container several times with a weak solution of Triton X-100 and add the washing to the centrifuge tubes.

4. Centrifuge for 10 min at 1000 rpm, and remove the supernatants as before and discard.

5. Add 3-4 ml of sodium nitrate solution to each tube. The sodium nitrate solution should have a specific gravity of 1.3.

6. Centrifuge for 3 min at 2500 rpm.

7. The supernatant, which now contains the helminth eggs, is carefully removed using a pipette and transferred to a beaker containing just less than 1 litre of tap water (the water dilutes the $NaNO_3$ so that the eggs will settle to the bottom of the beaker).

8. 3 ml of $NaNO_3$ solution are again added to the sediment in each tube and the whole centrifuged at 2500 rpm for 3 min. The supernatant is removed and added to the beaker containing the first supernatants.

9. The procedure in step 8 is repeated. The sediment will now have been centrifuged with $NaNO_3$ a total of 3 times.

10. The beaker containing all the supernatants diluted in water is left to stand for several hours to allow all the helminth eggs to settle to the bottom.

11. The supernatant from this beaker is carefully removed using a suction pump and discarded. The sediment is transferred to centrifuge tubes. The walls of the sedimentation beaker should be rinsed thoroughly using a wash bottle containing Triton X-100 and the rinsings added to the sediment in the centrifuge tubes. The whole is then centrifuged for 4 min at 2500 rpm.

12. The final centrifugate is placed on slides or in a counting chamber, depending on the amount of debris, and the number of helminth eggs counted under a high power microscope.

APPENDIX V

THE LIVERPOOL TECHNIQUE FOR THE ENUMERATION OF HELMINTH EGGS IN SEWAGE SLUDGE

This technique, which was developed at the Liverpool School of Tropical Medicine by Dr Crewe, comprises the following steps:

1. One hundred ml samples of sludge are mixed with water and sieved through a tea strainer. The suspended sample is left to sediment overnight and then the supernatant fluid is then poured off and replaced with one litre of clean water.

2. This process is repeated every 24 hours for a week, until the supernatant is clear.

3. The final sediment is divided between a series of 50 ml centrifuge tubes, 5-10 ml sediment per tube, and centrifuged at 600 g for 1 min. The supernatant is discarded and replaced with 40 ml flotation solution ($MgSO_4$ sp. gr. 1.275, or $ZnSO_4$ sp. gr. 1.375).

4. The sediment and floatation solution are poured into a conical flask thoroughly mixed, returned to the centrifuge tube and allowed to stand for 5 min. The tubes are then centrifuged at 600 g for 1 min.

5. The supernatant solution containing the eggs is poured into a series of 15 ml centrifuge tubes such that a positive meniscus is formed and a coverslip is then placed on the meniscus.

6. The tube and coverslip are allowed to stand for at least 30 min and then the coverslip is lifted off the tube, placed on a microscope slide and examined under the microscope at x40 or x100 magnification.

Notes

Step 1. When raw sewage was being processed with this method the sample was poured straight into a 1 litre measuring cylinder without any sieving.

Step 3. It was found that only one 50 ml tube was necessary as much of the suspended solids had been removed during the washing stages.

Step 6. Usually only four coverslips are examined from each tube, as after this very few eggs continue to be recovered.

APPENDIX VI

EVALUATION OF THE RATE OF RECOVERY OF ASCARIS SUUM EGGS FROM RAW SEWAGE USING THE LEEDS I, TEICHMAN AND EXTRABES TECHNIQUES:RAW DATA

Table 29. Percentage recovery of Ascaris suum eggs using the Leeds I technique.

No. of eggs seeded	No. of eggs recovered	Percentage recovery
840	192	23
99	18	18
463	71	15
659	74	11
116	24	21
259	66	25
178	30	17
551	224	44
379	78	21
591	159	27
411	83	20
395	136	34
561	134	24
584	188	32

Mean % recovery = 23.7
95% confidence limits = ± 4.46

Table 30. Percentage recovery of Ascaris suum eggs using the Teichmann technique.

No. of eggs seeded	No. of eggs recovered	Percentage recovery
99	15	15
881	194	22
828	134	16
98	19	19
199	38	19
174	60	34
332	50	15
314	62	20
518	93	18
359	48	13
361	50	14
443	98	22
497	140	28
584	198	34
401	126	31
341	106	31
354	86	24

Mean % recovery = 22.05
95% confidence limits = ± 3.37

Table 31. Percentage recovery of Ascaris suum eggs using the EXTRABES technique.

No. of eggs seeded	No. of eggs recovered	Egg count per litre	Percentage recovery
405	4	100	25
	0	0	0
	5	125	31
516	0	0	0
	0	0	0
	1	25	5
497	12	300	60
	0	0	0
	8	200	40
432	1	25	6
	0	0	0
	0	0	0
541	2	50	9
	8	200	37
	4	100	18
349	0	0	0
	2	50	14
	0	0	0
620	6	150	24
	7	175	28
	4	100	16
674	1	25	4
	0	0	0
	1	25	4

Mean % recovery = 10.9
95% confidence limits = ± 6.098

Note: 3 subsamples of 0.2 ml were examined from each sample to look at the distribution of eggs within the pellet.

APPENDIX VII

HOOKWORM SURVIVAL IN WASTE STABILISATION PONDS

Table 32. Survival of filariform Nippostrongylus brasiliensis over a range of pH:raw data

Treatment	Time				
	30 min a/	1 h b/	2 h	4 h	6 h
$Ca(OH)_2$ pH 8.6	92.5, 89.1	92.1	95.4	96.1	95.5
final pH	7.5	8.4	8.7	8.0	9.3
$Ca(OH)_2$ pH 10.0	94.2, 89.9	87.3	96.9	93.8	95.5
final pH	9.8	9.9	9.9	9.7	9.7
$Ca(OH)_2$ pH 11.5	80.4, 88.7	61.4	51.7	24.2	10.9
final pH	11.5	11.4	11.5	11.3	11.4
Distilled water, pH 7.1	96.1, 96.2	90.6	96.7	96.4	96.4
final pH	7.1	7.5	7.6	7.8	7.5
NaOH pH 8.5	93.5, 92.6	96.3	85.7	96.0	93.8
final pH	8.1	8.6	8.3	8.3	7.7
NaOH pH 10.0	88.7, 88.1	98.4	93.7	97.1	98.5
final pH	9.8	9.9	9.9	9.7	9.7
NaOH pH 11.5	95.9, 91.8	83.8	94.9	91.1	94.7
final pH	11.4	11.5	11.5	11.4	11.4

a/ Two samples were examined after 30 min
b/ One sample was examined after 1, 2, 4 and 6 h.

APPENDIX VIII

STOKES' LAW FOR THE CALCULATION OF SETTLING VELOCITIES

Stokes Law can be used to calculate the time taken for helminth eggs to settle in water. It is necessary to know how long grab samples of various sizes should be left to sediment before the supernatant can safely be removed and the remaining sediment centrifuged. (Part (i) of the technique for the examination of final effluent for helminth eggs).

Stokes Law states that: $Us = g\ (p_s-p)d^2/18u$

where: Us = settling velocity, m/s
g = acceleration due to gravity (9.81 m/s^2)
p_s = density of particles (ie. sp. gravity x 1000, kg/m^3)
p = density of suspending fluid (water, 1000 kg/m^3)
d = characteristic linear dimension of the particle (take greatest linear dimension for a conservative estimate.
u = molecular viscosity of the suspending fluid, (1.518×10^{-3} Ns/m^2 for water at 5°C)

The settling velocity of eggs of A. lumbricoides in water can thus be calculated as follows:

$$Us = 9.18\ [(1129.9-1000)(75 \times 10^{-6})]/\ 18(1.58 \times 10^{-3})$$

$$= 2.62 \times 10^{-4}\ m/s$$

$$= 1.572\ cm/min$$

Stokes Law is strictly valid only if the particle Reynolds number (Re), is < 0.2 (Mara, 1976).

Reynolds number (R_e) is defined as: pU_sd/u

For A. lumbricoides:

$$Re = [1000\ (2.62 \times 10^{-4})\ (75 \times 10^{-6})]/\ (1.518 \times 10^{-3})$$

$$= 0.0129$$

In this case Stokes Law is valid and the settling velocity of A. lumbricoides eggs in water is calculated as 1.572 cm/minute.

Settling velocities for eggs of Trichuris suis (this being the best indicator of Trichuris trichiura for which the relevant data could be found) and Ancylostoma caninum (similarly an indicator for A. duodenale and Necator americanus) can be calculated as 0.935 cm/min and 0.433 cm/min respectively.

APPENDIX IX

DEVELOPMENT OF A TECHNIQUE FOR ENUMERATING EGGS OF INTESTINAL NEMATODES IN RAW SEWAGE: DETAILS OF METHODS

This Appendix gives details of the experimental work done to develop the Leeds I technique as described in Section 2.

Primary cleaning

Rat faeces containing large numbers of the tapeworm Hymenolepis diminuta were ground up using a pestel and mortar. They were then added to samples of raw wastewater from Pudsey sewage treatment works in Leeds and poured through a series of 180 μm, 106 μm and 45 μm Endecott brass sieves. Debris collected on each sieve was examined for the presence of H. diminuta eggs by centrifuging the sample, removing the supernatant and floating any eggs away from the debris in a saturated solution of NaCl (specific gravity 1.19).

Debris from the two larger mesh sieves was found to contain H. diminuta eggs, although in theory they should have passed through. A few eggs were also found in the filtrate.

Secondary cleaning

One litre samples of raw wastewater collected from Pudsey sewage treatment works were seeded with known numbers of Ascaris suum eggs and left to stand in 1 litre measuring cylinders. Every 24 h the supernatant was removed using a suction pump (rather than by pouring) and then a litre of tap water was added. This washing procedure was carried out six times, after which the supernatant was seen to be clear. The final sediment was divided between six 15 ml tubes and centrifuged for 1 min at 600 g. The supernatant was removed and saturated $MgSO_4$ added as a flotation solution. The sediment was thoroughly mixed with the solution and then centrifuged again for 1 min at 600 g. The solution was made up to a positive meniscus by adding more salt solution and a coverslip placed on the top for harvesting the helminth eggs (see Appendix I for a full explanation of this technique).

The percentage recovery of Ascaris suum eggs from wastewater and tapwater after using repeated washing, using the Liverpool technique described in Appendix V, is shown in Table 33.

Table 33. Percentage recovery of Ascaris suum from raw sewage using the Liverpool technique.

Sample	No. of eggs seeded	No. of eggs recovered	Percentage recovery
Wastewater	363	8	2.2
Wastewater	208	8	3.8
Tapwater	273	96	35.0

During each washing stage it was observed that a large amount of material remained in suspension; these flocs which may well have had eggs attached to them, were then discarded with the supernatant. (The technique was originally designed to look at sludge samples from which much of this low density material may have already been removed during the preceding sewage treatment processes).

Primary treatment

Floc dispersal

Two 1 litre samples of raw wastewater were centrifuged, the supernatant removed and a solution of 5% Calgon added. The solution was left to stand for 4-5 hours and the sediment and floating material examined in a watch glass under a microscope.

There was no visible difference in the size or number of flocs after treatment.

Destruction of solids using cellulase

Tan powder from the fungus Aspergillus niger (Sigma Ltd) was used as the source of cellulase; it is completely soluble in water. One litre samples of raw sewage were taken at 0900 h from Pudsey sewage treatment works; these contained about 1.5 ml solid matter after centrifugation, of which an estimated maximum of 10% may be cellulose. It is known that 1 unit of enzyme will liberate 1.0 μm of glucose from cellulose in 1 hr at pH 5.0 and 37°C, and that Sigma cellulase contains 0.5 - 1.0 enzyme units per mg solid material. The sediment from 400 ml samples of raw wastewater was buffered at pH 5 using a phosphate buffer and three amounts of cellulase added. It can be calculated that the assumed concentration of cellulose in 400 ml of sewage (0.06 g) will be degraded into 375 units of glucose in 1 h by 0.375 g Sigma cellulase. Thus 0.015 g, 0.0375 g, and 0.375 gm were added to the sediments of three 400 ml samples and incubated overnight at 37°C.

All three concentrations of cellulase reduced the amount of solid matter to 0.25 - 0.5 ml.

Recovery of helminth eggs using cellulase

Four 1 litre samples of raw sewage were seeded with known numbers of Ascaris suum eggs using the seeding method described in Appendix VI. The samples were centrifuged at 2500 rpm and the sediments from each sample saved separately in 100 ml centrifuge tubes. The supernatant was removed and 50 ml of phosphate buffer (pH 5) and 0.03 g of Sigma cellulase were then added to the centrifugates which were then incubated at 37°C for 2 h. After incubation the tubes were again centrifuged at 2500 rpm for 10 min and the supernatant removed. The sediment from each tube was divided between four 15 ml glass centrifuge tubes and centrifuged for 10 min at 2500 rpm. The supernatant was removed and the eggs harvested using the coverslip recovery method.

Table 34 shows the percentage recovery of eggs from raw sewage after this treatment with cellulase. The mean percentage recovery is not significantly greater than that achieved by either the Teichmann or Leeds techniques.

Table 34. Percentage recovery of Ascaris suum eggs from samples of raw sewage after treatment with cellulase.

Eggs seeded	Eggs recovered	% recovery
591	159	27
411	83	20
395	136	34
561	134	24
mean recovery		26

Destruction of cellulose using Schwiezers reagent

Four 400 ml samples of raw wastewater were centrifuged and the debris, about 1.5 ml from each sample, was resuspended in 10 ml of water. 1 ml, 2 ml, 5 ml and 10 ml of cuprammonium solution were added respectively to each sample and left overnight at room temperature. The following day the samples were centrifuged and the debris quantified and examined under the microscope for changes in quality.

The amount of organic matter was decreased to 0.5-0.25 ml per sample, but the work was not continued due to the toxicity and abrasiveness of the reagent.

Recovery of helminth eggs using cellulase

Four 1 litre samples of raw sewage were seeded with known numbers of Ascaris suum eggs using the seeding method described in Appendix VI. The samples were centrifuged at 2500 rpm and the sediments from each sample saved separately in 100 ml centrifuge tubes. The supernatant was removed and 50 ml of phosphate buffer (pH 5) and 0.03 g of Sigma cellulase were then added to the centrifugates which were then incubated at 37°C for 2 h. After incubation the tubes were again centrifuged at 2500 rpm for 10 min and the supernatant removed. The sediment from each tube was divided between four 15 ml glass centrifuge tubes and centrifuged for 10 min at 2500 rpm. The supernatant was removed and the eggs harvested using the coverslip recovery method.

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Destruction of cellulose using Schwiezers reagent

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The amount of organic matter was decreased to 0.5-0.25 ml per sample, but the work was not continued due to the toxicity and abrasiveness of the reagent.

Use of a coating material on glassware and plasticware

One litre samples of raw sewage were seeded with Ascaris suum eggs and the eggs recovered using the Leeds I technique described in Appendix I. All beakers and centrifuge tubes used during the procedure were cleaned with a 10% solution of dimethyl-dichlorosilane (DD) in toluene. This procedure was carried out in a fume cupboard using plastic gloves as it is a highly toxic substance.

The percentage recovery of helminth eggs after treatment of equipment with DD is shown in Table 36 below. There did not seem to be any advantage in coating the equipment with DD.

Table 35. Percentage recovery of helminth eggs after treatment of equipment with dimethyl-dichlorosilane (DD)

Technique used	No. of eggs seeded	No. of eggs recovered	Percentage recovery
Leeds	379	78	21
Leeds + DD	494	51	10
Leeds + DD	345	48	14
mean % recovery			12