

Title The Viability of Some Helminth Parasites in Sewage Sludges Applied to Agricultural Land

Name R. Ann Dean

This is a digitised version of a dissertation submitted to the University of Bedfordshire.

It is available to view only.

This item is subject to copyright.

THE VIABILITY OF SOME HELMINTH PARASITES IN SEWAGE SLUDGES APPLIED TO AGRICULTURAL LAND

BY R. ANN DEAN

A thesis submitted to the Council for National Academic Awards in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Sponsoring establishment: LUTON COLLEGE OF HIGHER EDUCATION

Collaborating establishment: ROYAL VETERINARY COLLEGE, UNIVERSITY OF LONDON

SEPTEMBER 1981

ABSTRACT

THE VIABILITY OF SOME HELMINTH PARASITES IN SEWAGE SLUDGE APPLIED TO AGRICULTURAL LAND.

R. Ann Dean.

The public health and veterinary significance of sewage-transmissible helminth infections is largely underestimated. Since land application is the ultimate destination of much of the sludge produced, this study was designed to investigate the longevity of helminth parasites in such material.

The initial aim of the investigation was to develop reliable and quantitative isolation techniques. After determining specific gravity values for the ova of Taenia and Ascaris, several methods of isolating these were compared and modified to improve the efficiency of recovery.

The effectiveness of sewage treatment for the removal of these ova was examined by measuring sedimentation rates. These values indicated that the retention times currently used in treatment plants must often be inadequate. Anaerobic mesophilic digestion had no observable effect on the removal of ova.

A number of criteria for judging the viability of ova were used to investigate the persistance of helminth ova stored in raw, digested and dried-cake sludge. Treated sludges were found to provide the least favourable conditions for egg survival.

The influence of ultraviolet light, temperature and humidity on egg survival was studied. Of these, humidity had the most detrimental effect.

In situ lysimeter experiments were conducted to examine both persistance and viability of helminth ova under field conditions. Under the cirumstances tested Ascaris ova were found to persist longer than Taenia ova.

Calves were infected experimentally with ova recovered from raw sludge in order to obtain conclusive proof that such eggs were infective; ova retained infectivity upto 3 months.

This work contributes to the understanding of the fate of animal parasites in sludges applied to agricultural land and may therefore be used to assist in the drafting of responsible policies for the safe use of sludge in agriculture.

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

I gratefully acknowledge the help and advice of colleagues and staff of Luton College of Higher Education, The Royal Veterinary College, and the Commonwealth Bureau of Helminthology: in particular my supervisors, Dr. Gordon Storey of Luton College and Dr. Dennis Jacobs of The Royal Veterinary College.

I extend sincere thanks to Miss Pegg and Alison (R.V.C.) and to Eric (L.C.H.E.)

Dr. Lesley Harrison of Edinburgh University Ve terinary Field Station kindly demonstrated the E.L.I.S.A. technique for screening sera.

Thanks to Ann and Cathy for typing. The work was funded by a Bedfordshire County research grant.

TABLE OF CONTENTS

ABSTRACT

ACKNOWLEDGEMENTS

GENERAL INTRODUCTION 1

AIMS OF THE INVESTIGATION 23

METHODS

Determination of Specific Gravites for Helminth Ova 24

I. Extraction Methods for Isolation of Helminth Ova from sludge, soil, leachate, and herbage. 30

II. Preliminary Evaluation of Flotation Methods;

1. Sucrose Gradient Flotation 34

2. Combined Flotation Method 36

3. Single Flotation Method 37

4. Quantitative Recovery by the Antiformin-Sucrose Method 39

5. The Formal Ether Method 46

III. A Comparison of the Relative Efficiencies of the Antiformin-Sucrose and Formol Ether Methods 51

IV. Investigation of Extraction Methods for Ova in Herbage and Soil leachate samples 56

Summary 66

DETERMINATION OF SETTLING RATES FOR HELMINTH OVA

1. Design of Apparatus

2. Experimentation

EVALUATION OF VIABILITY CRITERIA FOR HELMINTH OVA 81

THE SURVIVAL OF HELMINTH OVA STORED IN SLUDGE 88

THE EFFECT OF ANAEROBIC, MESOPHILIC DIGESTION ON THE SURVIVAL OF HELMINTH OVA IN SLUDGE 98

THE EFFECT OF TEMPERATURE ON THE SURVIVAL OF HELMINTH OVA IN SLUDGE APPLIED TO PASTURE 102

PHYSICAL FACTORS AFFECTING THE SURVIVAL OF HELMINTH OVA: TEMPERATURE: HUMIDITY: ULTRVIOLET IRRADIATION 109

RECOVERY OR ASCARIS AND TAENIA OVA FROM IN SITU LYSIMETERS 124

EXPERIMENTAL INFECTION OF CALVES WITH TAENIA OVA 145

REFERENCES 151

APPENDIX 1 DISEASES ASSOCIATED WITH SEWAGE CONTAMINATION

APPENDIX 2 CONSTRUCTION OF COUNTING CHAMBERS

GENERAL INTRODUCTION

HELMINTH PARASITES AND THE ROLE OF SEWAGE AND SEWAGE TREATMENTS IN TRANSMISSION OF PARASITIC INFECTIONS

Parasitic worms of the groups:

CESTODA (Tapeworms)

NEMATODA (Roundworms)

TREMATODA (Flukes)

are called helminth parasites. Certain of the infective stages of these parasites, the ova and larvae, can be recovered from sewage. It follows therefore that infections caused by these parasites should be able to be controlled where sewage treatment adequately destroys the infective stages. As will be discussed however, such thorough treatment of sewage is not always practicable.

The species of helminthic parasites that have been recovered from sewage in this country include: Ascaris lumbricoides, the roundworm parasite of man; Taenia saginata, the very important beef tapeworm which may infect more than 0.1% of cattle in the United Kingdom (Crewe & Owen, 1978), Trichuris sp. which cause whipworm infections, and Enterobius vermicularis, the common pinworm infection of children. Other helminths present in sewage on occasions include dog and cat taeniids which may be washed into stormwater drainage systems, and soil nematode ova and larvae from the same sources receiving soil run off water. The eggs of Toxocara canis, the ascarid parasite of dogs are infective to man and eye involvement can cause loss of vision.

The occurrence of normally tropical parasitic infections in this country has increased with the advent of international travel - particularly air travel, where infected hosts can arrive at their destination well within the incubation period for the infecting parasite. Hookworm for example are reported only from immigrants (Public Health Lab. 1980) although transmission would be theoretically possible in a good British summer. It is interesting that Taenia saginata infections - called Cysticercus bovis when it occurs in the encysted form in the cattle infection - were almost unknown in Britian prior to the 2nd World War. Similar increases in parasitic infection coincident with the return of servicemen from abroad have been observed in the United States.

Other infectious agents transmitted by sewage include bacteria (e.g.) Salmonellae spp., Enterovirus Hepatitus virus and bacteriophages, and the parasitic protozoa Entamoeba histolytica and Giardia lamblia (Carrington, 1978)

Helminth infections may cause loss of essential constituents (e.g.) Iron, protein from the infected host, who may also exhibit a failure to absorb nutrients. Additionally, helminths may produce toxins or other harmful substances (WHO Technical Report No. 277 1964) Ascaris lumbricoides for example, may contribute to anaemia by interfering with protein digestion, induce allergic states, cause endocrine upsets, electrolyte disturbances etc (WHO report No. 277 1964). Ascariasis infections are usually symptomless in man however, and most of the damage caused is a function of the size of the adult worm. In heavy infections ascarids can cause lung damage due to pulmonary migration of larvae. Intestinal obstruction and ascarid wastes may, in heavy infections be toxic to the host. The Public Health Laboratory

Service of the U.K. reports on average 3 cases daily, however being non-notifiable (like most helminths), this cannot be a true reflection of its endemic status. The adult worm is 20 - 40cm long, the eggs are ovoid, being 47 - 70 x 35 - 50 um. The eggs have a thick protein coat which makes the eggs extremely resistant to chemicals and desiccation.

Taenia saginata, the beef tapeworm is the most important common cestode parasite of man in the U.K. (Smyth 1976).

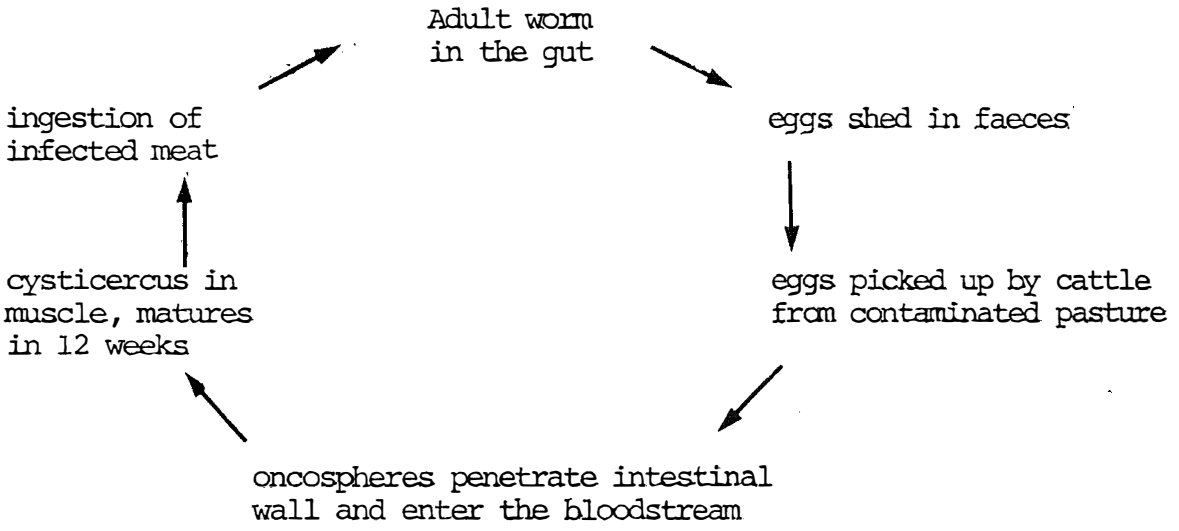
Taeniasis is a true zoonosis in which man is essential to the parasitic life cycle. Taeniasis is rarely a serious disease in man. In the case of T. solium there are recorded instances of reverse peristalsis permitting hatching of larvae which encyst in the spinal cord but even if this could happen with T. saginata it would be a freak accident and most unlikely. The Public Health Laboratory Service reports about 100 cases per annum, but as taeniasis is not a notifiable disease, this figure probably represents only a small proportion of cases.

The adult tapeworm lives only in the intestine of man and the infection is acquired by eating infected meat containing cysticerci. These appear not to affect the health of the cattle carrying them. They may be destroyed by freezing the carcass at -10°C for 10 days or by heating above 56 C. The cysts are whitish and about 1cm in diameter. When ingested the cysts evaginate and the scolex attaches to the wall of the ileum. Segments are produced from the protoscolex, the worm attaining full size in about 12 weeks.

The scolex has 4 suckers for attachment and 1000 or more segments (proglottids). Their size is about 4 metres, although much longer worms have been found. Each segment has both male and female

reproductive organs which develop at different rates. (Protandy- (ie) testes mature earlier than ovaries). It is therefore not unusual to have intrasegmental fertilization, sperm are transferred from one segment to a more mature segment. Mature (gravid) segments containing up to 10,000 ova, are shed in faeces.

Life cycle of Taenia saginata:-



Taenia ova are extremely resistant, they have a thick outer layer the striated embryophore - which encapsultates the embryo. The eggs measure 43 x 31 μm and are infective only to cattle. When the larvae hatch they encyst in muscles of the heart, diaphragm, tongue, oesophagus and in the masseter muscles (Geerts et al 1977). Obviously a compromise must be reached between thorough meat inspection and minimal damage to carcasses so public health officers employed by abbatoirs have a set inspection procedure concentrating on heart and masseter muscles -(eg) 3 cuts are made into every heart. Rickard (1977) estimates that only 30% - 40% of infections are detected at slaughter. Heavy infections are rarely seen in this country and if any cysts at all

are picked up at inspection the carcass is frozen with consequent loss of value to the beef producer. In 1955 Silverman estimated the nationwide loss to be £500,000. Regulations in the United States are at least as rigorous; if cysts are found in at least 2 specified locations the carcass is condemned. Carcasses with a single cyst are cooked or frozen. Jurameck et al (1976)

The transmission of parasitic diseases of man and livestock depends upon a host excreting a viable infective stage or an ova which becomes an infective stage, which subsequently comes into contact with a new host - be this final or intermediate. Diseases assocated with sewage contamination are summarised in appendix 1.

It is known that primary and secondary sewage treatments and mesophilic anaerobic digestion are not sufficient to destroy all parasitic ova and it is of special concern that ova of human intestinal parasites are commonly isolated from sludge.

Attempts have been made to estimate the probable numbers of helminth ova in sewage, from the level of infection of the community and rate of egg production of the parasite:- (eg.) if it is assumed that 1% of the population is infected with a worm producing 10^6 ova daily and wastewater flow is 200 gallons per capita daily it would be expected that 50 ova were found in each gallon of sewage (after Greenberg & Dean, 1958). Since sewage composition is so variable, however, such calculations are probably without value. Egg input into sewage is necessarily variable (eg.) if one person is infected with the beef tapeworm Taenia saginata 10^6 ova may arrive at the sewage works in a few short periods making detection difficult, and estimates unreliable. Sewage inputs have been examined in many areas:-

Liebmann (1965) estimated 80 helminth ova per litre of sewage in a Bavarian works.

* Silverman (1955 estimated 1 - 2 Taenia ova per litre of sewage (U.K.).

* Greenberg & Dean (1958) estimated 50 ova per gallon or 11 per litre (U.K.).

The density of most parasitic ova and cysts is such that they usually settle out during primary sedimentation - Liebmann (1965) estimated 700 ova/litre in primary sludge. Settling rates for ova range between 1 - 3 ft per hour but operational settling times can be below optimum (e.g. 1) where plants must periodically cope with large volumes of stormwater drainage.

The Sewage Treatment Process

Sewage treatment plants are designed to separate the suspended solids and many soluble constituents from both domestic sewages and industrial wastes. The Royal Commission's standard for sewage is 30:20 (i.e.) 30 mg/litre suspended solids and 20 mg/litre Biochemical Oxygen Demand Value.

Initially, coarse solids such as rags and wood are removed from the incoming sewage by wire mesh screens. Grit is frequently removed by allowing sewage to flow through narrow channels at a rapid rate so that grit, but not the lighter sewage solids, will settle out.

Sewage flows next into sedimentation tanks usually 8ft - 10ft deep containers with sloping floors. During this primary sedimentation approximately 50% - 70% of the total solids settle out as primary (raw) sludge with an average solids content of 4% - 7%.

The Biochemical oxygen demand (B.O.D.) is reduced by 25% - 50% (Mosey, 1979). At the end of this stage the suspended solids content which is approximately 300 mg/litre for an 'average' sewage is reduced

to 30 mg/litre.

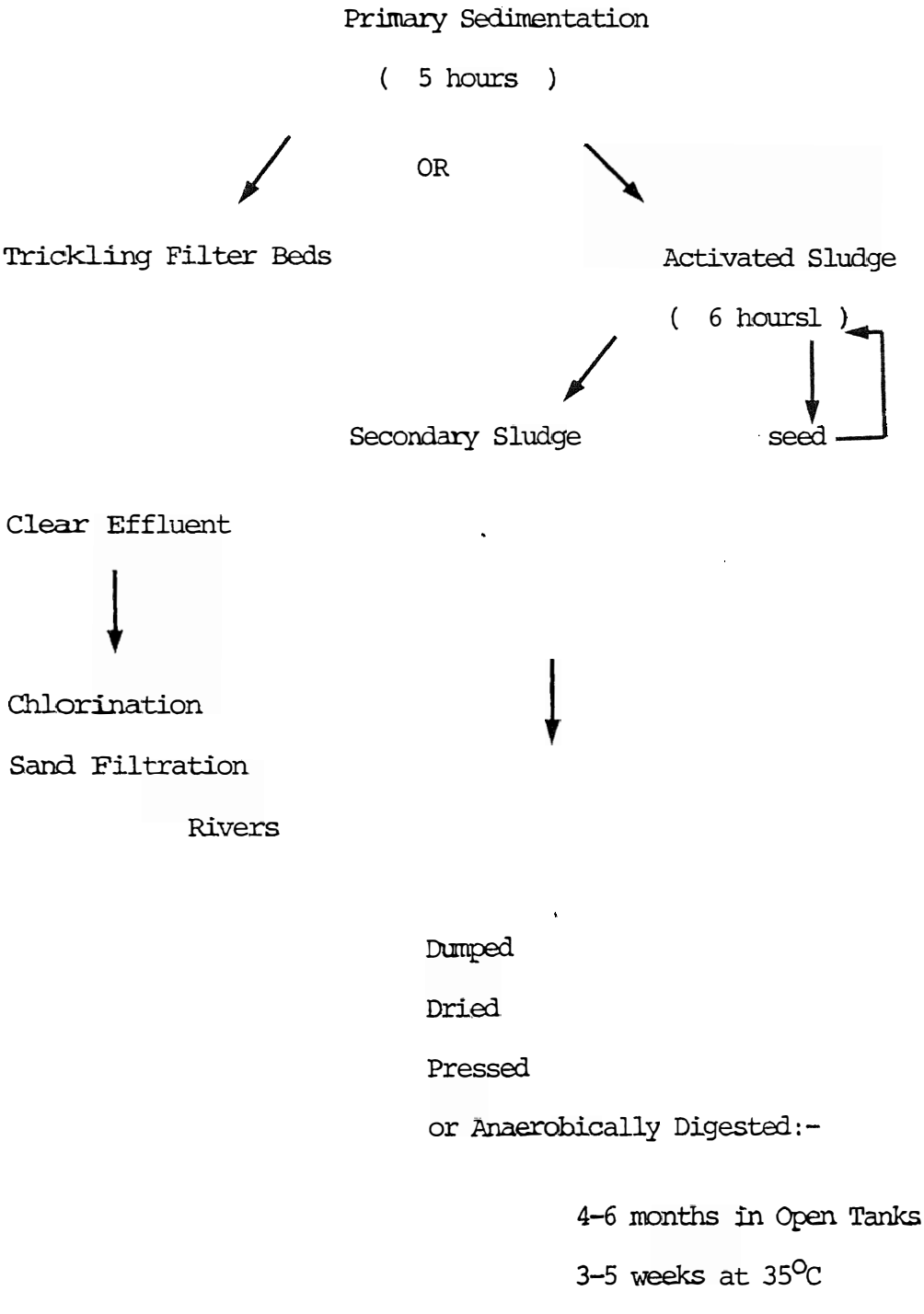
In the next stage of treatment organic solids and liquids not removed during the primary sedimentation are microbially oxidised by either the activated sludge process or the biological filtration process. Activated sludge plants offer the advantage of requiring far less land to operate. In the process, liquid effluent is mixed with flocs of microorganisms by air diffusion from the base of the activated sludge tank. Suspended and organic solids are absorbed by the biologically activated sludge and any organic matter is oxidised by the microorganisms. The overflow is allowed to settle and a fraction of the active sludge is returned to the tank to be mixed with fresh sewage. Efficiency of removal of suspended solids and organic matter is variable, but can approach 90%.

In the trickling or biological filter process sewage is sprayed over 6ft - 8ft deep beds of clinker (broken stones) which serve as an inert filter medium. The stones are coated with a 2 - 3 mm bacterial/algal slime (zooglea) which purifys the sewage as it percolates through the bed. In East Europe, filter beds are seeded with Enchytraediae worms to kill Ascaris ova.

Removal of suspended solids is 85% - 95% by trickling filter process.

The mixture of primary and secondary sludges from these treatment stages may be disposed of at this stage but more often receives further treatment. The sewage bulk is reduced either by anaerobic digestion or by dewatering; by air drying, vacuum filtration, or filter pressing. Compounds used to assist dewatering include $FeSO_4$, $FeCl_3$, $AlClO_3$, lime and polyelectrolytes (organic coagulents). Sludge 'cakes' produced have a total solids content of 30% - 60%. A summary of the sewage treatment processes is given in figure 1.

Fig: 1. Summary of Sewage Treatment Unit Processes



In anaerobic mesophilic digestion sludge undergoes fermentation at $30^{o}C$ - $35^{o}C$ in the absence of oxygen. The retention time is usually 20 - 30 days. During this time solids are reduced by approximately 45%. The substrates in sludge, (starches, fats and proteins), are broken down into simpler substances (sugars, fatty acids and amino acids), by a mixed population of anaerobic bacteria. Methanogenic bacteria in the digestors use hydrogen, CO_2 and acetates from the 'acid forming' or nonmethanogenic bacteria and produce CO_2 and methane which is used as a fuel to heat the digesters.

The process is sensitive to pH and temperature, the pH optima being in the range 6.4 - 7.2 and buffering being achieved by NH_4^+ ions and HCO_3^- ions in the system.

Effectiveness of Sewage Treatment for Helminth Removal

Primary Treatment

Effective sedimentation is essential if effluents are to receive only physical pretreatment before agricultural use. Under suitable conditions, viz. minimum turbulence and adequate time, most helminth ova in sewage settle out in the sludge. Flocculating agents may be used to aid sedimentation.

Cram (1943) found settlement removed most Ascaris ova although cysts of E. histolytica remained in effluent after 3 hours settling. Newton (1949) reported 98% removal of Taenia ova after 2 hours settling. Wang and Dunlop (1954) reported 50% removal of Ascaris but gave no indication of settling rates. Greenberg and Dean (1958) cited the results of Russian studies which obtained removal rates for helminth ova of 97% by settling sewage in an Imhoff tank. Rowan (1964) reported 35% - 74% removal of Ascaris ova by sedimentation.

Actual settling rates found by experiment were 2 - 3ft. per hour for Ascaris ova (61 - 91.5cm/hr) 1 ft. per hour for Taenia ova (30.5 cm/hr). Such removed ova remain a potential source of infection unless the sewage sludge is to receive further treatment sufficient to destroy the ova that have been deposited. In Britain, primary sedimentation tanks are normally designed to cope with a surface loading of $30m^3/m^2/day$ and a minimum retention time of 2 hours at maximum flow (Mosey 1979). As will be discussed, however, such retention periods are not sufficient to remove all species of helminth ova.

Secondary Treatment

Secondary treatment is the biological oxidation of organic liquids and solids which were not removed by sedimentation.

Newton et al (1949) obtained 62% - 70% removal of Taenia ova on a 3.6ft deep trickling filter bed made up of 1.5 inch gravel. Removal rates of nearly 100% were obtained by sand filtration. Kabler (1959) cites studies giving 18% - 26% removal of tapeworm ova and 70% - 76% removal of Ancylostoma, Toxascaris and Ascaris ova. In activated sludge systems Newton et al (1949) found no obvious damage to Taenia ova. Cram (1943) found E. histolytica cysts, A. lumbricoides ova and Ancylostoma ova were not killed by activated sludge treatment.

Digestion

The resultant sludges may be anaerobically digested, in which case the digesting temperature is the critical factor for helminth destruction. Nearly all plants in the U.K. operate below 50°C, a temperature at which ova can be expected to survive. Cram (1943) found that 3 months of 'cold' digestion, (i.e.) at 20°C and 30°C had little apparent effect on Ascaris ova, whereas after 60 days 'cold' digestion Newton et al (1949) recovered 50% apparently unaffected Taenia ova. The results of work done by Silverman and Guiver (1960) conflict with those of other workers in finding that mesophilic digestion killed Taenia ova, but such discrepancies may be due to differences in digestor design and digestion conditions.

Smith et al (1975) reported that all ova in sludge were killed by 2 hours aerobic digestion at 55°C. Ascaris ova were reported by Rudolfs et al (1950) to be unable to withstand temperatures above 60°C for periods above 5 minutes and this was confirmed by failure of embryonation after such heat treatment.

For anaerobic digestion processes, W.H.O. (1964) recommendations were 30 days at 38°C and for aerobic digestion 20 days at 45°C which, on the evidence of the above studies is inadequate to kill the helminth ova of at least some species (e.g.) Ascaris lumbricoides and Taenia saginata.

Tertiary Treatments

Chemicals and composting have been used to remove ova from sludge and have proved effective although economic considerations preclude their use except where a specific and contained infection occurs. The World Health Organisation Technical Report No. 277 (1974) reported that Ascaris ova were killed by ultraviolet rays from a mercury vapour lamp at 1 metre distance over 10 - 15 days when the irradiation lasted 3 - 10 minutes on 12 - 19 occasions. Trichuris ova were killed by gamma rays with 8 doses of 1000 rads for 22 minutes over a 53 day period. The report suggested these techniques could be applied to sewage treatments. Ascaris and Trichuris ova were killed by damp heat for 200 seconds at 70°C - 90°C or 2 hours at 50°C. Ascaris and Trichuris ova on soil were killed by 90 minutes at 48°C - 50°C in direct sunlight. Ancylostoma ova were killed by 45 minutes at 39°C - 42°C or by 12 hours freezing. Chlorination has been used to kill Schistosomes, e.g., Rowan (1964) found 0.2 ppm chlorine killed schistosome miracidia in 30 seconds. Chlorination was ineffective against Ascaris ova Wang and Dunlop (1954). Sludges have been disinfected with a range of chemicals: for example with Taenia saginata, Chilikin (1976) obtained 99% kill using 10% carbathione/24 hours or 5% - 7% for 3 days. Kutsumi (1965) obtained 100% kill of Ascaris ova using thiabendazole at 6.25 ppm/25°C or 100 ppm/10°C. Ancylostoma ova were killed in 3 days by NH_4NO_3 added to sludge at the rate of 1 kg/m^3.

(W.H.O. report, 1964)

Sand filtration or microstraining appear to be most effective in removing helminth ova; with 12 inch depth of sand Cram (1943) reported 100% removal of Ascaris ova and Ancylostoma ova and Newton et al (1949) recorded 100% removal of Taenia ova by this method.

Alternative modes of sludge application to land have been advocated to reduce public health risks (e.g.) Romanenko (1972) suggested deep layer ploughing of sludge into farmland. Such specialised applications are expensive however and not widely used.

Although it is possible to substantially reduce the overall number of parasitic ova in sludge it is uneconomic to remove all pathogens. It is more difficult to set an 'acceptable' level of contamination with parasitic pathogens than for bacterial pathogens in sludge because infective doses vary with different species and with host factors. Theoretically just one egg in some cases could constitute an infectious dose.

It has been repeatedly confirmed that a certain percentage of Ascaris and Trichuris ova in particular retain viability throughout all stages of sewage treatment; see Figure 2 . As will be later discussed however, viable ova may not necessarily be infective.

Fige 2 Persistance of Ascaris and Taenia ova throughout Sewage Treatments

I

Primary Treatmente

Newton et al (1949)	98% Taenia removed by 2hre settling
Wang and Dunlop (1959)	50% Ascaris removed by settling

II

Secondary Treatmente

(a) Trickling Filter Newton et al (1949)	62% - 70% Taenia removed
(b) Activated Sludge Newto et al (1949)	No effect on Taenia viability
Cram (1943)	No effect on Ascaris viability

III

Anaerobic Digestion : (Mesophilic)

Cram (1943)	No effect on Ascaris in 3 months 10% viable Ascaris in 6 months
Rudolfs (1959)	No effect on Ascaris viability in 3 months.
Silverman and Guiver (1960)	No effect on Taenia in 20 days
conflicting* Kutsumi (1958)	* Ascaris killed by 6 - 8 days at 32°C -33°C

IV

Other Methods:

(a) Sandfiltration Newton et al (1949)	99e6% - 100% Taenia removed

(b) Microstaining Silverman and Griffiths (1960)

95% Taenia removed

95% - 100% Ascaris removed

(c) Chemical Treatments

Chilikin (1976) Lime	Ascaris survived
Chilikin Carbathione	10% helminth ova killed in 1 day
	5% - 7% killed in 3 days

Survival of Helminths in Sludge and Soil

Parasitic infective stages are well adapted to survive outside their host species and this can present a major problem in relation to the health risk from sludge treated pasture or crops to man and livestock, should ova remain viable after undergoing standard treatment practices.

Survival of ova in soils and sludges generally, depends on the maintenance of a certain minimum moisture and temperature. Ova are more likely to survive in clay soils which retain moisture; the effects of temperature being moisture-dependent. Low temperatures tend to be survived, although development may be retarded; Ascaris ova for example were found viable after 3 months in drying sludge but none were viable in sludges with less than 4% moisture (Rudolfs et al 1974). Brown (1929) showed that 90% of Ascaris ova remained viable after 35 days on shaded, sandy soil and after 54 days there still remained 69% viable, but on exposed sandy soil only 21% of ova were viable after the shorter time of 20 days. Forstner (1968) found that 25% of Ascaris ova remained viable on crops after 6 months and 90% retained viability after 6 months in soil. Longer survival times have been recorded (eg) 2 years in field conditions (W.H.O. report No. 277, 1964), 7 years in drying sludge (Kozlova, 1968), and 4 years in sandy soil (Oganov 1977).

Jepsen and Roth (1952) found Taenia ova still viable after 159 days on soil. After more than 4 months on soil Sweatman and Williams (1963) recovered Taenia ova which were still viable.

Jepsen and Roth (1952) found Taenia ova still viable after 159 days on soils. After more than 4 months on soil Sweatman and Williams (1963) recovered Taenia ova which were still viable.

There is evidence to suggest that infective ova may arrive on pasture by means other than sludge applications. Silverman and Griffiths (1955) showed that Taenia ova remain infective after passing through a seagull's gut indicating that birds can be a source of field contamination. Birds can transport whole proglottids from filter beds or from seashores contaminated with sewage effluent washed inshore from outfalls by tides. Gotzsche (1951) demonstrated that calves became infected by eggs of Taenia saginata in seagull droppings. It is possible that the same mechanism could operate for the transmission of other helminth infections.

Applying sludges to land returns water and valuable nutrients to soil. Risks associated with the practice depend not only on undesirable components in sludge (microbial pathogens and toxic metals) but upon the treatment to which the sludge has been subjected, the type of land treated and the use to which the land is put, crops, grazing or fodder crops.

Treatment processes suggested by the World Health Organisation for Wastewater reuse are as follows.

	Crops not for Human Consumption	Crops eaten cooked	Crops eaten raw
Health Criteria*	1 and 4	2 and 4 or 3 and 4	3 and 4
Primary Treatment	Essential	Essential	Essential
Secondary Treatment		Essential	Essential
Sand Filtration (or Equivalent)		Sometimes required	Sometimes required
Disinfection		Sometimes required	Essential

* Health Criteria; (1) Free from gross solids, significant removal of parasite eggs.

(2) As (1) plus significant removal of bacteria.

(3) No more than 100 coliforms per 100 ml in 80% of samples.

(4) No chemicals that lead to undesirable residues in crops.

after W.H.O. report no. 517 (1973)

As a consequence of recent concern about the increasing use of sewage sludges as soil conditioners and the associated health risks, the Department of the Environment have issued advisory guidelines for those responsible for disposing of sludges to agricultural land. As will be discussed later these recommendations seem arbitrary and unrelated to the probability of parasite survival under standard disposal practice.

Extracts from the Guidelines for the Disposal of Sewage Sludge to Land (D.O.E. National Water Council, 1977)

6.1.35 Where no odour nuisance will be caused, untreated sludge may be applied to land for the production of animal feedcrops or ....; crops which are always cooked before human consumption. Ploughing should take place after application. Land should not be used for grazing within a period of 6 months ..;.. unless adequate monitoring for pathogens shows that a shorter period is acceptable. Untreated sludges likely to contain certain longlived pathogens ..... should not be used.

6.1.36 Sludges ..... should preferably be well digested so that upwards of 40% of the original organic matter or 30% of the total solids has been destroyed ..... (air or mechanically dried). Sludges may be used (if) stockpiled to give an overall sludge age of 1 year; lagooned for 2 years or more; lime or otherwise treated

whereby the risk of transmission of disease is reduced to a low level.

6.1.37 Sludges from secondary treatment ..... which have been kept separate from the primary sludges are acceptable ..... since they have been subject to a degree of aerobic digestion. Surplus activated sludge from partial treatment ..... should not be used unless first stabilised by further aerobic digestion.

6.1.38 Salad or other crops which may be eaten raw should not be sown until 12 months after the application of treated sludge to land, but animal feed, or crops to be cooked, may be grown in the interim.

6.1.40 Animals should not be grazed until 21 days after application of treated sludge to land ..... 5 weeks for dairy cattle to supply milk not to be pasteurised.

6.1.41/2 approval of the district veterinary officer is required before applying sludge in brucellosis free areas.

The Working Party stated that 'it is our opinion that the application of treated sludge to agricultural land is good practice ..... this is an economical method of disposal which utilises the nutrients in sewage sludge ..... under proper control ..i.e..there is little effect on the environment and the benefits far outweigh any possible disease hazards.'.

The Manurial Value of Sewage Sludge

Dealing with the sludge produced during the sewage treatment can cost up to 40% of the total expenses incurred (Mosey 1979)a The total yearly cost for sludge treatment and disposal is approximately £100 million in the United Kingdom. Land disposal is a widely used method and of increasing popularity. In 1970 40% of all sludge was applied to agricultural land; 20% was used for land fill. Presently the Yorkshire water authority dispose of most of their untreated sludge to land (Jeger Report 1970)a

The value of sludge as a fertiliser is twofold; it provides both a water source for irrigation and a source of nutrientsa in particular nitrogen and phosphorusa to soila The evaluation of sludge as a fertiliser is usually based on its nitrogen content. Nitrogen is essential for cellular materials including protein and any defiency is rapidly shown by stunting and yellowing of crops. With escalating costs of chemical fertilisers (e.g. 'nitram' - ammonium nitrate, and 'Nitrochalk' - calcium nitrate) sewage sludges in either liquid or dried 'cake' form have become economical alternative sources of plant nutrientsa Given the increasing use of sludge fertilisation it is therefore most important to monitor the use of sludge as a fertiliser in order to establish any potential for disease transmission to humans and livestocka

Liquid sludges characteristically contain nitrogen in a form which is readily available to plants (e.ga nitrates). Dried sludges lose mineral nitrogen but do contain bound (organic) nitrogen which must be broken down in the soil before becoming available for plant growth. Activated sludge in particulara is often air dried (to a moisture content of 5% - 10%) and contains

4% - 6% nitrogen

3% - 4% phosphorus

The nitrogen in activated sludge is largely bound up in the bodies of the dead bacteria which constitute a large part of the sludges Once the readily mineralised nitrogen has been utilised, organic nitrogen is then released slowly. Such gradually released nitrogen is especially useful to crops such as grasses which can use nitrogen over a long growing period although the nitrogen released from dried sludge may be too slow for crops with a short growing season (Johnston 1974). For crops with a continual nitrogen demand, raw sludge offers a nitrogen-bank effect similar to expensive chemical 'slow-release' fertilisers. Anaerobically digested sludges have more soluble nitrogen than raw sludges and so are a more immediate supply of nitrogen than raw sludge whose nitrogen must be first broken down in the soil.

Phosphorus in sludges is in a combined form which is not immediately available to plants. It is, however, readily absorbed and stored within the plant so a source of phosphorus is less important than a source of available nitrogen.

Sludges are low in potassium compared with most chemical fertilisers but are comparable in effectiveness with potassium from any other sources Coker (1977) on the basis of field trials in which sludge was supplemented with potassium fertiliser concluded that for most purposes the sludge application alone is adequate.

Applications of sludge to land are of the order of 5000 to 12000 gallons of sludge per acre, rising to 20000 gallons in winter when pastures are not grazed and sludge is less of a 'nuisance' to the farmer (Bellingham pers. comm).

Although sludge application is a useful way of nutrient recovery, problems arise since undesirable contaminants such as heavy metals and microbial pathogens are also present. The problem presented by

pathogens in sludge have already been considered. The heavy metals found in sludge include zinc and chronium. Toxic metals such as these, and Cadmium, Copper, Nickel are found in sludges at concentrations well in excess of normal soil levels. The Department of the Environment proposed limits in 1977 for these metals and for arsenic, boron, mercury, molybdenum, lead and selenium; on the basis of sludge application over periods of over 30 years. Toxic metals may restrict crop growth or accumulate in the crop, thereby being transferred to animals eating the crops.

Aims of the Investigation

1. To develop reliable techniques for the isolation and enumeration of parasitic ova from
   (a) soil;
   (b) soil leachate;
   (c) vegetation;
   (d) sewage sludges.

2. To develop criteria for assessing the viability of parasite infective stages.

3. To establish survival times for parasitic infective stages under field conditions.

4. To establish criteria upon which recommendations can be made for the safe application of sewage sludges to agricultural land.

DETERMINATION OF SPECIFIC GRAVITIES FOR HELMINTH OVA

A. Introductions-

To be extracted from the sludge and soil samples helminth ova must be separated from organic materials and mineral particulate matter. The range of specific gravities for the settleable solids found in sewages is 1.01 - 1.20 (Sepp 1963) which corresponds to the probable range of specific gravities of the ova to be recovered, thereby indicating the difficulties involved in separating ova from sewage debriss

Currently used techniques for separating ova from organic materials depend on differential flotation. To discover the most suitable solutions to use in suchsmethods it was necessary to determine the specific gravity of ova of each species which might be recovered during the study.

Previous work on specific gravity determinations on helminth ova has been reported by Sawitz, Tobie and Katz, (1939) working with Necator americanus ova and by Sawitz (1942) working with eggs of Enterobius vermicularis (pinworm), Trichuris trichuria (whipworm), Trichuris vulpis (dog whipworm) and Ascaris lumbricoides for which he determines the following ranges of specific gravity: 1.115, 1.15 - 1.16, 1.15 - 1.16, 1.1 - 1.25. The lower end of the range for Ascaris ova relating to fertilized ova which have lower densities than unfertilized eggs.

B. Method:-

Standard sucrose solutions of specific gravity valuess 1.05, 1.15, 1.175, 1.23, 1.25 were prepared and kept at 4°C at which temperature water achieves its maximum density, in order to reduce

mixing when the solutions were later layered into centrifuge tubest The specific gravity values were checked by hydrometer at ambient temperatures on the day of each experiment, when any necessary adjustments were madet

Ova of the species: Taenia saginata, Trichuris suis, Dipylidium caninum and Ascaris lumbricoides were used in the experiment. For each species in turn a sucrose gradient was prepared by layering 2ml volumes of sucrose solution into a centrifuge tube, beginning with the solution of 1.25 specific gravity. The solution of 1.15 specific gravity was mixed with the ova suspension in order to overcome surface tension effects which could hold eggs to the surfacet The solution of 1.05 specific gravity and 2ml distilled water of 1.00 specific gravity were layered on last, making a total of six layers in all. After centrifuging at 2000 rpm for 15 minutes with a run up period of 5 minutes allowed to prevent turbulence in the gradientt the six layers were removed separately with a drawn out pasteur pipettet All samples were examined microscopically and the numbers of ova found in each layer recordedt The method was repeated for each speciest

The method used was a modification of that described by Marquardt (1960) for removing Nematodirus eggs from faecal debris. Instead of layering eggs onto the surface of the gradient as described by him, they were mixed with the solution of 1.15 specific gravity for the reason mentionedt

To confirm the results obtained by this method, ova of the four species were subjected to upward flotation in tubes each containing solutions of sucrose with different specific gravitiest The method was that described by Sawitz (1942) but sucrose replaced $ZnSO_4$ solution as the flotation medium. The suspension of ova

(100 - 450 ova in 1ml normal saline) were pipetted into centrifuge tubes then mixed well with the selected sucrose solutiona Sufficient of the appropriate sucrose solution was added to form a convex meniscus above the rim of the tube and topped with a coverslipa Samples were centrifuged at 3000 rpm for five minutes and the ova collected by upwards flotation onto the coverslip which was then examined and the eggs counted. For each technique 20 replicates of the specific gravity determinations were madea

Table (1)

(a) Determination of Specific Gravities of ova by the modified method of Marquardt

Specific Gravity		1.25	1.23	1.22	1.175	1.15	1.05
Ova recovered (+) = found (-) = absent	Taenia	+	+	+	+	+	-
	Ascaris	+	+	-	-	-	-
	Trichuris	+	+	+	-	-	-
	D. caninum	-	-	-	+	-	-

(b) Determination of Specific Gravities of ova by the modif: method of Sawitz

Specific Gravity		1.25	1.23	1.175	1.15	1.05	1.0
Ova recovered (percentage of original moculum recovered)	Taenia	92.89	85.78	89.89	67.44	-	-
	Ascaris	100	48.0	-	-	-	-
	Trichuris	64.0	21.0	3.33	1.0	-	-
	D. caninum	100	39.0	19.5	9.0	-	-

(c) Minimum Specific Gravity of solution required to support ova:-

	Marquardt's method	Sawitz's method
Taenia	1.15	1.15
Ascaris	1.23	1.23
Trichuris	1.22	1.15
D. caninum	1.175	1.15

C. Resultsa-

Using Marquardtás modified method it can be seen that Taenia ova appear to be more variable in density than Ascaris ovaą being recovered in solutions of specific gravity 1.15 to 1.25 whereas Ascaris ova were recovered only in solutions of specific gravity 1.23 and 1.25. Only a 64 percent recovery of Trichuris ova was obtained in solutions of specific gravity 1.25ą suggesting that a solution of higher specific gravity would be required to achieve greater recoverya The use of solutions of higher specific gravity howevera would cause osmotic damage to more fragile ova such as those of Taeniaa By this method the specific gravity value for the ova concerned was taken to be the lowest specific gravity solution capable of supporting the ova. These were as follows- Taenia = 1.15ą Ascaris = 1.23ą Trichuris = 1.22 and D. caninum = 1.175a

By the method of Sawitz the same specific gravity values were obtained for Ascaris ova and for Taenia ova as were obtained using the method of Marquardta Howevera when the specific gravity of Trichuris ova was determined by the method of Sawitz a lower value (1.15à was obtained than by the method of Marquardt which gave a value of 1.22. Similarly D. caninum ova were recovered (in low numbers) in solutions of specific gravity 1.15.

D. Discussion-

No data could be found in the literature for comparison of the minimum specific gravity values found for T. saginata and D. caninum ova; determined as 1.15 and 1.175 respectively in this experiment.

The specific gravity value of 1.23 found for Ascaris ova confirms that reported by Sawitz who found that unfertilized ova required solutions of specific gravity 1.25 in order to float. Lane (1925) found that twice as many Ascaris ova floated in $CaCl_2$ solution of specific gravity 1.30 than in NaCl solution of specific gravity 1.20.

The specific gravity value obtained for Trichuris suis ova (1.22) is higher than that determined by Sawitz for Trichuris trichuria ova of 1.15 - 1.16 although he reported 90% recoveries when those ova were floated in solutions of specific gravity 1.20. Lane (1925) found solutions of specific gravity 1.15 insufficient to float "whipworm" eggs and that, as in the case of Ascaris ova twice as many floated in solutions of specific gravity 1.30 than in those of specific gravity 1.20.

In selecting an appropriate solution for recovery of ova by flotation a compromise must be reached between efficiency of recovery and the selective isolation of ova from debris of similar densities without causing osmotic damage to ova.

As can be seen from the range of specific gravity values obtained for these ova; (1.15 - 1.23) there is considerable overlap with the specific gravities of settleable sewage solids (1.01 - 1.20). To separate ova from other particulate matter therefore presents considerable practical difficulties, as will be discussed later in the chapter on isolation methods.

EXTRACTION METHODS FOR ISOLATION OF HELMINTH OVA FROM SLUDGE, SOIL, LEACHATE AND HERBAGE

A: Introduction

For medical and veterinary diagnoses the demonstration of the presence or absence of parasitic ova or cysts in a faecal sample is usually sufficient. Consequently most of the currently used diagnostic techniques are qualitative, distinguishing at most between light or heavy infections. The samples in such diagnoses often have high parasitic burdens, infected faecal samples can contain in excess of 10^8 ova per gram of faeces (Spindler 1929), although more usual limits are about 5×10^4 ova. Sewage samples however contain much lower concentrations of helminth ova, (Silverman 1955) for example, received from 1 to 6 Taenia ova per gallon of sewage, (1 per litre), and Liebmann 1965) estimated 80 Ascaris ova per litre of sewage.

For these reasons a major objective of the investigation was to develop reproducible and quantitative methods of isolating ova from samples containing relatively low numbers of ova in a liquid with a high suspended organic matter content.

The materials from which ova were to be recovered were, sewage sludges, soils, herbage and soil leachates. Initially, studies were directed towards evaluating methods that had previously been used to isolate ova qualitatively from these or other materials: to quantify, and where possible improve the techniques to increase the efficiency of recovery. The samples being investigated need to be concentrated before quantitative examinations can be undertaken. Methods for concentrating ova in a sample prior to their separation from the suspending mediumare usually based on either the principle of

differential flotation, or differential sedimentation.

Spindler (1929) made qualitative isolations of Ascaris ova from soil using a sodium dichromate flotation solution. Faust et.al (1938) used a variety of techniques to isolate parasitic ova and cysts from faeces, but their quantitative comparison of methods was confined to the recovery of protozoon cystse

Sawitz (1942) used a Zinc Sulphate flotation for determining the buoyancy of nematode ova and for their subsequent extraction. Ritchie, Pan and Hunter (1952) compared the formol-ether method of Ritchie (1948) with the Zinc Sulphate flotation and concluded that the former method was both more efficient and caused less distortion of ova. Allen and Ridley (1969) adapted the formol-ether method by using faster centrifuge times and by using a formalin solution rather than formol saline for the suspending medium. With these modifications they achieved an increase in positive diagnoses of Ascaris and Taenia ova in faecal samplese Steer et al (1974) proposed that electrostatic charges held ova to soil and other particles, and by adding 1.0% anionic detergents they were able to achieve high ($\triangleq$ 100%) recoveries of Ascaris ova from inoculated refuse and sludge mixturese using an adaptation of the formol-ether method and a seeding rate of 300-600 ova per ml of sample. Bartlet et al (1978) compared Zinc Sulphate flotation with the formol-ether methods, concluding that the Zinc Sulphate method was superior for recovery of all but Schistosome ova. The formol-ether method was more efficient in terms of numbers recovered but caused distortion of ova. Meyer et al (1978) combined Zinc Sulphate flotation with membrane filtration, achieving high (50% - 83% depending on initial inoculum) recovery of Ascaris ova

from sludge. Dada (1979) achieved a recovery rate of 67.5% for Toxocara ova in soil, using a range of flotation solutions. Bailenger (1979) observed that mercury ions (Hg^{++}) affected flotation of ova, flotation increasing in proportion to (Hg^{++})s concentration. He explained this by interaction between (Hg^{++}) ions and groups on the egg surfaces, altering the hydrophilic/lipophilics balance between ova and flotation solution.

Owing to the probability of sparse and patchy egg distribution and adhesiveness between organic material and eggs, special methods of treatment with surfactants were needed in order to release ova from debris. Isolations from soil or sewage sludge pose similar problems. Both necessitate the separation of low concentrations of ova from particulate organic matter of low specific gravity. Isolation from soil leachate presents the problem of separating relatively small quantities of solids - the ova - from a large volume of suspension. Isolations from herbage have the attendant problem of egg adherence to organic materials. This is particularly a problem for those members of the genus Ascaris which have a roughened coat and therefore adhere strongly to organic particles in the sample.

III Preliminary Evaluation of Flotation Methods

The use of differential flotation methods to recover ova from samples will depend on the buoyancy of the ova of each particular species. A flotation solution must be chosen so that the specific gravity of the solution is higher than that of the ova, but preferably not greater than that of organic debris, in order to achieve a good separation. As mentioned in the chapter on specific gravity determinations however, there is considerable overlap with the specific gravities of settleable sewage solids.

When using centrifugal flotation to concentrate ova in a sample, the centrifuge time is a function of the difference between the specific gravity of the ova and the specific gravity of the medium in which they are suspended. Too great a difference between the values could cause osmotic damage to some ova such as those of flukes. Cysts too, tend to collapse in concentrated solutions. To separate ova from a solution of closely similar specific gravity however, requires a longer spin period.

The methods of concentrating ova in samples examined were; (1) sucrose gradient flotation, (2) a combined flotation method and (3) single flotation methods. To recover ova from samples quantitatively it was necessary to concentrate the ova in the sample and then separate them from surrounding debris. Centrifugation was used in conjunction with surfactant treatments to achieve this, in the antiformin-sucrose and formol-ether methods of extraction.

The samples from which ova were to be recovered were soil and raw sewage sludge. The sludge samples were collected from the primary settling tanks of a local sewage treatment plant. This particular

plant processes sewage received from the Harpenden, Wheathampstead and Luton areas, classified as "mixed urban" sewage. The collected sludge was found to have a total solids content of 4.68 percent.

The sludge was passed through a 100 mesh (0.15 mm aperture) sieve in order to remove coarse debris whilst still retaining ova and all other particles with diameters not exceeding 0.15mm. The ova likely to be recovered from these investigations have diameters in the range 0.03 - 0.07 mm. When surfactants were used however, (sections 4,5) it was possible to dispense with sieving which had resulted in considerable egg losses from samplese

1. Sucrose Gradient Flotation

A. Introduction

Marquardt (1961) described a technique for separating nematode ova from faeces by centrifugation in a sucrose solution of graded specific gravity. This method was investigated to gauge its potential usefulness for separating ova from sludge samples.

B. Method

Marquardtés method entailed sieving a faecal suspension to remove coarse debris and then allowing the material to sediment. The sediment was spun on a prepared sucrose gradient so that the ova form a layer at a position on the gradient determined by their density.

For this study raw sewage sludge was collected from primary settling tanks of a local treatment plant and passed through a

100 mesh sieve to remove coarse debris. A gradient was prepared by layering into a centrifuge tube; 2 ml saturated sucrose solution (s.g. = 1.275) and 1 ml each of 1/2, 1/4, 1/6, 1/8 dilutions of the saturated sucrose solution having specific gravity values of 1.23, 1.11, 1.06, 1.05 respectively. A 3 ml sample was layered onto the top of the gradient.

After centrifuging at 1500 rpm for two minutes the opaque layers of ova were visible between the sucrose layers of the specific gravity range 1.06 - 1.23. These layers were withdrawn using a finely drawn out pasteur pipette. Recovered ova were washed three times in saline at a centrifuge speed of 1500 rpm for five minutes.

C. Results and Discussion

In the sludge samples used it was found that not all ova could be confined to a single clearcut layer, free from sewage debris of similar density. For this reason it was decided that the method would not be suitable for quantitative recoveries of ova. Despite this limitation the method could be of use when examining samples previously concentrated by a different method. The concentrated sample could then be layered onto the gradient, and where more than one type of ovum was present in the sample, provided that their densities were sufficiently different, these would be recovered from different positions on the gradient after centrifugation: thus enabling several layers of ova to be extracted from a single centrifugation.

2. Combined Flotation Method

A. Introduction

Having rejected a gradient method for extraction it was decided to investigate the recovery of ova by centrifugal flotation in a range of different flotation solutionst A method which was first described by Parfitt (1958) and entailed sequential centrifugation and resuspension of sediments in the different flotation solutions was studied for this purpose.

B. Method

Sludge samples were collected from the primary settling tanks of a local treatment plant. Samples were presieved as already describedt A 10 ml sample of the sieved sludge was then centrifuged at 1500 rpm for two minutest The sediment from this sample was then resuspended in 10 ml saturated Na Cl solution (specific gravity = 1t20)t Fraction (A) was pipetted from this suspension. After the remainder had been spun at 1000 rpm for two minutes fraction (B) was pipetted from the upper layer. The sample was recentrifuged at 1500 rpm for two minutest To provide fraction (C) the sediment was resuspended in saturated Zn SO_4 solution (specific gravity = 1.364) and centrifuged at 1500 rpm for two minutes; the sediment providing fraction (D). All fractions were examined microscopically.

Observations

Since by this method, a range of flotation conditions are provided it could be used to recover ova of more than one typet Protozoon cysts for example float most readily in Zn SO_4 solution and so predominate in fraction (C)t. The drawback to this method was that since the method of taking fractions A - D precluded total

removal of ova from samples it was not really suited to quantitative examinations. In an attempt to overcome this limitation flotations of ova involving only one set of conditions at a time were chosen for the next series of investigations.

3. Single Flotation Method

A. Introduction

In an attempt to make quantitative recoveries of ova from samples a range of flotation solutions was used separately rather than in the combination described in Section (2). As previously mentioned in the general introduction, sludge contains low concentrations of ova. To evaluate the efficiency of a particular isolation method it was therefore desirable to inoculate the sludge or soil samples with ova and determine how much of the initial inoculum was recovered after treatment.

B. Method

Sludge was collected and sieved as described in previous sections. The sludge samples were then inoculated, using as the seed, Ascaris and Trichuris ova at a density of 24 and 6 ova respectively per ml of sludge. The sieved and inoculated sludge sample was divided into 10 ml aliquots and centrifuged at 1500 rpm for two minutes. The supernatant was not discarded but retained and reprocessed after transferring to another centrifuge tube. This additional spin was incorporated to reclaim any ova not sedimented by the first centrifugation. All the sediments were collected and resuspended in the selected flotation solution; either saturated Zn SO_4 or Na Cl solution, and then topped up with the solution until a meniscus formed

slightly above the rim of the tuber A clean 7/8 coverslip was placed on the top of the tube so that only a very small airbubble was trapped underneath. After centrifugation at 1500 rpm for five minutes the coverslip was carefully removed onto a microscope slide for examination. Any eggs adhering to the inside of the glass tube were dislodged by scratching the inner rim with an inoculating needle and topping up with fresh flotation medium. The centrifugation was repeated until four coverslip recoveries had been mader All coverslips were examined microscopically and the four counts totalled.

B. Results and Discussions

The recoveries of Ascaris and Trichuris ova from inoculated sludge are shown in Table (2). The sludge was seeded at the rate of 24 Ascaris and 6 Trichuris ova per ml.

Table (2)

Recovery of Ova from Sludge					
	n	$\bar{x}$	s	C.V.	Percentage Recovery
Ascaris	30	3.27	2.21	67.6	13.6 ± 0.81
Trichuris	30	1.50	1.41	94.0	25.0 ± 0.51

Where n = the number of trials

$\bar{x}$ = the sample mean

s = the standard deviation of the mean

C.V. = the coefficient of variation $\frac{s}{\bar{x}}$ 100 expressing the variability of the sample relative to the mean. The recovery percentages are expressed with their 95 percent confidence limits.

The high coefficients of variation of these results; 67.6% for recovery of Ascaris ova and 9410% for recovery of Trichuris ova, indicate that these results are widely dispersed. Efficiency of recovery was low (13.6% recovery of Ascaris ova, 25.0% recovery of Trichuris ova)1.

The low rate of recovery may have been due to eggs adhering to the organic matter in the sludge, Ascaris ova in particular having a rough, adhesive outer coating. To attempt to overcome this problem the next stage was to study the possibility of using a surfactant to release ova from adhering particles. These investigations are discussed in the following section.

4. Quantitiative Recovery of Ova by the Antiformin-Sucrose Method

A. Introduction

Spindler (1929) described a technique involving surfactant treatment of a faecal sample which made the subsequent flotation stage a more efficient method of recovery. Surfactant properties are also made use of in the standard formol-ether method described in the section which follows, in which method formalin or formol saline acts as a surfactant.

In his original method Spindler employed sodium dichromate as the flotation solution, but as it was desired to use the method for recovering viable ova, sucrose solutions were deemed to be more appropriate. A similar method is used by E. J. Pegg (personal communication) for recovery of Toxocara ova from soil and faecal samples. In both of these methods the recovery of ova after a centrifugation stage is by collecting loopfulls from the liquid's

surfacee For quantitative recoveries this method of recovery needed to be adapted as not all of the ova would be retained in this way.

B. Method

Sludge samples were collected and inoculated as described previously. The samples were not sieved however, as it was hoped that surfactant treatment would obviate the need for this stage which was responsible for losses of eggse The samples were divided into 5 ml aliquots, plastic syringes being used to dispense the samplese These were mixed with an equal volume of a surfactant, (30% antiformin substitute) and then thoroughly mixed. Blunt glass rods were used to mix the samples violently in order that the surfactant solution could act on all particlesé surfacese At the end of the mixing the stirring rod in each tube was left in position, and the mixing procedure repeated periodically for approximately 1 houre

After 1 hour had been allowed for the surfactant to effect separation the sample was mixed with the flotation medium; sucrose solution with a specific gravity value of 1.23 was added to the sample/antiformin mixture. After these were thoroughly mixed the stirring rods were washed with more sucrose solution so that no egg losses would occur through eggs adhering to the stirrerse The tubes were then topped up with sucrose solution to form a convex meniscus and covered with a clean coverslip. The coverslips were found to sometimes slip off the rim of the tube during centrifugation and so the rims of these tubes were ground to a flat surface to prevent the coverslips dislodging. After centrifuging at 1500 rpm

for 15 minutes the coverslip was removed onto a slide for counting (still inverted) and the final flotation was repeated twice, making a total of three coverslip recoveries.

The method as developed for isolating ova from sludge was used for isolations from soils, with the following modifications:-

1. A 10 g sample of dried soil was used.
2. 50 ml centrifuge tubes were used in place of the 15 ml tubes used before.
3. 25 ml of sucrose solution was used.

For both soil and sludge recoveries preliminary trials were undertaken and a statistical test applied to indicate the required number of replicates to give a decimal accuracy of 0.05 (or 95% confidence limit). The choice of the number of samples to be taken depends on the degree of precision required. With continuous data the required number of samples is given by the formula:-

$$N = \frac{t \times s}{D \times \bar{X}}$$

t = Student's "t" statistic, derived from 't' tables for (n) degrees of freedom

s = standard deviation of the trial sample

$\bar{X}$ = mean of the trial sample

D = preset decimal accuracy

n = number of items in a trial sample

This may be regarded as a comparison of the standard error of the observations with the standard error acceptable. The larger this is with respect to the acceptable level of accuracy, the more samples will be required. In three trials, each of 20 items, the calculated values for N were 12.97, 18.0, 16.9

therefore N average = 15.96.

This means that 16 replicates of the technique are required for a decimal accuracy of 0.05.

Breakdown Analysis of the Antiformin Sucrose Method

During preliminary investigations only low recoveries of ova were obtained (29.5% recovery from 60 trials)s Because of this it was decided to examine the technique in more detail. For satisfactory quantitative recoveries it was important to know where in the technique these losses were taking place. Separate counts were therefore made at stages in the method.

Table (3)

Results of Breakdown Analysis of the Antiformin-Sucrose Method			
	Initial two coverslip counts	Further two coverslip counts	Syringe rinsings
Ascaris	$\bar{x}$ = 7.563±0s848	$\bar{x}$ = 0.563±0.356 t_1 = 3.375 p < 0.001	$\bar{x}$ = 0.313±0.235 t_2 = 4.867 p < 0.001
Taenia	$\bar{x}$ = 5.625±1.04	$\bar{x}$ = 0.5±0s25 t_1 = 4.411 p < 0.001	$\bar{x}$ = 1.5±0.669 t_2 = 3.770 p < 0.001

Table (3) shows the results obtained from a stage by stage analysis of the antiformin-sucrose method of ova recovery from samples of sewage sludges The sludge samples were inoculated so that

each ml should contain 10 Ascaris ova or 10 Taenia ova. To determine whether or not the incorporation of further coverslips rinsings did give a significant increase in recovery a "t" test of significance was applied to the results. The aim of the test was to find out if there was a worthwhile increase in recovery to be gained by including the extra two coverslip recoveries. This was found by the 't' test (t_1) to be significant at a probability level of 0.001, for both Ascaris and Taenia ova. A second comparison of recovery was made by comparing the recovery of coverslips 1 and 2 and the recovery when syringe rinsings were included, where plastic syringes had been used to dispense samples. This was found by the 't' test (t_2) to be significant, again at a probability level of 0.001. On the basis of this analysis it is clearly an advantage to include additional coverslip recovery stages and to include syringe rinsings to achieve the best possible recovery of ova. This was therefore adopted as a standard procedure.

C. Results

To check that the formula used to find the required number of replicates of the technique was in fact adequate, a further series of extractions from sludge was performed, using the information gained from the breakdown analysis described. The results of these extractions are summarised in table (4). It can be seen from the table that, by incorporation of the information about minimising egg losses gained from the breakdown analysis of the extraction method it was possible to increase recovery rates from 29.5 percent to a final best recovery rate of 56.2 percent for Taenia ova and 76.6 percent for Ascaris ova.

Recovery of Ova from Sludge by the Antiformin-Sucrose Method						
Trial	No. of Trials	Inoculum	S	$\bar{x}$	CV(%)	Recovery (%)
Initial	60	10/ml (Ascaris)	6.669	6.95±1.69	95.6	29.5±7.04
Improved Surfactant Mixing	60	10/ml (Ascaris)	5.670	11e8±1e43	48.5	48.7±6.04
Improved Surfactant Mixing and Coverslip Placement	60	10/ml (Ascaris)	1.730	7.56±0.85	22.5	76.6±4.05
	60	10/ml (Taenia)	0.52	5.63±1.04	37.9	56.2±1.04

where S = standard deviation

$\bar{x}$ = mean (given with 95% confidence limits)

C.v. = coefficient of variation $\frac{S}{\bar{x}}$ 100

and recoveries are expressed with 95% confidence limits

D. Discussion

Using the basic technique as described by Spindler (1929) the initial trials of the antiformin-sucrose method yield low (< 30%) recoveries of ova from soil or sludge samples (Table 4). Improvements to the technique in the areas of:-

(1) Application and removal of coverslipsa

(2) Attention to thorough mixing of sample and surfactant.

(3) Incorporation of syringe and coverslip rinses in the total countsa

are reflected in the higher and more consistent count obtained in later trials. The coefficient of variation for example, which is a measure of variability within a sample, is reduced from 95.6% in initial trials to the more acceptable figures of 22a5% for recoveries of Ascaris ova and 37.9% for recoveries of Taenia ova.

From the adaptations made to the basic technique recoveries of ova from sludge were increased to up to 76.6%. The surfactant treatment was evidently effective in releasing ova from debris so that ova could then be supported by the flotation medium (sucrose) and hence be recovered by upward flotation onto a coverslipa

A method of recovering ova from faecal material which is widely used in medical laboratories is the formol-ether technique which again makes use of surfactants. Instead of the subsequent flotation stage however, the technique uses the sedimentation principle to separate ova from debrisa It was planned to study this method in order to compare the relative effectiveness of the antiformin-sucrose technique with that of the formol-ether technique.

5. The Formol-Ether method

A. Introduction

This technique, which is a standard diagnostic tool in medical laboratories is based on an original method of Ritchie, Pan and Hunter (1952), but was modified in (1970) by Allen and Ridley who reported that their method gave from 15 - 50 times higher concentration of helminth ovae The method has been used essentially unchanged in public health laboratories since 1952 for diagnosis of helminth infection.

B. Method

The sludge samples were collected and inoculated with Ascaris and Taenia ova as previously described, then divided into 10 ml aliquots. Each sample was then centrifuged at 3000 rpm for two minutese The supernatants, which are normally discarded were retained and centrifuged for a second timee The sediments were combined, then resuspended in a 7 ml volume of 10% formalin-saline. The action of formalin is similar to that of the antiformin substitute used in the previous method. Both work on the surfaces of particles, promoting release of ova from organic debris. After mixing the sample well, 3 ml of diethylether was added. This is an organic solvent which dissolves greasy materials viz. soaps and mineral oils found in sewage sludges. After a thorough mixing by covering the tube with parafilm and rapidly inverting several times the sample was centrifuged at 2000 rpm for two minutese All sediments and tube rinses were combined and transferred to microscope slides for counting.

The number of replicates required was calculated as previously described for the antiformin-sucrose method. In four trials, each of

20 counts., N (the minimum required replicates) was found to have the values; 14.38, 22.57, 12.15, 13.31.

N average = 15.60. Therefore to obtain a decimal currency of 0.05 16 replicate samples are required.

Breakdown Analysis of the Formol-Ether Method

To ascertain where in the method egg losses could occur., separate counts were made at each stage of the isolation in the same way as previously applied to the antiformin-sucrose method. Results of the analysis are summarised in table (5).

Results of Breakdown Analysis. of the..Formol-Ether Method

	Sedimentation	Syringe Rinses	Supernatant	Tube and Pipette Rinsings
Ascaris	$\bar{x}$=13.8 ± 2.3	$\bar{x}$=0.9 ± 0.98 t = 1.8 p < 0.05	$\bar{x}$=0.1 ± 0.196 t = 10 p < 0.001	$\bar{x}$=0.9 ± 0.85 t = 4.41 p < 0.001
Taenia	$\bar{x}$=3.5 ± 0.6	$\bar{x}$=0.3 ± 0.3 t = 1.0 not significant	$\bar{x}$=0.3 ± 0.3 t = 2.0 p < 0.05	$\bar{x}$=0.2 ± 0.26 t = 6.15 p < 0.001

Table (5) show the result of a stage by stage analysis of the formol-ether method of ova recovery from sewage sludges. The sludge

samples were inoculated so that each ml should contain 20 Ascaris ova or 5 Taenia ova. As previously described for the antiformin-sucrose technique, a "t" test of significance was applied to the results to determine whether or not a significant increase in numbers of ova recovered could be obtained by the inclusion of syringe rinsings and by processing the supernatant. To this end the values obtained from the basic method of sedimentation were compared with the increase in recovery when syringe rinsings, tube and pipette rinsings and a repeated sedimentation performed on the normally discarded supernatant were included.

For recoveries of Ascaris ova the losses of ova at each stage were shown to be significant indicating the value of incorporating these additional stages into the basic technique.

For recoveries of Taenia ova, losses of ova were not significantly high from discarded syringe rinsings. The reason for this could be that Taenia ova are less likely to adhere to the syringe surfaces than Ascaris ova which have a characteristically adhesive, rough coat. Loss due to tube and pipette rinsings were significant however, possibly because glass surfaces have a greater tendency than plastic to hold eggs.

Table (6)

Recovery of ova from sludge by the formol-ether method						
	Trials	Inoculum	$\bar{x}$	s	C.V.(%)	Recovery (%)
Trichuris	40	10/ml	7.78±1.52	4.90	63.0	100
Ascaris	40	20/ml	14.18±1.67	5.38	37.9	60.6±8.3
	40	20/ml	13.80±2.30	3.70	26.8	69.0±2.3
	40	30/ml	29.44±2.54	1.09	3.7	98.0±2.5
Taenia	40	10/ml	7.06±0.83	1.69	23.9	69.0±1.6
	40	5/ml	3.50±0.60	0.97	27.7	70.0±0.6

Where;

$\bar{x}$ = mean, expressed with 95% confidence limits

s = standard deviation

C.V. = coefficient of variation

recoveries are given with their 95% confidence limits

Using the information gained from the breakdown analysis of the formol-ether method recoveries of up to 70% were obtained for Taenia ova and up to 98% for Ascaris ova. Nearly complete recovery of Trichuris was achieved. The improvement in recovery technique is reflected in the decrease in (s) and (C.V.), both measures of variability of results.

D. Discussion

Quantitative recoveries of helminth ova had been achieved by both the antiformin-sucrose and the formol-ether method. Because the formol-ether method involves the use of an organic solvent (diethyl ether) which can damage ova it was decided that the method would not be suited to obtaining viability data which was especially important in this study where the effect of differentesewage and agricultural practices on the longevity of helminth ova was being examined. Where total recoveries were concerned however, the formol ether could be an appropriate method to use and it was therefore planned to compare the recovery efficiencies of the two methods.

III A Comparison of the Relative Efficiencies of the antiformin-sucrose and formol-ether methods

Fewer than 25% of the ova in sewage sludge were able to be recovered by single flotation methodse By comparison, recoveries of Ascaris ova of up to 76.6% and 98% were obtained by the antiformin-sucrose and formol-ether techniques respectively. To establish the relative efficiency of each method a statistical comparison was made, using sludge samples inoculated with Ascaris and Taenia ova. The test used was an analysis of variance, the variance under consideration being the efficiency of recovery and the two factors being simultaneously tested were types of ova and method of recovery used. Bulk sludge samples were seeded with ova at the rate of 10 ova per ml and then subdivided so that the techniques were compared on the same materiale

The extractions were made as described previously using the required number of replicates for a 0.05 level of accuracy in the counts, already determined as 16. The results of these counts are summarised in table (7). From tables of the F distribution, F 0.05 (1) 3,63 = 3.15, however the calculated value of F (the ratio of the group mean squares to the error mean squares)

= $\frac{35e8}{3.1}$ = 11e569 which is greater than the critical value (3e15) derived from tablese This establishes that the means are significantly different.

The next step is to determine whether or not there is any interaction between type of ova and the method of recovery used.

Table (7)

Recovery of Ova by antiformin- sucrose and formol-ether methods

Ova	Method	$\bar{x}$	S	S^2	Totals
Ascaris	antiformin-sucrose	7.56	1.79	3.20	121
Ascaris	formol-ether	9.25	1.34	1.80	148
Taenia	antiformin-sucrose	5.63	2.13	4.54	90
Taenia	formol-ether	7.06	1.69	2.86	113

where $\bar{x}$ = mean

S = standard deviation

S^2 = variance

Again, from table (7) the following data can be computed;-

"Methods" sums of squares = 39.063

"Ova" sums of squaresl= 68.063

"Interaction" sums of squaresl= 0.25

"Method" F = 12.626 ($p << 0.0001$)

"Ova" F = 22.0 ($p << 0.0001$)

The results obtained, (i.e.) "Method" F and "Ova" F both significantly higher than the F value derived from tables of the F distribution, indicate that there is a significant interaction. This means that the efficiency of recovery is affected by the type of ova being recovered, as well as by the method employed.

Having now established that a significant interaction exists, the final stage is a multiple comparison. The test applied was the Newman-Keuls test (Newman 1939, Keuls 1952) a multiple range test. Acceptance of the fact that interaction exists means that not all population means are equall The multiple range test is called for to determine between which population means differences exist. The first step is to arrange sample means in order of increasing size;- 5.63, 7.06, 7.56, 9.25 these beinglμ, μ_2, μ_3 and μ_4 respectively.

The means are now compared in pairs, μ_1 against μ_2, μ_2 against μ_3 and so on, making a total of six different comparisons. The overall conclusion from this comparison is that;

at a significance level of $p = 0.0001$, $\mu_1 = \mu_2 = \mu_3 \neq \mu_4$

at a significance level of $p = 0.01$, $\mu_1 = \mu_2$ $\mu_3 \neq \mu_4$ and $\mu_2 = \mu_3$

at a significance level of $p = 0.05$ $\mu_1 \neq \mu_2 = \mu_3 \neq \mu_4$

For "methods", this means that, by the antiformin-sucrose method, recovery of Ascaris ova is superior to the recovery of Taenia ova. By the formol-ether method, recovery of Ascaris ova is again superior to that of Taenia ova. For "ova", this means that, the recovery of Ascaris ova is more efficient by the formol-ether method at all probability levelse Recovery of Taenia ova is also more efficient by the formol-ether method at all probability levels.

These results show a clearcut superiority in recovery by the formol-ether method over the antiformin-sucrose method. Selection of the antiformin-sucrose method for use in field trials was therefore based solely on avoidance of egg damage caused by the organic solvent (diethyl ether) used in the formol-ether method.

For "methods", this means that, by the antiformin-sucrose method, recovery of Ascaris ova is superior to the recovery of Taenia ova. By the formol-ether methods, recovery of Ascaris ova is again superior to that of Taenia ova. For "ova"s, this means thats, the recovery of Ascaris ova is more efficient by the formol-ether method at all probability levelss Recovery of Taenia ova is also more efficient by the formol-ether method at all probability levelss

These results show a clearcut superiority in recovery by the formol-ether method over the antiformin-sucrose method. Selection of the antiformin-sucrose method for use in field trials was therefore based solely on avoidance of egg damage caused by the organic solvent (diethyl ether) used in the formol-ether method.

IV Investigation of extraction methods for ova in herbage and soil leachate samples

A. Introduction

In order to recover ova which might have passed through the soil profile of the *in situ* lysimeters used for field trials it was necessary to have a method of separating ova from dilute suspensions. The same requirement was present when recovering ova which may have remained on herbage growing on the *in situ* lysimeters.

Whilst well established methods exist for extraction of larvae from herbage; Parfitt (1955), Lancaster (1970), Ahluwalia (1975), fewer techniques deal specifically with extraction of the ova.

B. Method

The method for extraction of infective nematode larvae from herbage described by Lancaster entailed agitation of a maximum of 500 g herbage in 20 litre of water containing a few drops of a wetting agent. After removing the herbage for dry weight analysis, the wash fluid was passed through a 100 mesh sieve, then through two 400 mesh sieves. All the material which had been collected on the 400 mesh sieves was washed into centrifuge tubes and spun at 3000 rpm for five minutes. After the supernatant had been discarded the remaining deposits were resuspended in a saturated $MgSO_4$ solution (specific gravity = 1.29) or in a saturated $ZnSO_4$ solution (specific gravity= 1.364).

The tubes were topped up to form a convex meniscus and then covered with a clean coverslip so that ova (or larvae) could be collected by upward flotation onto the coverslip in the same way as

described for the antiformin sucrose technique. The centrifugation was performed at 1000 rpm for two minutes, after which time the coverslips were carefully removed and the washings pooled. These were then recentrifuged at 1500 rpm for two minutes. All but 0.1 ml was removed, leaving a final deposit which was examined microscopically.

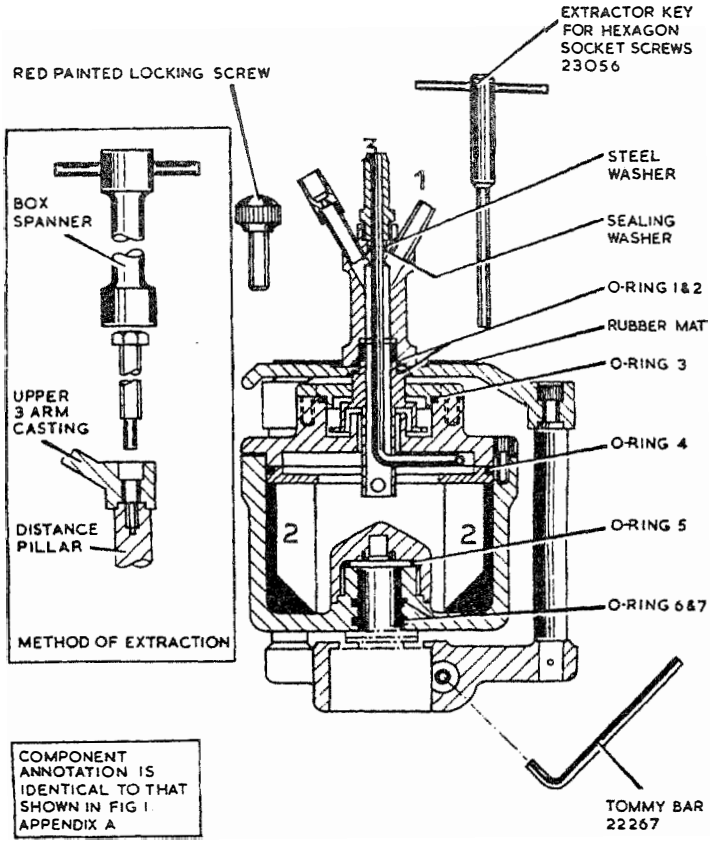
It was hoped that this method, with adjustment of centrifuge times and speeds, could prove useful for recovery of ova. The 400 mesh sieve, with its apertures of 0.037 mm is able to retain ova whose diameters exceed this value. Ascaris ova, for example, have a minimum size of 0.06 mm and Taenia, 0.04 mm.

Lancaster compared his technique with a soaking/sedimentation procedure described by Parfitt (1955) and achieved a marked increase in recovery of larvae (a minimum of 200 percent increase).

Ahluwalia (1975) recovered Cooperia larvae from herbage by a soaking/sedimentation method in which 5 g samples of herbage were soaked for 24 hours in 500 ml water. After washing, the herbage was passed through a 60 mesh sieve and allowed to sediment for 12 hours. A 25 ml subsample of sediment was centrifuged at 500 rpm for 10 minutes and from this, a 5 ml subsample was taken for examination. Ahluwalia attained 64.5 percent recovery of larvae using this method.

In addition to the methods involving sieving followed by centrifugation, the use of a continuous action rotor was investigated. This apparatus is designed to separate small quantities of solid matter from large volumes of suspension and it was hoped to develop extraction techniques for ova based on the action of this rotor. The rotor is shown in Figure (3).

Figure 3 The Continuous Action Rotor



SECTIONAL VIEW OF THE ROTOR

B. Methods

When attempting to isolate ova from herbage one of the more important problems to be overcome is that of adherence of ova to leaves and particulate organic matter. In this work the pretreatment used was that of Lancaster, viz. washing herbage in water containing 2 drops of Teepol per 10 litre. This acts a surfactant to release ova from the herbage.

To test the efficiency of the recovery of ova from herbage, samples were inoculated with ova by pouring a suspension of ova onto herbage and collecting any excess inoculum for a comparative count. Both the method of Lancaster and that described by Ahluwalia were tried out. The suspension used to inoculate the herbage samples contained 150 Taenia ova, 625 Ascaris ova.

The continuous action rotor was used to extract ova from both herbage washings and soil leachate samples. For this, the herbage was subjected either to the pretreatment described by Lancaster viz, using teepol as a wetting agent, or that described by Ahluwalia, in which herbage was soaked in water for 24 hours. Instead of further processing, the washings were next passed through a 100 mesh sieve and put through the continuous rotor as described in figure 3. The forces generated at the high speeds of centrifugation (15,000 rpm was used), deposit any solids in the suspension onto a polypropylene sediment collector inside the rotor, (no. (2) in the diagram). The deposit can be washed from the rotor and then centrifuged at 2000 rpm for 15 minutes to obtain a sediment for counting, using specially constructed chambers (appendix 2).

To recover ova from leachate by this method no sample pre-treatment was required. Soil leachate samples could be placed in a reservoir above the centrifuge and run directly into the rotor inlet (no. (1) in the diagram). Rate of flow was controlled by a screw clip on the tube leading to the rotor inlet.

In order to establish the efficiency of each technique samples containing known inocula of ova were processed.

C. Results

For recovery trials of ova from herbage samples 150 Taenia ova and 625 Ascaris ova were used per sample. For recovery trials of ova from soil leachate 10 litre samples of water containing inocula ranging from 350 - 750 Ascaris or Taenia ova were used.

Results of recovery from leachate are shown in figure 8 . Results of herbage recoveries using the continuous flow rotor are shown in figure 9 . Very low recoveries were obtained by both Ahluwalia's method (16.9%) and that of Lancaster (23.1%). Although it could have been possible that recoveries by these methods could have been improved with further work, it was decided that since t continuous flow rotor gave more efficient recoveries in prelimi trials that it would be more profitable to follow up this method exclusively.

Table (8a)

Recovery of Taenia ova from soil leachate using the continuous rotor

inoculum (l)	flow (ml/ min)	$\bar{x}$ (/ml)	S.E.	recovery (%)
750	300	118.1 ± 18.98	8.39	78
750	300	149.4 ± 41.94	18.54	90
750	300	114.8 ± 18.39	8.13	76
750	300	128.5 ± 31.06	13.73	86
750	300	116.9 ± 10.79	4.77	84
750	300	133.1 ± 17.85	7.89	89
750	300	84.8 ± 11.48	5.07	90
750	300	187.5 ± 15.99	7.07	≥100
750	300	138.9 ± 9.66	9.66	87
				Range1 = 76% - 100%
				Mean = 86.66%

where: $\bar{x}$ = mean, expressed with 95% confidence limits

S.E. = standard error of the mean

(counts are averages of 10 trials)

Table (8b)

Recovery of Ascaris ova from Soil Leachate using the continuous action rotor				
Inoculum	Flow (ml/min)	$\bar{x}$ (/ml)	S.E.	Recovery (%)
680	300	34.7 ± 2.53	1.12	72
680	1000	19.1 ± 1.56	0.69	42
680	400	75.2 ± 5.54	2.45	83
350	300	48.8 ± 13.89	6.14	70
350	300	65.2 ± 20.87	9.23	90
350	300	56.2 ± 23.05	10.19	80
350	300	47.5 ± 12.35	5.46	63
350	300	45.1 ± 14.84	6.56	66
350	300	71.7 ± 13.64	6.03	98
350	300	62.7 ± 9,43	4.17	90
350	300	76.0 ± 13.55	5.99	76
350	300	60.5 ± 10.88	4.81	87

where: $\bar{x}$ = mean, expressed with 95% confidence limits

S.E. = stamdard error of the mean

(counts are average of 10 trials)

Mean Recovery (all trials) = 76.4%

Table (9a)

Recovery of Ascaris ova from Herbage using the continuous action rotor			
Herbage washings	Inoculum excess	S.E.	Recovery (%)
700 s = 0.48	3100 s = 1.37	0.59	16.9
1182 s = 46.52	1278 s = 40.33	27.47	23.1

where: s = standard deviation

S.E. = standard error of the mean

no. of trialsl= 10

Table (9b)

Recovery of ova from Herbage using the continuous action rotor					
ova	inoculum	flow (ml/min)	$\bar{x}$ (/ml)	S.E.	recovery (%)
Ascaris	63/ℓ	300	1.7 ± 0.59	0.26	97
	63/ℓ	300	4.5 ± 1.31	0.58	72
Taenia	15/ℓ	300	0.5 ± 0.375	0.17	≏100
	15/ℓ	300	1.2 ± 0.816	0.36	80

where $\bar{x}$ = mean, expressed with 95% confidence limit

S.E. = the standard error of the mean

D. Discussion

Initial trials run with the continuous flow rotor to determine a suitable rate of throughput of sample indicated that a rate of 1000 ml/minute was too fast to obtain satisfactory recovery rates, at this rate only 42% of Ascaris ova were recovered (Table 8b). The flow rate was controlled by means of a screw clip on the tube leading into the inlet pipe (no. 1 in the diagram, figure 3). A rate of 300 ml/minute was selected, at which rate the recovery of Taenia ova from leachate ranged from 76% - 100% and for Ascaris ova, from 63% - 98%. Mean recovery rates were 86.66% and 76.4% for Taenia and Ascaris respectively. The lower rate of recovery of Ascaris ova could be due to some tendency of Ascaris ova to adhere to the sediment collector and thereby fail to be recovered.

The technique of continuous flow centrifugation was also used to recover ova from herbage; the soil leachate being replaced by the herbage washings. By this method the mean recoveries of Ascaris and Taenia ova were 84.5% and 90% respectively (table 9b).

Having developed methods of ova recovery able to give reasonable returns of ova, these could now be used to recover ova from samples derived from field and laboratory survival trials.

Summary

On the basis of this evaluation of extraction methods it was concluded that for the following studies extraction of ova from sludge and soil samples should be performed using the improved antiformin-sucrose technique which gives mean recovery efficiencies;-

76.6 % for Ascaris ova

56.2% for Taenia ova

and that extraction of ova from herbage and soil leachate samples should be performed using the continuous action rotor at 15000 rpm and throughput of 300 ml/minute, which gives mean recovery efficiencies;-

76.4% for Ascaris ova

86.7% for Taenia ova.

DETERMINATION OF SETTLING RATES FOR OVA IN SLUDGE

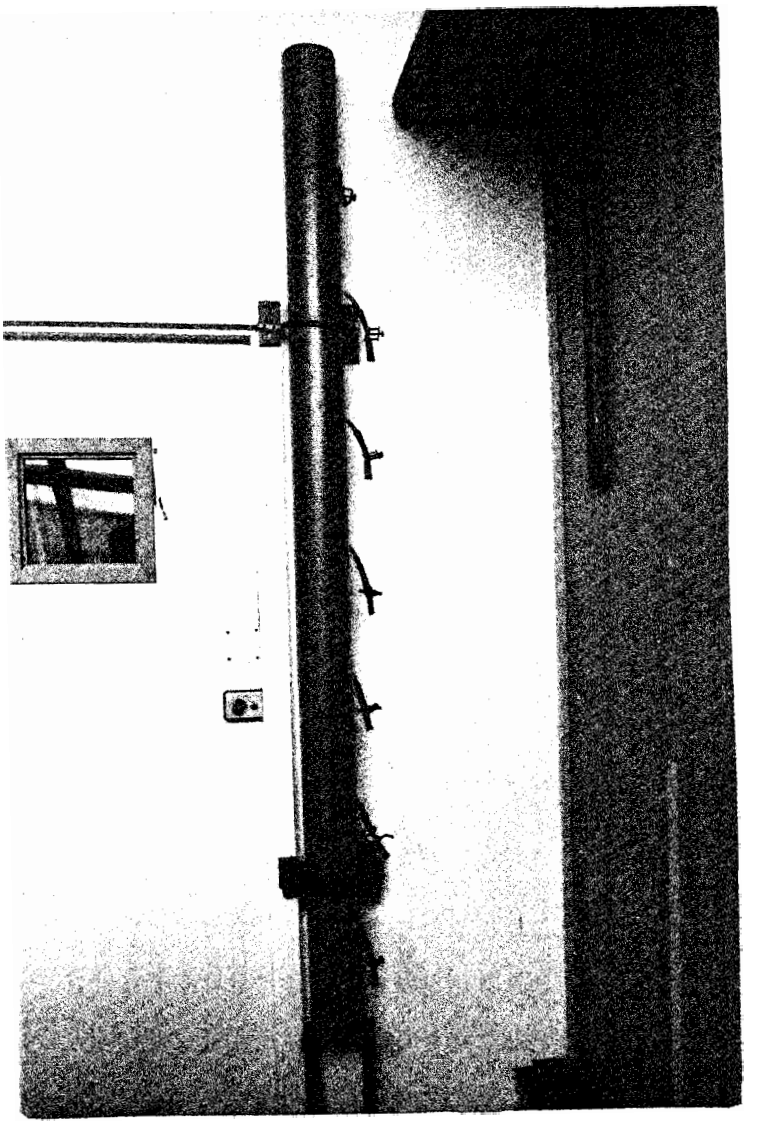
A. Introduction

As discussed in the general introduction the practice of sewage treatment in Britain requires mechanical pretreatment and sedimentation with a minimum two hour retention period at maximum flow (Mosey 1979). This minimum retention is not always achieved; seasonal variations (e.g.) snow and heavy rain, can overload facilities, particularly where many of the sewage treatment plant systems were designed to cope with smaller volumes of sewage than they now receive. When sewage input is 6 times greater than dry weather flow plants are permitted to discharge untreated waste. Clearly, retention periods are often inadequate to permit effective sedimentation. Kruse (1962) recommended 2 hours sedimentation for sewage, although Cram (1943) reported that hookworm ova and amoeba cysts had not all settled in 3 hours. Bush (1960) stated that all Ascaris eggs settled out in 1 hour.

B. Methods

(1) Apparatus:- Figure (4) shows a laboratory sedimentation column which was designed to determine settling times for helminth ova in sludge. A 1.75 metre length of PVC pipe, 10cm diameter, was used to construct the settling column. Holes of 1cm diameter were drilled along the pipe at 25cm intervals. Into these were inserted tuberculin syringe cases to reach the centre of the pipe. The cases were fixed to the outer surface of the pipe with an epoxy resin

Figure 4 The Sedimentation Column



adhesive. Polyethylene tubing was then inserted and glued into each syringe casing. A length of flexible rubber tubing with an attached screw clip was then fitted over each piece of polyethlyene tubing so that samples could be drawn off as required from any outlet along the main pipe.

The column base was sealed by glueing a section cut from PVC sheet to one end of the pipe. An aspirator tap was inserted into the base and the column was attached to a wall support as shown in figure (4).

The column volume was 13.7 litre with a volume of 1.7 litre between each outlet.

(2) Procedure:- Sewage sludge was collected from the primary settling tanks of a local sewage treatment plant. The sludge was adjusted by dilution to contain a total solids concentration equivalent to the sewage treatment works' input, viz: 0.5 gram/litre. (0.75% volatile, Stokes personal communication).

The column was filled with diluted sludge and 10ml of a suspension containing 1.5×10^5 Ascaris or Taenia ova was layered onto the surface of the column. The inoculum was gently stirred into the top layer in order to prevent surface tension forces holding eggs at the surface.

Samples were taken from each outlet at intervals over 6 hours. To take a sample the screw clip closing the outlet was released and the first 20ml taken was discarded to compensate for the dead volume of the tube and syringe casings. The next 20ml was retained as the sample and examined in the modified Fenwick counting chambers for which construction and use are described in appendix 2 .

C. Results

The settling rates which were determined for Ascaris and Taenia ova during 5 separate experiments are shown in tables 10a and 10b, where settling rates are expressed as metre per hour for each outlet with time. From a comparison of these tables it is clear that Ascaris ova sediment faster than Taenia ova; for example, Ascaris ova were recovered from the 1 metre outlet in 0.25 hours, but 3 hours elapsed before Taenia ova were recovered at this depth. A trend towards a decline in settling rate was observed. both species' ova initially settled rapidly (8.3m/hr for Taenia ova and $\leq$ 25m/hr for Ascaris ova) but after the first hour the rate had dropped to 0.5 m/hr for Taenia ova and 1.25 m/hr for Ascaris ova. In order to follow this trend the average settling rates at different depths are presented graphically in figure 5, which show that Ascaris ova appeared to settle at a rate which initially increased with depth, but after the ova had travelled through a distance of about 0.75 metre, the average rate started to decline, and continued to do so until the maximum depth of the column was reached. Taenia ova showed a more consistent decline in settling rate, without the initial increase which had been observed for Ascaris ova. The decline in settling rates could be accounted for by the settlement of other particles present in the sludge which would therefore increase the viscosity of material through which ova fall. This would also explain the observed nonuniform settlement of ova.

In order to make the trends in sedimentation more clear, the

Settling Rates for Taenia ova in sludge:

expressed as metres per hour

Outlet number	I	II	III	IV	V	VI	VII
distance (metre)	0.25	0.5	0.75	1.0	1.25	1.50	1.75
time (hour)							
0.03	8.3						
0.08	3.1						
0.17	1.4						
0.25	1.0						
0.33	0.75	1.52	2.3				
0.42	0.6						
0.50	0.5						
0.58	0.43	0.86					
0.67	0.37	0.75					
1.0	0.25	0.5	0.75				
3.0	0.08	0.17	0.25	0.3	0.4		
4.0	0.06	0.13	0.2	0.25	0.3	0.38	
6.0	0.04	0.08	0.1	0.16	0.2	0.29	

Results consist of combined and averaged data from five separate trials.

Settling Rates for Ascaris ova in sludge:

expressed as metres per hour.

Outlet number	I	II	III	IV	V	VI	VII
distance (metre)	0.25	0.50	0.75	1.0	1.25	1.50	1.75
time (hour)							
0.03	8.3	16.6	25				
0.08	3.1	6.3	9.4				
0.17	1.4	2.9	4.4				
0.25	1.0	2.0	3.0	4.0			
0.33	0.75	0.78	2.3	3.0			
0.42	-	1.2	1.8	-	2.9		
0.50	-	1.0	1.5	2.0	2.5		
0.58	-	0.86	1.3	1.7	2.2		
0.67	-	-	1.1	1.5	1.86	2.2	2.6
1.0	-	-	0.75	1.0	1.25	1.5	1.75
3.0	-	-	-	0.3	0.4	0.5	
4.0	-	-	0.2	0.25	0.3	0.38	
6.0	-	0.08	0.1	0.16	0.2	0.25	

Results consist of combined and averaged data from five separate trials.

FIG: 5

CHANGE IN SETTLING [illegible] WITH DEPTH FOR OVA

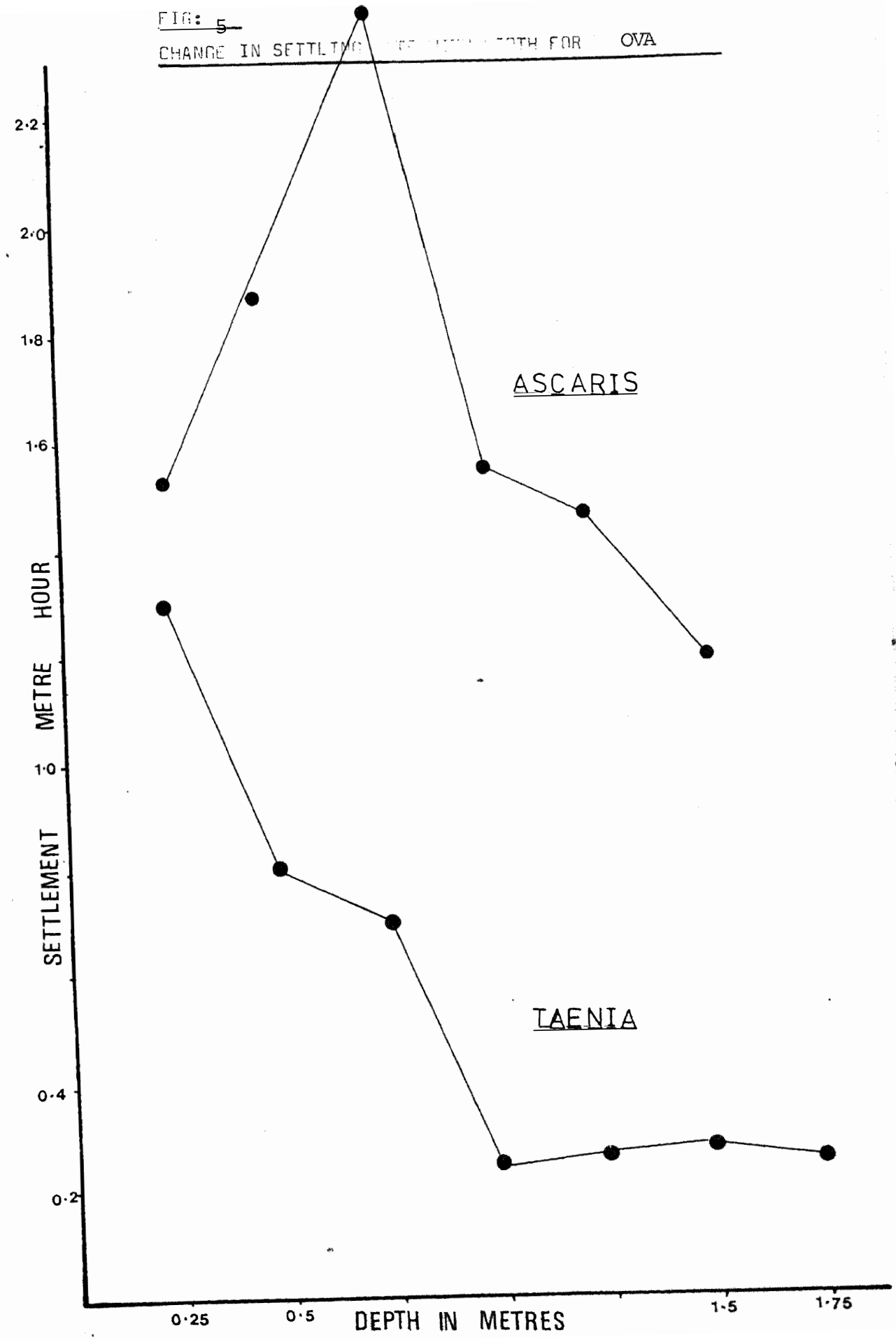


FIG: 6a

CHANGE IN SETTLING RATE WITH TIME FOR TAENIID OVA

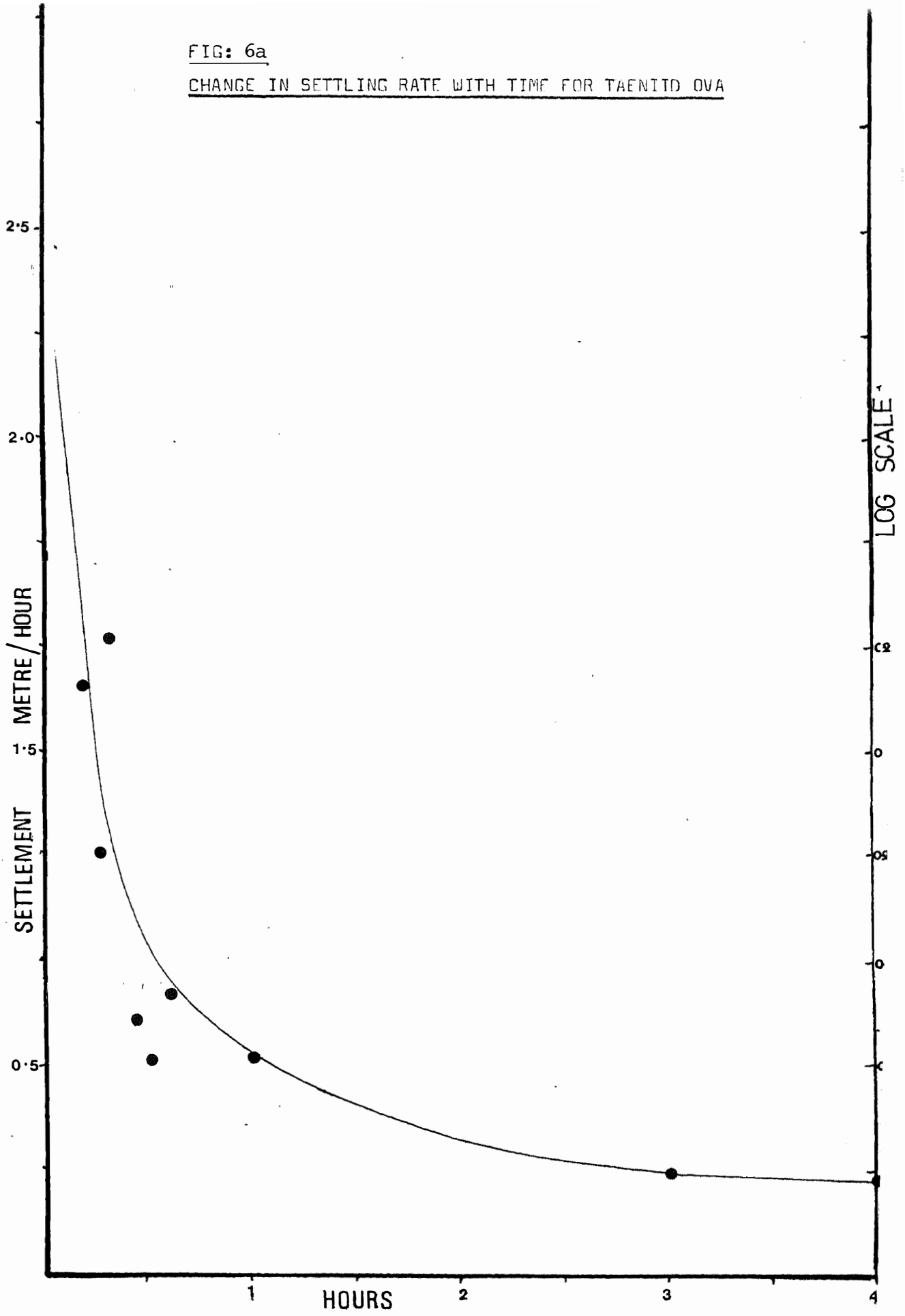


FIG: 6b

CHANGE IN SETTLING RATE WITH TIME FOR ASCARID OVA

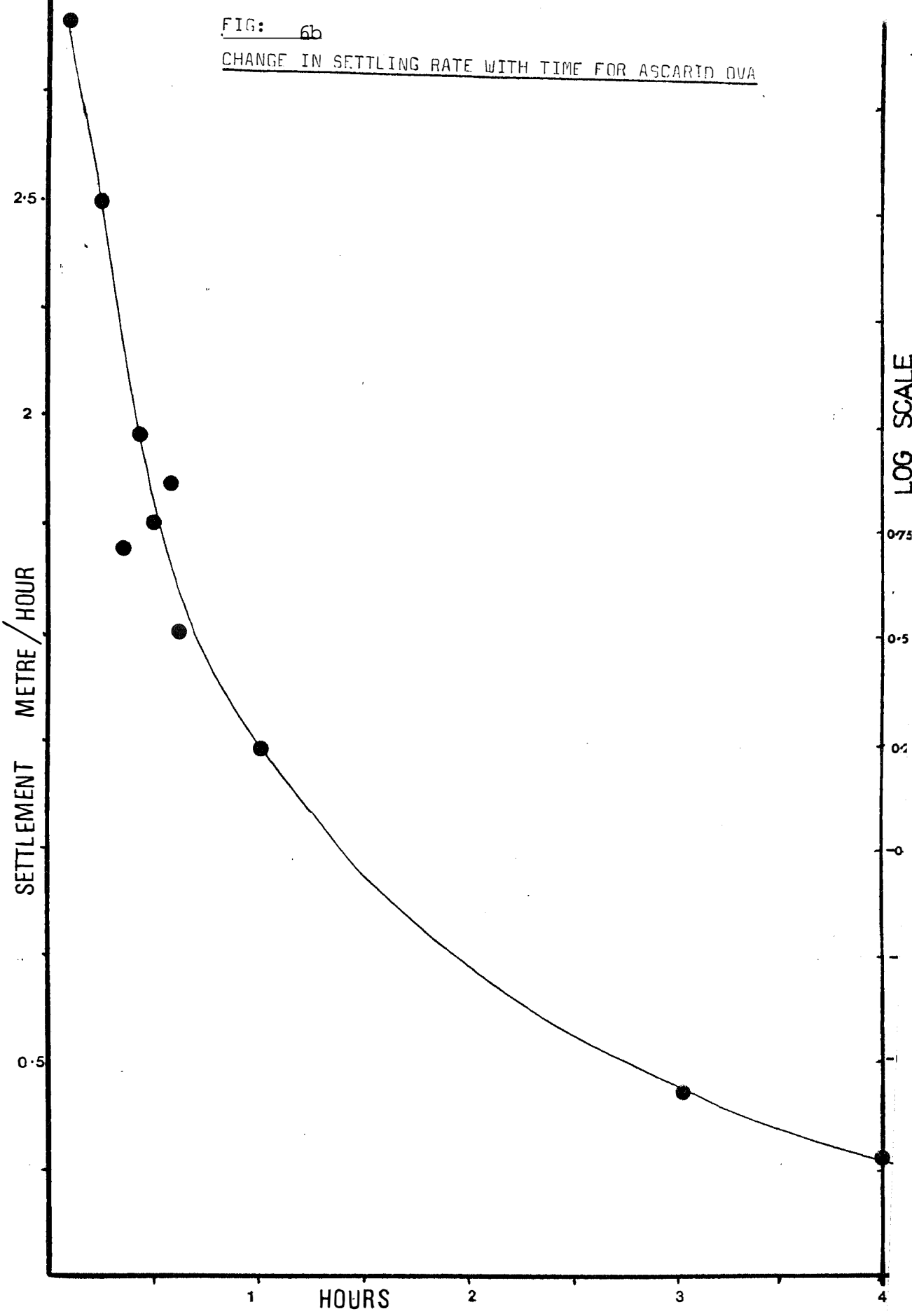


Table (11à

Settlement of Ascaris ova in sludge.

Percentage of inoculum per ml recovered.

Outlet number distance (metre) time (hour)	I 0.25	II 0.5	III 0.75	IV 1.0	V 1.25	VI 1.50	VII 1.75
0.03	79	43	18	-	-		
0.08	61	27	20	-	-		
0.17	53	38	38		-		
0.25	24	33	71	75	-		
0.33	12	33	45	25	-		
0.42	-	29	76	-	33		
0.50	-	16	48	63	25		
0.58	-	22	43	35	25		
0.67	-	-	26	15	54	25	57
1.0	-	-	10	36	24	39	30
3.0	-	-	-	-	80	48	82
4.0	-	-	-	100	38	57	66
6.0	-	-	1	9	45	65	75

Results consist of combined and averaged data from five separate trials.

Table (11b)

Settlement of Taenia ova in sludge:

Percentage of Inoculum per ml recovered

Outlet number distance (metre)	I 0.25	II 0.50	III 0.75	IV 1.0	V 1.25	VI 1.50	VII 1.75
Time (hour)							
0.03	12						
0.08	17						
0.17	8						
0.25	7						
0.33	5	0	1.7				
0.42	12						
0.50	8						
0.58	25	2					
0.67	35	12					
1.0	82	35	5				
3.0	23	50	75	84	25		
4.0	-	25	83	88	70	30	
6.0	10	-	-	18	37	55	10

average rates of settlement over the time period of the experiment are also presented graphically. The settling pattern of Taenia ova with time (figure 6a) shows an initial rapid rate of settlement which after the first hour decreased sharply until after 3 hours a rate of approximately 0.25 metre per hour was maintained. Ascaris ova settled at a more rapid rate than did Taenia ova, and the settling pattern for Ascaris ova (figure 6b) showed no levelling off in settlement rate within the time period of the experiment.

The concentration of each species' ova initially present in each ml of sludge and in each ml of recovered sample is known. From these data an average settling rate can be determined. The average number of ova recovered in a sample is multiplied by the time the ova take to reach the outlet from where the sample was taken. These, "egg-hours" are totalled and divided by the total ova per ml recovered, assuming that only insignificant numbers of ova go undetected. The result is an estimate of the time taken for the "average egg" to travel a known distance. From these data, summarised in tables 11a and 11b , for ova settling in sludge containing 0.5 g/l total solids, Taenia ova settled at approximately 50cm/hr and Ascaris ova at approximately 120 cm/hr.

D. Discussion

The results obtained are consistent with known specific gravities of Ascaris and Taenia ova. The specific gravity of Taenia ova was found to be 1.15 and that of Ascaris ova to be 1.23. The specific gravity values for Ascaris ova however, vary with the stage of development. Fertilized ova may have an S.G. = 1.10 while unfertilized can have a value of 1.25 (Sawitz 1942). In a mixed

population of ova therefore, they will be settling at different rates. These facts could explain the nonuniform settling behaviour observed, for example, initial recoveries of low density ova followed by recoveries of higher density ova could account for the apparent increase in rate of settlement of Ascaris ova.

Edge effects altering the settlement of ova falling near to the sides of the column should have been negligible, since the syringe casings through which samples were drawn protruded into the centre of the column. During sedimentation however, there would also be upward flow rates for settling sewage particles to be overcome (Newton 1949). These upward flow rates would have most effect on the low density ova. Newton also stated that primary sedimentation would remove 98% of Taenia ova in 2 hours, but his studies referred to settlement in an 18 inch deep column. Liebmanns (1964) reported a 68% removal of Taenia ova in 2 hours and 89% removal after 3 hours but gave no details of depths involved.

Although Ascaris ova which settle fasters are more efficiently removed from sewage it is clear that these ova are now concentrated in sludge and must therefore be inactivated if this sludge is to be used in agriculture. Primary sedimentation is unlikely to remove all Taenia ova into sludge, according to the results obtained in this experiment. The conditions inside the column would have been conducive to rapid settlement, with a minimum of the turbulence which can reduce the efficiency of the sedimentation stage of sewage treatment. Liebmann (1964) has stated that ova of specific gravity= 1.1 should settle at 2ft - 3ft/hour (61cm - 91cm/hour) in

static conditions. In this study, Taenia ova (specific gravity 1.15) did not settle so rapidly; however, there would certainly be sufficient ova concentrating in the sludge to be a problem if such sludge were applied to land without storage and/or treatment. If the effluent from settling tanks were used to irrigate agricultural land it is highly probable that such effluent would contain Taenia ova and could potentially expose cattle to the risk of bovine cysticercosis.

EVALUATION OF VIABILITY CRITERIA FOR HELMINTH OVA

A: Introduction

Although helminth ova may be recovered from land which has been treated with sewage sludge this need not imply that such eggs would be infective to cattle. To establish the potential risk presented by these ova it was necessary to have a laboratory method to determine egg viability.

In vitro methods for distinguishing between viable and nonviable ova can be divided into three categories, namely: direct examination, staining and activation.

Direct examination can be used for certain species such as *Ascaris lumbricoides* which has an infertile ovum which is morphologically distinct from the viable ovum whereas *Taenia* species have ova with thick, radially striated walls which make visible examination unreliable for distinguishing between viable and nonviable ova unless the latter are grossly distorted.

Staining is a well established method for distinguishing between dead and live ova. Shepherd (1962) used "new blue R" to stain a variety of nematode ova and larvae. Le Jambre, Crofton & Whitlock (1970) used acridine orange to determine hatchability of nematode ova.

Ova of *Taenia* species are particularly difficult to stain, since the already dark embryophore must be digested before stain uptake is evident.

Hatching and activating agents which, in contact with ova stimulate them to develop into larvae, may also be used to distinguish between viable and nonviable ova. *Ascaris* ova may be hatched simply

by incubation as demonstrated by Tarczynski (1963a In this method ova are incubated at 35°C - 37°C for 10 - 14 days in distilled water. Essentially the same technique was used by Alicata (1934) and by Pitts (1948) except that lower incubation temperatures of 23°C - 25°C were used and 2 percent formalin replaced distilled watera

The activation of Taenia ova requires specific "hatching" solutions, the term "hatching" being used here to refer to the disintegration of the embryophore. It is only after this stage that the embryo is activated to tear through the onchospheral membranea The method used by Silverman (1954) to activate Taenia ova requires a pretreatment of incubating in acidified pepsin solution whichawill dissolve the embryophore. Following this stage, the embryos are stimulated by specific hatching solutionsa

In this study it was decided to evaluate staining and activation techniques rather than direct examination. The inherent difficulties presented by the latter method rendered it unsuitable for use with samples containing much particulate debris of the same size as the ova.

B: Methods

(i) Activation: Prior to activation washed Taenia ova were incubated in acidic pepsin solution made by dissolving 1 percent pepsin in a 1 percent solution of hydrochloric acid (1 ml of concentrated HCl made up to 100ml with normal saline). Approximately 50 eggs per bijou bottle were used. After 90 minutes incubation at 37°C the ova were transferred to an activating solution containing 1 percent pancreatin which, in the presence of bile, is known to affect the permeability of the onchospheral membrane (Silverman 1954). A comparison of this method used by Silverman, and a later modification described by Heath and Smyth (1970) is made in table (2). Heath and Smyth used a shorter pretreatment time and a higher concentration of bile,

Table (12s)

Method of Silverman		Method of Heath & Smyth
90 min in acid pepsin		60 min in acid pepsin
Pancreatin	1%	1%
Bile Salts	1%	-
Na HCO_3	1%	1%
Cholesterol	0.04%	-
Trypsin	0.04%	-
Bile	-	5%

Table (13)

Response of Ova to Vital Stains

Percent ova viable	0	50	75	100
Stain	Percentage Stain Uptake			
0.1% Trypan Blue	0 0 0 S = 0 S.E.= 0	17 21 17 S = 5.8 S.E.=6.57	38 36 34 S = 3.5 S.E. = 3.96	86 78 77 S = 7.6 S.E.=8.6
0.1% Meldola's Blue	0 0 0 S = 0 S.E.= 0	21 19 19 S = 2.3 S.E.=2.6	47 47 46 S = 0.58 S.E.= 0.66	74 73 75 S = 1.5 S.E.= 1.69
0.1% Neutral Red	76 72 76 S = 6.9 S.E. = 7.81	1 79 55 S = 55.8 S.E. > 3000	24 33 31 S = 7.9 S.E. = 8.94	36 29 35 S = 6.9 S.E. = 7.81

(ii) Staining: To obtain Taenia onchospheres for staining, eggs were teased from gravid proglottids and kept in sterile saline at 4°C. Heath and Smyth (1970) added antibiotic solutions as they required bacteria-free suspensions for ova to develop into cystic larvae. As this study was concerned with separating ova from samples with very high microbial burdens it was unnecessary to treat the suspensions of ova with antibiotics.

Ova were subjected to acid-pepsin treatment and artificial digestion as described by Silverman (1954)t A series of suspensions containing 10 000 ova, each suspension having a known proportion of fresh to killed ova was prepared. The dead ova were obtained by exposure to 56°C for 10 minutes, sufficient to kill ova without causing obvious physical damage. To each series was added a different vital staint Trypan bluet Meldola's blue and neutral red. From a preliminary trial in which known viable eggs were stained it was found that concentrations of 0.1 percent were most suitable. Below this concentration eggs did not stain, and higher concentrations made eggs difficult to distinguish from their background.

C: Results

As can be seen from table (3) Meldola's blue stain at a concentration of 0.1 percent in aqueous solution showed the closest correlation to the actual proportions of heat-inactivated ova in the suspensions. One anomalous result was obtained with 0.1 percent neutral red stain. A suspension containing dead and live ova in the ratio of 1:1 failed to stain ova in one trial.

Ova subjected to digestion and then placed in either of the two hatching solutions showed visible darkeningt A maximum response of only about 30% was achieved with these ova.

D: Discussion

Using the technique of vital staining it was observed that once the thick, outer striated shell of Taenia ova was broken down by the pepsin solution the membrane underneath took up stains over a 1 - 2 hour period. Dead ova stained darkly instantaneously indicating a complete loss of the semipermeable nature of the membranes The proportion of stained eggs in each series of trials correlated with the proportion activated in hatching solutionss Eggs from batches which failed to respond to stains also failed to show signs of activations

When Taenia ova were treated by the activation method described by Silvermans hooks became visible in a few embryos after 60 minutes activation in hatching solution. Even after 90 minutes only about a third of the ova showed any signs of development. No significant differences in efficiency in promoting activation were noted between the two methods. Ova which failed to show activation also showed no response to stainings

The low apparent viability of the experimental method could be accounted for by the difficulty in obtaining very fresh ova. Since response to staining seemed to be the most practicable of the two methods this technique was selected for use in examining eggs recovered from field trials.

Eggs which are apparently viable are not necessarily also infective. Calf-feeding eperiments were therefore initiated to establish infectivity of ova.

SURVIVAL OF HELMINTH OVA STORED IN SLUDGE

A. Introduction

Survival periods for helminth ova in sludge have been investigated by many workers in the field of effluent disposal. Their results indicate that of the helminth ova present in sewage sludges, Ascaris ova survive longest under all conditions. Cram (1943) for example, recovered viable Ascaris ova from drying sludge after 170 days and Schatzle (1969) found that both Ascaris and Taenia ova were viable after 7 months in sludge, being destroyed finally only by composting. Alexandre et al (1975) were able to recover viable Ascaris ova from sludge which had been treated with lime in order to destroy bacteria and less resistant ova.

In the present study it was important to know for how long ova of intestinal parasites could persist in sludge in order to establish the potential risk of disease transmission when such sludges are applied to land.

B. Method

Two experiments were set up; the first to compare the persistance of three different helminth ova after storage for different lengths of time in raw sludge, and the second to investigate the effect on the viability of Taenia ova, of storage in three different sludge types.

(a) Storage in raw sludge. For the first trial raw sludge was collected from the primary settlement tanks of a local sewage

treatment plant and was passed through an 8mm geological sieve in order to remove rags and other coarse debrise The sludge was analysed by the methods described in the Department of the Environment handbook and was found to contain 3.0% total solids and to have a pH value of 6.0e The sludge was inoculated with 1.7×10^4 Taenia ova, 9×10^3 Ascaris ova and 6×10^3 Toxocara canis ova per litre. After thorough mixing the sludge was divided into 100ml aliquots so that each container held 1700 Taenia ova, 900 Ascaris ova and 600 Toxocara ova. Samples were taken each month and the containers were discarded after sampling.

(b) Storage in raw, digested and e"cake"esludge: A second trial was set up to examine the persistance of Taenia ova stored in three different sludge types. The raw sludge and dried "cake" sludge, consisting of mixed raw and activated sludge treated with lime and copperas ($FeSo_4$), dried and filter pressed, were obtained from the East Hyde Sewage Works, Harpenden. The digested sludge came from a conventional anaerobic treatment system at the Maple Lodge Sewage Works, Rickmansworth. The dried "cake" sludge was crumbled and mixed to a slurry with tap water so that it could be inoculated in the same way as the raw and digested sludges. All sludges were divided into 100ml aliquots, each containing 2×10^3 Taenia ova.

Samples were taken each week, the containers being discarded after sampling. Recoveries of ova were made by the previously described antiformin-sucrose method, taking 16 replicates of each sample in order to achieveea decimal accuracy of 0.05 (Southwood 1966)e

C. RESULTS

(i) Experiment 1.

Recoveries of ova, expressed as a percentage of the initial inoculum recovered are given in table 14 , and as a semi-logarithmic graph of absolute numbers of ova recovered, in figure 7 . Taenia and Ascaris ova were still present in low numbers after 12 months storage in sludge, but Toxocara ova could not be detected in sludge sampled after 5 months. The gradual disappearance of ova presumably resulted from degradation of ova. Alternatively the long storage in sludge may have made these ova more susceptible to osmotic damage during the extraction of ova from sludge by the antiformin-sucrose method. Ascaris ova appeared to be the least affected by storage, with 9.2% of the Ascaris ova being recovered intact after a year stored in raw sludge. The high (93%) recovery of Toxocara ova in the second month is attributed to the breakdown of clumps of ova which had not been evenly distributed in this sample.

The regression equations plotted in figure 7 show that the plot of recovered Toxocara ova had the largest negative gradient, indicating that of the three species the numbers of Toxocara ova declined the most rapidly. A modification of Student's t test was carried out to establish whether the gradient of each regression line was significantly different from zero.

for Taenia ova $t = 5.952$, [significant at a probability level of 1%]

for Ascaris ova $t = 3.259$, [significant at a probability level of 5%]

for Toxocara ova $t = 3.465$, [significant at a probability level of 1%]

FIG: 7

LONGEVITY OF DIFFERENT HELMINTH OVA STORED IN RAW SLUDGE

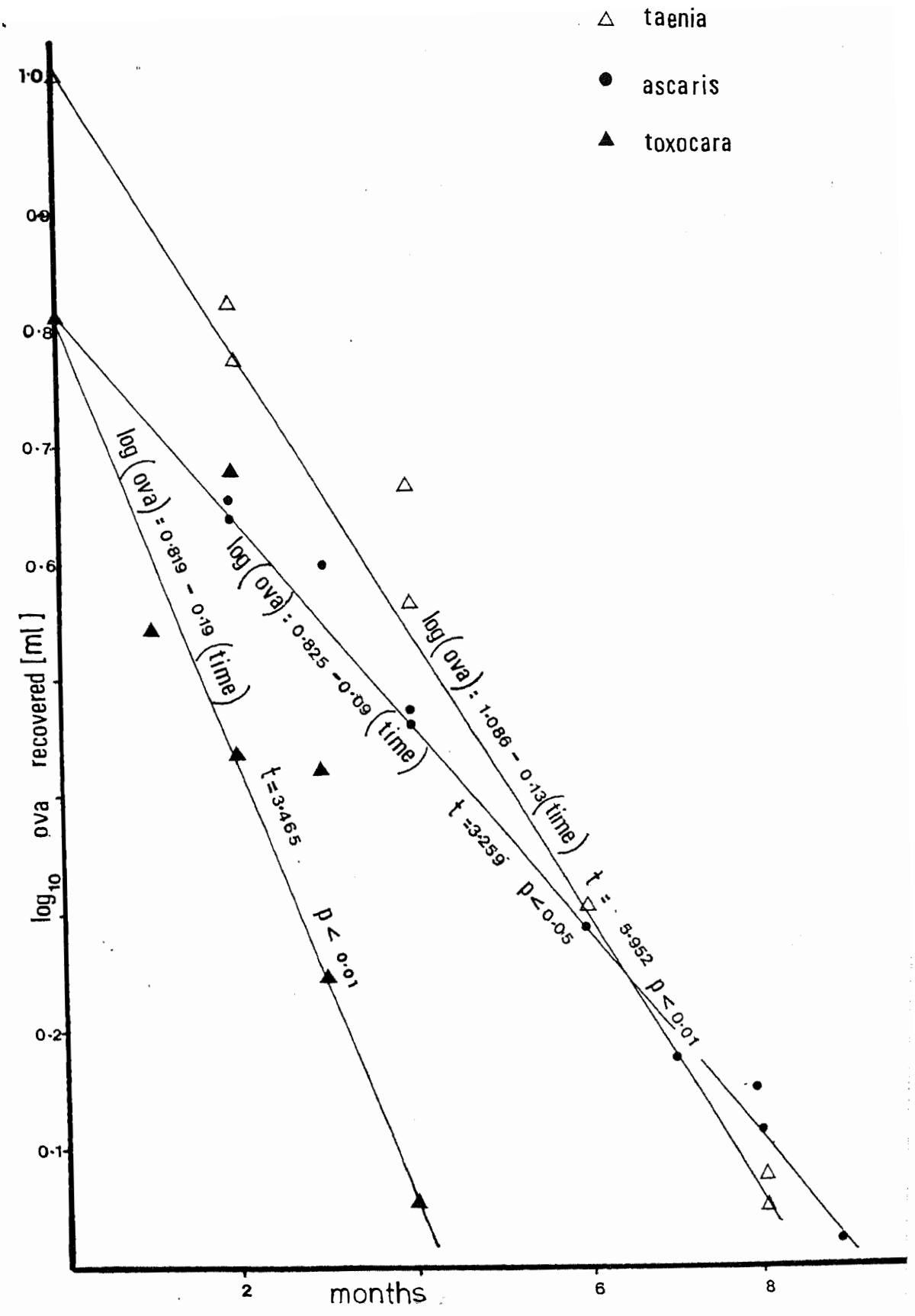


Table 14

Percentage of initial inoculum recovered from raw sludge				
Storage Time (months)	Mean Temperature (°C)	Ova recovered		
		Taenia	Ascaris	Toxocora
1	15.1	70.6	67.3	66.7
2	14.2	48.7	67.2	93.0
3	11.8	36.1	59.1	51.2
5	6.0	38.1	44.5	13.1
7	6.1	15.2	22.7	-
8	5.0	9.7	20.9	-
9	8.7	8.6	15.4	-
10	11.3	6.1	11.8	-
11	14.8	4.1	10.0	-
12	14.6	2.5	9.2	-

Since the calculated values for t exceeded those derived from t tables, all gradients show a statistically significant difference from zero.

(ii) Experiment 2.

Recoveries of Taenia ova from the three sludge types are presented in the form of semi-logarithmic graphs in figures 8a , 8b and 8c . An attempt was made to distinguish between viable ova recovered and totalrecoveriest Uptake of Meldola's Blue Stain was taken as the criterion of viabilityt as described in the previous section.

To determine whether or not there was any statistically significant difference in survival of Taenia ova stored in each sludge typet a t test of comparison was carried out, at a minimum probability level of 5%;

For "cake"	vs	digested sludge	t = 2.16 (significant)
For "cake"	vs	raw sludge	t = 2.46 (significant)
For digested	vs	raw sludge	t = 2.05 (not significant)

Survival of Taenia ova in "cake" sludge was therefore significantly different from survival in raw or digested sludge. It is interesting that total recoveries of Taenia ova did not diminish significantly in "cake" or digested sludges, but declined at a significant rate in raw sludge.

D. DISCUSSION

Of the ova used in this experiment, Toxocara ova persisted for the shortest time during storage in raw sludge, and none could be detected in any samples after 5 months. Taenia ova were recovered over a longer period, and a 2.5 percent recovery of ova was achieved

FIG: 8a

RECOVERY OF TAENIID OVA FROM SEEDED RAW SLUDGE

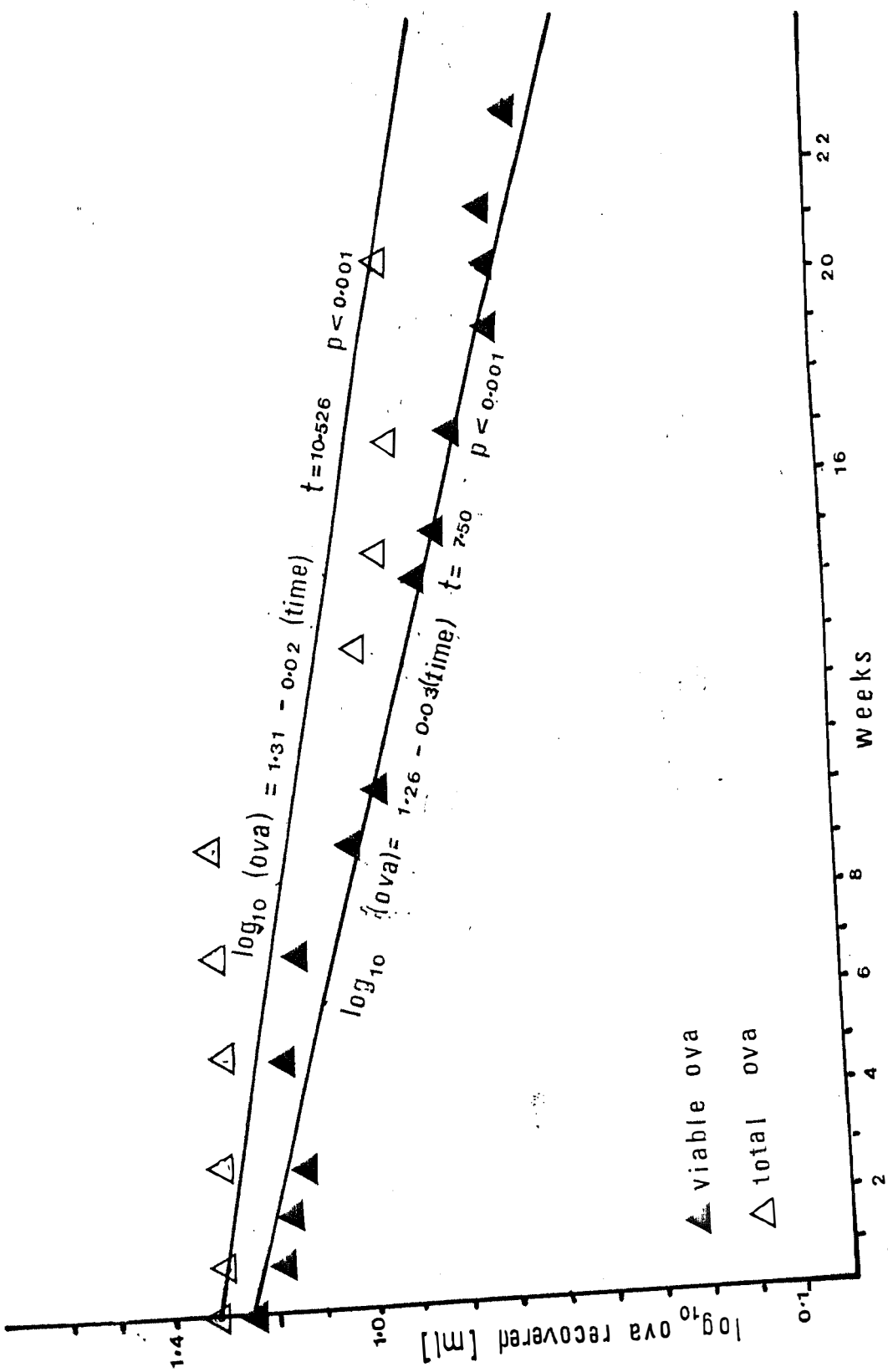
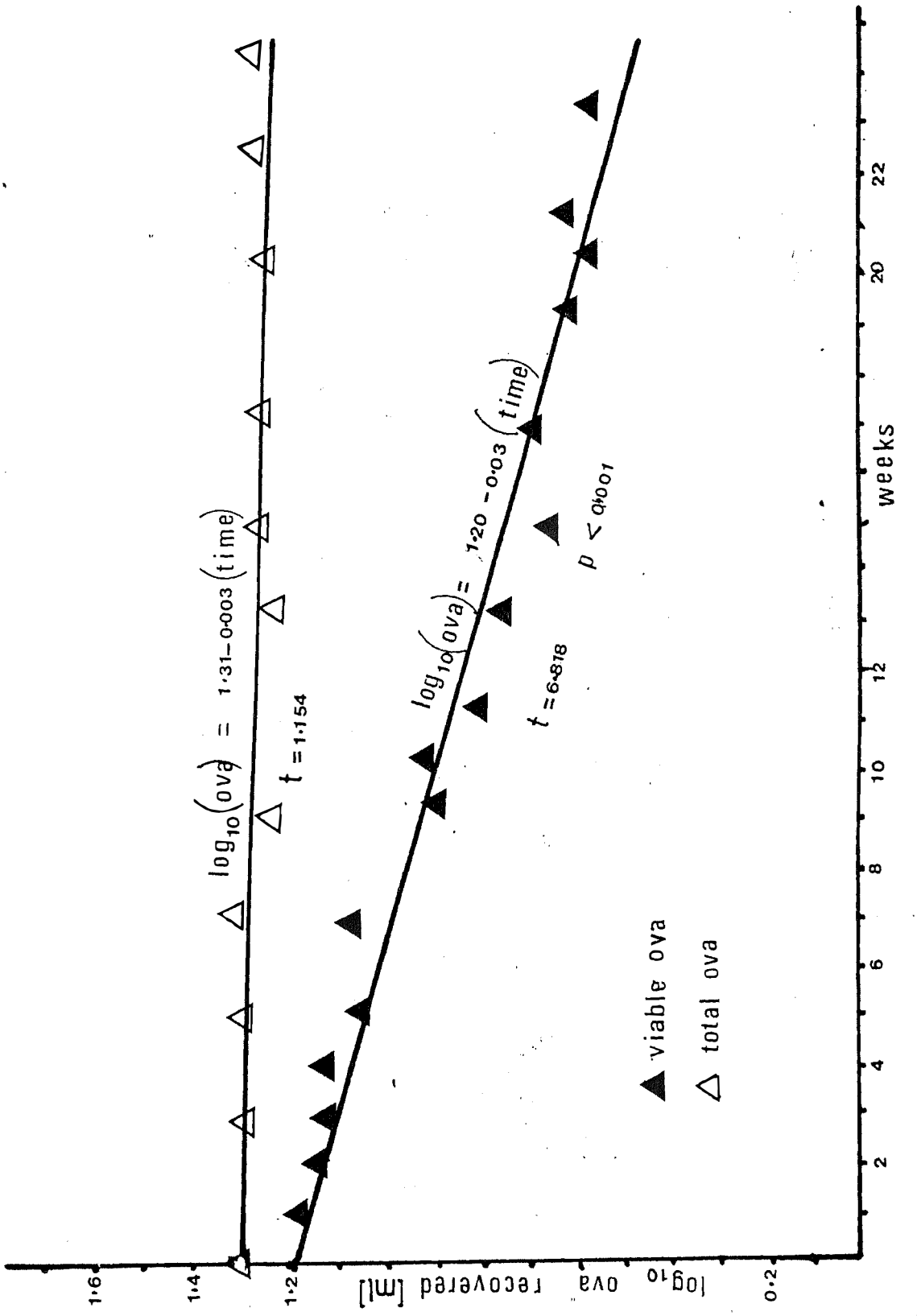
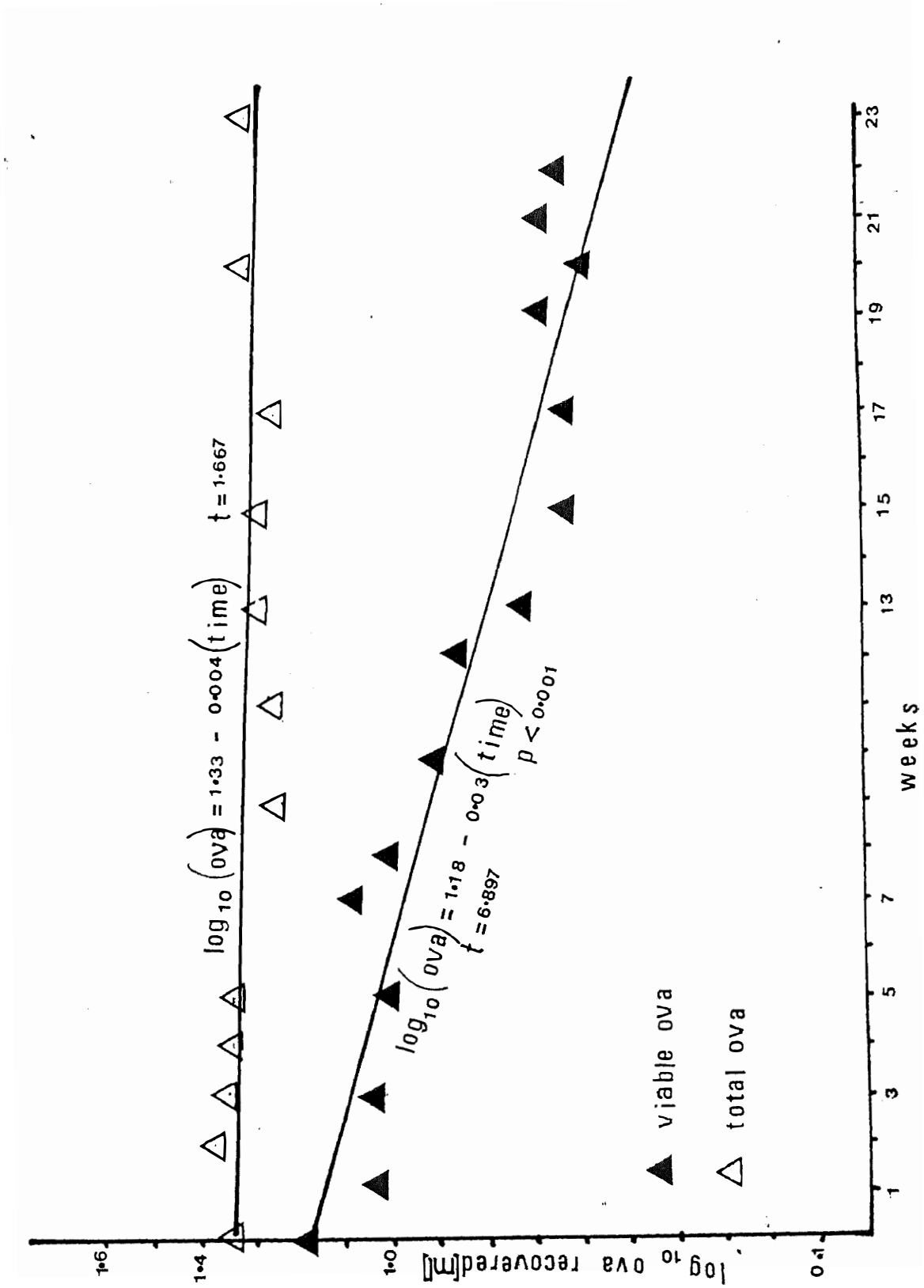


FIG: 8b

RECOVERY OF TAENIID OVA FROM SEEDED DIGESTED SLUDGE




log10 (ova) = 1·33 - 0·004 (time) t = 1·667
log10 (ova) = 1·18 - 0·03 (time)
t = 6·897
p < 0·001
viable ova
total ova
log10 ova recovered[ml]
weeks
1·6
1·4
1·0
0·1
1
3
5
7
13
15
17
19
21
23

after 12 months, which was the maximum storage period. Ascaris ova appeared to withstand storage with the least damage; after 12 months storage a 9.2 percent recovery of ova was achieved. These recoveries were for total numbers of ova recovered, so in the second experiment an attempt was made to distinguish between total recoveries and recoveries of viable ova.

For the second experiment figure 8 shows the regression lines for both viable and total recoveries of Taenia ova. The closeness of these regression lines indicates that loss of viability was a gradual process. The distinction between viable and total recoveries of Taenia ova from digested sludges and from dried "cake" sludge is more pronounced. It was deduced from these observations that Taenia ova survive better in untreated, than in treated sludge.

Ova were found to persist several months even in treated sludges, and although more rigorous treatments (e.g.) heat or irraditation would completely remove ova, these are often impracticable. Forstner (1970) for example, reported that 80 days - 90 days ensilage (composting) killed Taenia ova. Silverman (1956) reported that 10 minutes exposure to a temperature of 59°C would kill Taenia ova. Thermophilic digestion is a costly process however, and only cold digestion and mesophilic digestion is used in British sewage treatment plants.

Since the most effective treatments are too slow or expensive for practical application the only feasible alternative for minimising the risks to livestock must centre on the time between treatment and the release of cattle onto the land.

THE EFFECT OF ANAEROBIC, MESOPHILIC DIGESTION ON THE SURVIVAL OF HELMINTH OVA IN SLUDGES

A. Introduction

All sewage treatments are designed to remove suspended solids, including helminth ovat Most plants follow sedimentation by some form of aerobic digestion using trickling filters or activated sludge processes. In a few plants this is augmented by anaerobic digestion, which breaks down organic compounds to carbon dioxide and methane gases, thereby reducing the bulk of the sludge, and destroying many of the pathogens in sludge. A considerable body of evidence howevert exists to show that ova of intestinal parasites can survive this latter sewage treatment process. Cram (1943) found that viable Ascaris ova could be recovered from sludges subjected to 12 months anaerobic, mesophilic (up to 37°C) digestion. Rudolfs et al (1950) found that Ascaris ova survived 3 months of sludge digestion and their results were confirmed by the findings of Fitzgerald and Ashley (1977). The ability of Taenia ova to withstand sludge digestion is less well documented. Newton et al (1949) found that 10% - 15% of Taenia ova were not affected by 203 days in an anaerobic digester. The viability criteria used however was microscopic examinations of ova and it is therefore possible that at least some of the apparently unaffected ova may have being nonviablet Silverman and Guiver (1960) reported that Taenia ova subjected to 5 days mesophilic digestion could not be activated by their acid-enzyme treatment which is described in a previous section. These latter results, which conflict with the findings of

many other workers, might have been due to differences in digestion conditions or design of digesters.

B. Methods

Two 5 litre batch feed continuous digestion vessels containing 4 litre of sludge were set up at an operating temperature of 35°C and with a daily feed of 400ml of raw sludge. The retention time was therefore 10 days. The digesters were inoculated with 7×10^4 Ascaris ova and 3.2×10^4 Taenia ova. Daily checks on pH and gas production were made to ensure the digesters were operating efficiently, the gases being collected and measured by water displacement.

A second series of small scale batch digesters was set up. Each vessel contained 150ml of sludge; 50ml of raw sludge and 100ml of digested sludge as the "seed" containing an established population of a diverse collection of fermentative bacteria. The digesters were inoculated with 3.5×10^4 Ascaris and 1.6×10^4 Taenia ova and then incubated at 35°C. Sampling from the 4 litre batch feed digesters was achieved by drawing off 400ml of sludge each day before feeding with fresh sludge. Subsamples were taken from the 400ml and processed by the antiformin sucrose methodrpreviously described.

Sampling from the batch digesters was achieved by subsampling from one vessel daily and discarding the vessel's contents after sampling. Sixteen replicate samples were used for a decimal accuracy of 0.05 (Southwood 1966).

C. Results

ne 4 litre batch feed continuous digestion vessels, Taenia ova were recovered for only 6 dayss Dilution would account for part of this decline in numbers, as 400ml of sludge was removed daily to compensate for the daily addition of 400ml of fresh sludge. A second, and probably more important factor in failure to recover eggs could be the collection of ova in the surface scum at the top of the digester contentss The "sludge crust" is a common feature of digesters and it has been established that micro-organisms concentrate in the scum (Summers et al 1979)s Finally, the stirring mechanism which was designed to prevent pockets of undigested materials, may have been insufficient to provide an even distribution of ova in the digestion vessel, so that samples would not be representative of the digester contents.

Ascaris ova were detected up to 22 days, as might be expected from the higher initial concentration present in the digester. No disintegrating ova were detected prior to their disappearance. Of the eggs unaccounted for, a proportion would inevitably be lost by dilution, and an indeterminable number lodged in the surface scum layer which could not be sampled.

D. Discussion

It is evident that the conditions provided by anaerobic digestion vessels operating at mesophilic temperature are not adequate to completely destroy all helminth ova present in sewage sludges. Digested sludge applied to land may therefore still have risk potential. To destroy all helminth ova, thermophilic conditions

would probably be required; for example Smith et al (1975) reported that all ova in sludge would be killed at temperatures of 55°C and above. In practice however, the heat input required to run a thermophilic digestion system means that it is not an economically feasible method to adopt. Sand-filtration and microstaining treatments are reported to be equally effective in removing ova; Cram (1943) achieved 100% removal of Ascaris and Ancylostoma ova by this method and Newton et al 1949 achieved 100% removal of Taenia ova.

THE INFLUENCE OF TEMPERATURE ON PERSISTANCE OF HELMINTH OVA IN SLUDGE APPLIED TO PASTURE

A. Introduction

Temperature is one of the most important physical parameters influencing survival of ova in the external environment. Sweatman and Williams (1963) found Taenia ova to be still infective after 4 months on pasture under mild (N.Z.) winter conditions, whereas Coman (1975) reported that although Taenia ova survived several months in winter and spring (Australia), their survival time dropped to 2 weeks in summer months. Ova in sludge applied as a fertiliser to pasture are to some extent protected against temperature fluctuations by the insulating effect of the sludge.

In order to examine the effects of temperature on persistance of helminth ova in sludge applied to pasture, inoculated sludge was applied to seed trays at a rate equivalent to large scale commercial applications, and the trays were held at known temperatures. All other physical parameters were controlled. The purpose of these studies was to ascertain under what conditions the numbers of helminth ova would most rapidly diminish.

The significance of the rate of decline in numbers lies in the link between sludge application and disease transmission. There have been cases of parasitic infections of cattle transmitted via vegetation contaminated with untreated sludge, Rickard and Adolph (1977), McPherson, Mitchell and McCance (1978). This link is well defined in the case of Taenia infections where the human host is necessary to the completion of the parasitic life cycle.

From the trials conducted it was hoped to establish the most favourable conditions under which to apply sludge with minimal risk to agriculture.

B. Methods

For the laboratory experiments, seedtrays were filled with turves measuring 0.03675 cubic metre (weight 6.36 kilogram). The turves were allowed several weeks to establish.

Raw sludge was collected from the primary settling tanks of a local treatment plant and passed through an 8mm geological sieve in order to remove rags or coarse debris. The sieving also ensured that the applied sludge could be evenly distributed over the turves. Dry weight analyses were carried out on sludge samples which were found to have a suspended solids content of 4.1 percent.

The analysed sludge was inoculated by adding 5ml of a suspension containing 2.46×10^4 *Ascaris* ova per ml and 5ml of a suspension containing 2.0×10^4 *Taenia* ova per ml, to a 4 litre volume of sludge.

In accordance with the commercial application rate of 8000 gallons per acre, 660ml aliquots of the inoculated sludge were applied to each seed tray. Duplicate seed trays were sealed in double layered polythene and incubated:

(i) at ambient temperature (in a glasshouse on a roof of the building)

(ii) at 5°C (in a temperature-controlled cold room, illuminated by a fluorescent striplight at 1 metre distance from the sealed trays).

(iii) at 25°C (in an environmental cabinet, illuminated by lights at 1 metre distance from the sealed trays).

Five gram samples of sludge and the top layer of soil were taken from the surface of the turves. These were prepared as 10% weight to volume samples in 30% antiformin solution and subjected to extraction by the antiformin sucrose technique previously described. 16 replicate 1 gram samples were taken for a decimal accuracy of 0.05 (Southwood 1966).

C. Results

The data for recoveries of ova from turves held at the three different temperature conditions are represented by semi-logarithmic graphs for each species (figures 9a, 9b, 9c). The larger negative gradients of the plots for recovery of Taenia ova, indicate that ova of Taenia species declined in numbers more rapidly than did the Ascaris ova. To establish that the gradients of the graphs for recovery of each species ova were significant values, a t test of gradients was carried out. All gradients proved to be significant values at a minimum probability level of 0.02.

To compare the rate of decline in numbers of ova recovered, the biological half life for each species ova, under each set of conditions was calculated. This quantity measures the time taken for a 50 percent drop in numbers of ova recovered. At 5°C the biological half life ($t_{\frac{1}{2}}$) for Ascaris ova was found to be 30 days, and for Taenia ova at the same temperature the $t_{\frac{1}{2}}$ was 5.8 days, or (5) times shorter than the half life period of Ascaris ova. At ambient temperature the $t_{\frac{1}{2}}$ for Ascaris ova was 8.3 days and for

Taenia ova was 4.3 days.

Ascaris ova were recovered for up to 60 days at 5°C and Taenia ova were recovered for up to 28 days at this temperature. At ambient temperature both species' ova were recovered for up to 21 days, although Ascaris ova were recovered in greater numbers. At 25°C both species ova were again recovered up to 21 days, but in lower numbers than had been recovered at 5°C.

A statistical comparison of the gradients of the graphs showing recovery of Ascaris and Taenia ova at each temperature was made. At 5°C, ambient temperature and 25°C the calculated t values were 2.16, 0.57 and 0.61 respectively. Only at 5°C was the rate of decline in numbers of Taenia ova significantly greater than for Ascaris ova.

D. Discussion

From these observations it appears that Taenia ova in sludge applied as a fertiliser are reduced in numbers more rapidly than are Ascaris ova. This result might be expected since Taenia ova have a much thinner outer layer than Ascaris ova and so presumably would disintegrate more readily.

The half life difference between the two species was more pronounced at 5°C than at ambient temperature or 25°C.

Commercially, sludge applications are made throughout the year, but with the heaviest applications of up to 20 000 gallons per acre being given during winter months (Bellingham, personal communication). Since ova persist longer under winter conditions

FIG: 9a

RECOVERY OF ASCARID AND TAENIID OVA FROM RAW SLUDGE APPLIED TO TURVES HELD AT 5° C

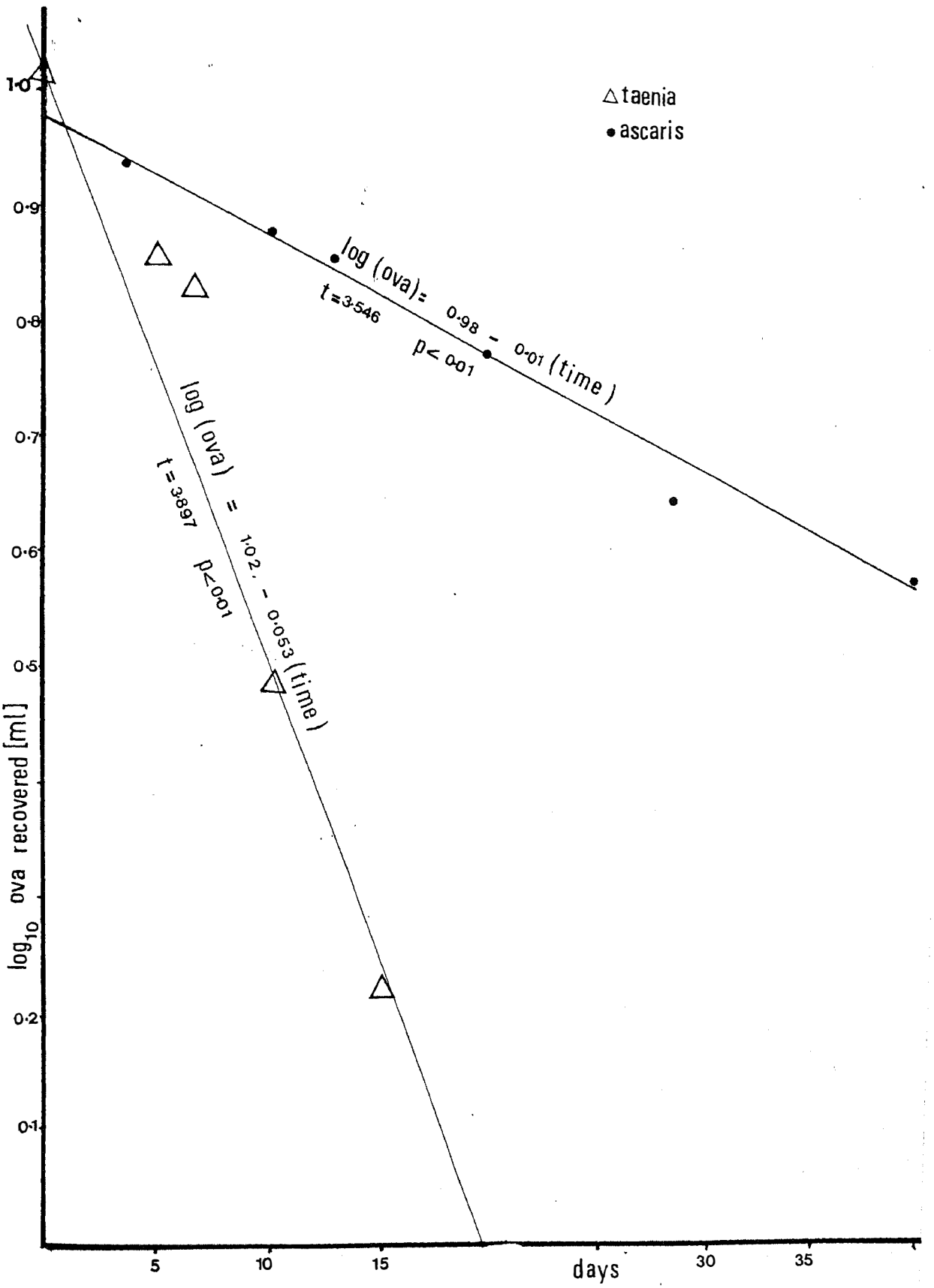


FIG: 9b

RECOVERY OF ASCARID AND TAENIID OVA FROM SEEDED RAW SLUDGE APPLIED TO TURVES HELD AT AMBIENT TEMPERATURE

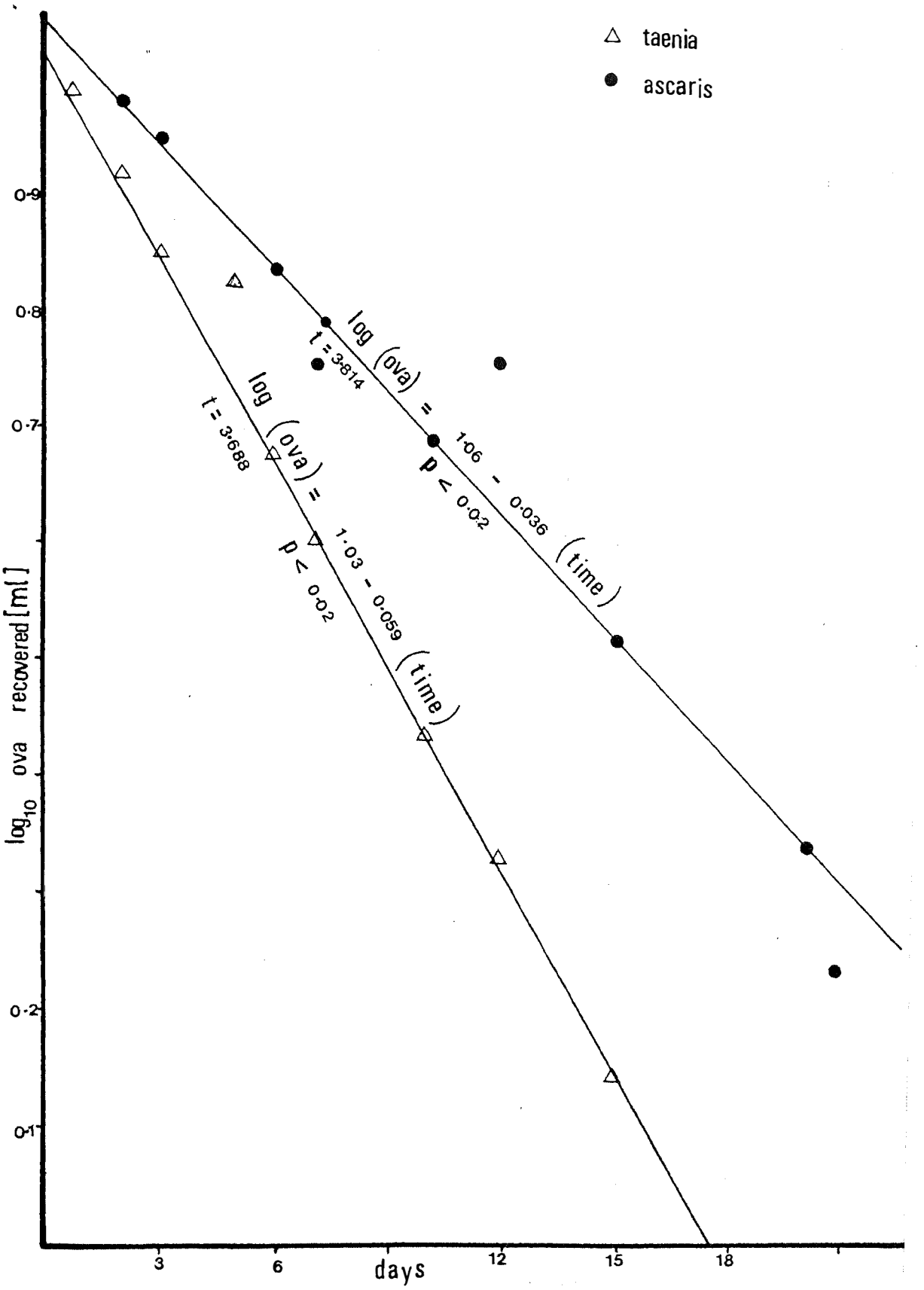
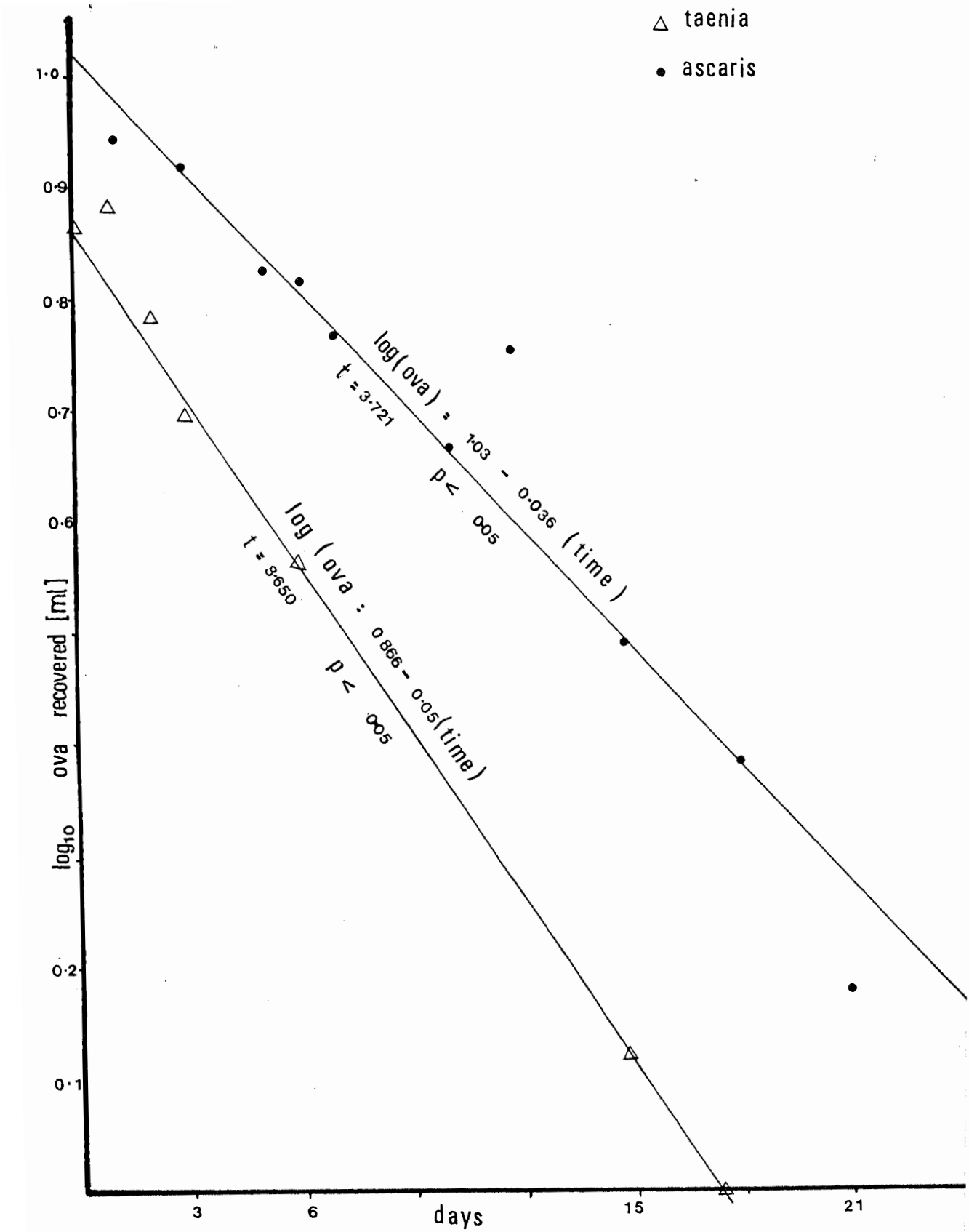


FIG:

RECOVERY OF ASCARID AND TAENIID OVA FROM SEEDED RAW SLUDGE APPLIED TO TURVES HELD AT 25° C



than during warmer months), a correspondingly longer withdrawal period is required to prevent animal contact with infective material.

As only total numbers of ova recovered from treated areas were considered in these experiments, a study of egg survival using viability criteria was instigated to determine what a "safe period" would be. These studies are discussed in a later section.

PHYSICAL FACTORS AFFECTING THE SURVIVAL OF HELMINTH OVA:

TEMPERATURE, HUMIDITY AND ULTRAVIOLET IRRADIATION.

A. Introduction

(1) The effect of temperature;- Transmission of parasitic infections, for example, cysticercosis, usually involves a period during which the infective stages of the parasite are exposed to the external environment. It is therefore important to know the conditions under which ova will develop, or at least remain viable, and the conditions which are not conducive to their survival.

There have been several investigations of thermal death points of ova, but it is difficult to draw general conclusions from these studies as they were carried out under different conditions. Silverman (1956) found that 10 minutes exposure to 59°C inactivated Taenia ova, which failed to develop when subsequently placed in hatching solutions. Silverman and Guiver (1960) inactivated Taenia ova in suspension by exposing them to 35°C for 20 days and Newton et al (1949) recovered "apparently normal" Taenia ova after 6 months in sludge at 24°C - 29°C. Suvarov (1965) found evidence that Taenia ova could survive very low temperatures. In his studies Taenia saginata ova survived 62 - 64 days at -4°C and for 16 - 19 days at -30°C. Although Ascaris ova are notoriously resistant to extremes of environmental parameters, their development is inhibited by high temperatures. Nolf (1932) inactivated Ascaris ova by exposure to 50°C for 5 minutes or by flash exposure to 56°C. Otto (1929) cited

the work of Cram (1924) who found Ascaris ova able to withstand a temperature of -17°C for 40 days, and Bakker (1924) who stated that -30°C always proved lethal. Seamster (1950) found 31.1°C was the optimum temperature for the development of Ascaris suum ova, and that 14.5°C was the threshold temperature below which the ova failed to embryonate. A more recent study of the influence of temperature on the rate of egg development confirmed Seamster's results and correlated rate of egg development with the incidence of A. suum infections in pigs (Stevenson 1979).

(2) The effect of humidity;- Moisture availability is a major limiting factor affecting the survival of helminth ova in the external environment. Ascaris ova are amongst the most resistant to desiccation and are therefore a suitable indicator organism to use to establish the conditions which will inactivate helminth ova.

Since they have a thinner shell than Ascaris ova, Taenia ova might be expected to withstand the rigours of desiccation less readily. Penfold, Penfold and Phillips (1937) however, produced evidence that Taenia saginata ova remained viable for 102 days on "dry" pasture in Australia. Jepsen and Roth (1952) also demonstrated the ability of Taenia saginata to survive for at least 159 days on grass. No temperatures were stated but their studies were carried out in Denmark between February and July which would probably mean warm, moist conditions. Danish winters are colder than U.K. and spring comes later. Summers are more stable than ours usually, but rainfall can occur in every month of the year. Silverman (1956) stated that Taenia saginata ova did not survive more than 14 days in

the absence of surface moisture, but that they survived at least 335 days in normal saline at 4°C. There is evidence of differential susceptibility to desiccation between species. Laws (1967) for example, working with canine tapeworms found that Taenia pisiformis and Echinococcus granulosus were less resistant to desiccation than those of Taenia hydatigena or Taenia ovis. The former failed to survive even brief exposure to humidities below 75 percent.

Otto (1929) found that the development of Ascaris ova was suspended at relative humidities below 80 percent and that they desiccated more rapidly at 30°C than at 22°C. Nolf (1932) found that at 40% - 50% relative humidity, Ascaris and Trichuris ova survived only 4 days but that survival was increased to 15 - 20 days at 77% relative humidity. Seamster (1948) found that Ascaris ova held at 31±1°C and 80% - 95% relative humidity were completely desiccated in 8 days. Although most results in the literature are given in terms of relative humidity it is important to consider the cases where ova are present in solid materials. Cram (1943) was able to recover viable Ascaris ova from sludge cake with a moisture content as low as 5.84% after 81 days. No ova survived in sludges with moisture contents below 5 percent.

Despite the many experiments relating viability of ova to variations of a single parameter, in practice ova are exposed to more than one parameter and it is relatively meaningless to isolate the effects of each parameter. It was therefore necessary in these studies to devise a multifactorial experiment for a realistic appraisal of egg survival.

The effects of temperature appeared to operate over a narrow range; ovalheld at 26.7°C and 25.9°C at 95% relative humidity were not desiccated andldeveloped normally. These findings conflict with those of the Australian meat research committee (1979) who found that at 30°C and 33% relative humidity a few eggs of Taenia hydatigena survived 7 days, while at 4°C and 99% relative humidity some eggs survived for as long as 300 days.

(3) The effect of ultraviolet irradiation:- Whilst in tropical countries U.V. light reaching the earths surface can be sufficient to kill parasite infective stages, the radiation level experienced in normal British summers would be unlikely to reach levels lethal to ova. Yoshida (1920) found that strong sunlight of Japanese summers "checks development, and if continued will kill (Ascaris) eggs". Laws (1967) found that exposure to U.V. light at 20°C and 10cm distance had no effect on Taenia pisiformis ova in 8 days. Brown (1927) found that Ascaris ova on sandy soil exposed to direct sunlight degenerated in 3 weeks. Nolf (1929) inactivated Ascaris ova by exposure to U.V. light at wave lengths of 280µm - 315µm and 180µm - 315µm. Dognon and Tsang (1925) exposed Ascaris megalocephala ova to U.V. light at 16°C and 40°C. At both temperatures 10% and 80% of the ova were destroyed by 60 seconds exposure at 1mm distance.

Whenever ova are exposed to extreme conditions they will not all die at the same time, but will decline in numbers according to a normal distribution. Although it is the usual practice to determine LD50's or LD90's to evaluate the effect of environmental extremes in practice it is important to know the maximum possible survival time; (i.e.) when it is safe to resume landuse.

B. Materials and Methods

Solutions of organic compounds or saturated solutions of some inorganic salts with a constant vapour pressure at a particular temperature may be used to keep an enclosed volume of air at a constant, known humidity. For these studies aqueous solutions of glycerol were used to provide the required humidities. The relationship between the concentration of glycerol and the corresponding relative humidity produced above the solution at 25°C is shown in figure 10. In particular;-

70% glycerol corresponds to 50% relative humidity

45% " " " 75% " "

15% " " " 95% " "

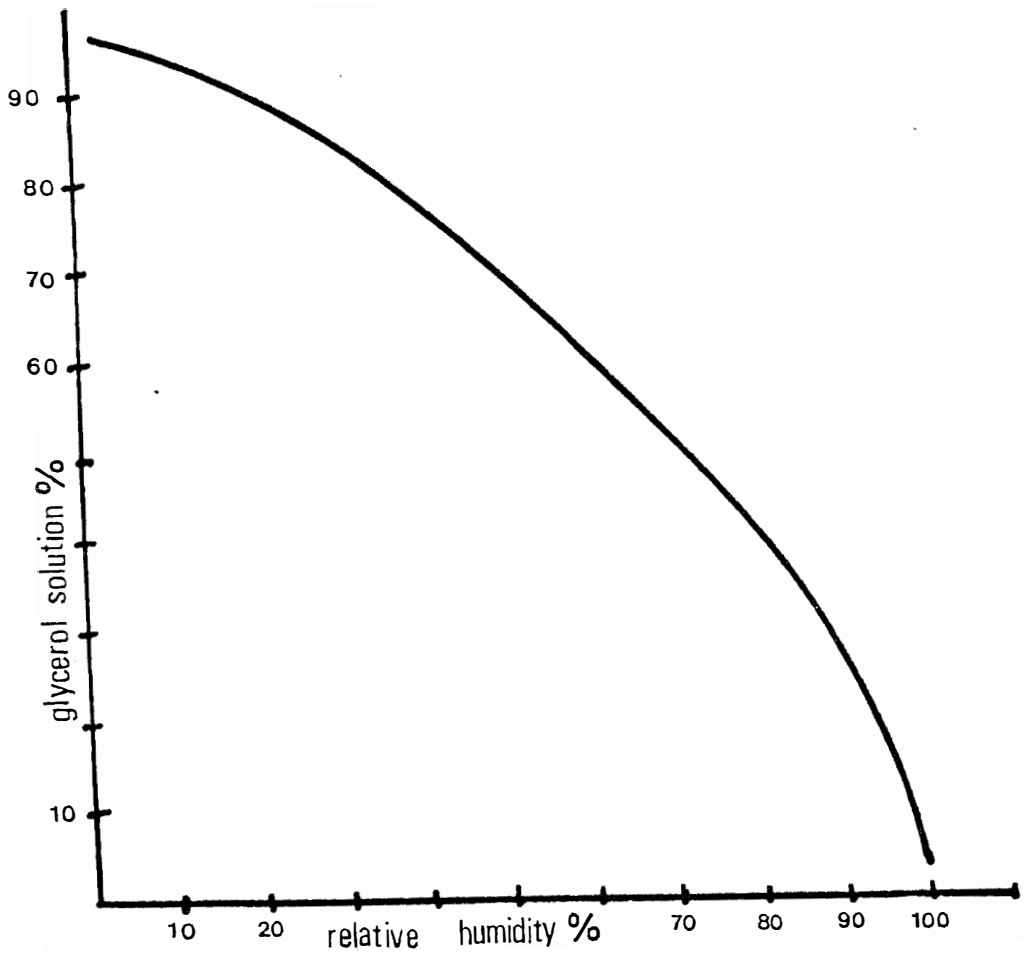
Although the vapour pressure of water alters with temperature so that there are different amounts of water in the vapour phase, the relative humidity should not alter significantly within the temperature range of the experiments viz. 11°C - 35°C.

A 50 dropper pasteur pipette was used to deliver 1 drop of a 10^3 ova/ml suspension of Ascaris ova or Taenia ova onto clean coverslips which had been ringed with "glyceel" sealant to contain the droplet and thereby prevent losses of ova. The suspensions were airdried until the free water had just evaporated. The coverslips were then placed inside 6cm petri-dishes which were set (uncovered) into 9cm petri-dishes partially filled with glycerol solution at concentrations of 70%, 45% or 15% giving a relative humidity inside the covered dish of 50%, 75% or 95% relative humidity respectively. The dishes were incubated at 11°C, 15°C or 35°C and allowed to equilibrate overnight. All experiments were

Fig 10

The relationship between the concentration of glycerol solution (weight per cent) and the relative air humidity above the solution at 25°C.

Slavik (1974)



in the dark. In addition, a parallel series of experiments was set up with the additional factor of exposure to U.V. light (280 Å - 315 Å) at 5cm distance.

On alternate days, one of the coverslips from each combination of conditions was removed for examination and the glycerol replaced with fresh solution of the same concentration. By leaving 48 hours between sampling the humidity of air surrounding the coverslips would be re-established.

When removing coverslips from the petri-dishes for examination, those bearing Taenia and Ascaris ova were treated separately. Those with Taenia ova were activated with 1% acid-pepsin as previously described. Ova which subsequently showed uptake of Meldola's blue stain within 1 - 2 hours were considered viable.

The coverslips holding Ascaris ova were first checked microscopically for evidence of morphological changes. The coverslip well was then filled with distilled water and the coverslip incubated at 35°C in a petri-dish lined with moist filter paper. During this incubation period coverslips were removed periodically and re-examined for signs of egg development.

C. Results

The survivorship curves for Ascaris and Taenia ova under all conditions are presented in figures 11 - 14 . Ova which had been exposed to U.V. irradiation under all conditions were apparently nonviable, since none of the Taenia ova responded to stain, and no Ascaris ova showed signs of development when returned to conditions

FIG: 11

THE EFFECT OF HUMIDITY AND TEMPERATURE ON RECOVERY OF ASCARIS LUMBRICOIDES OVA.

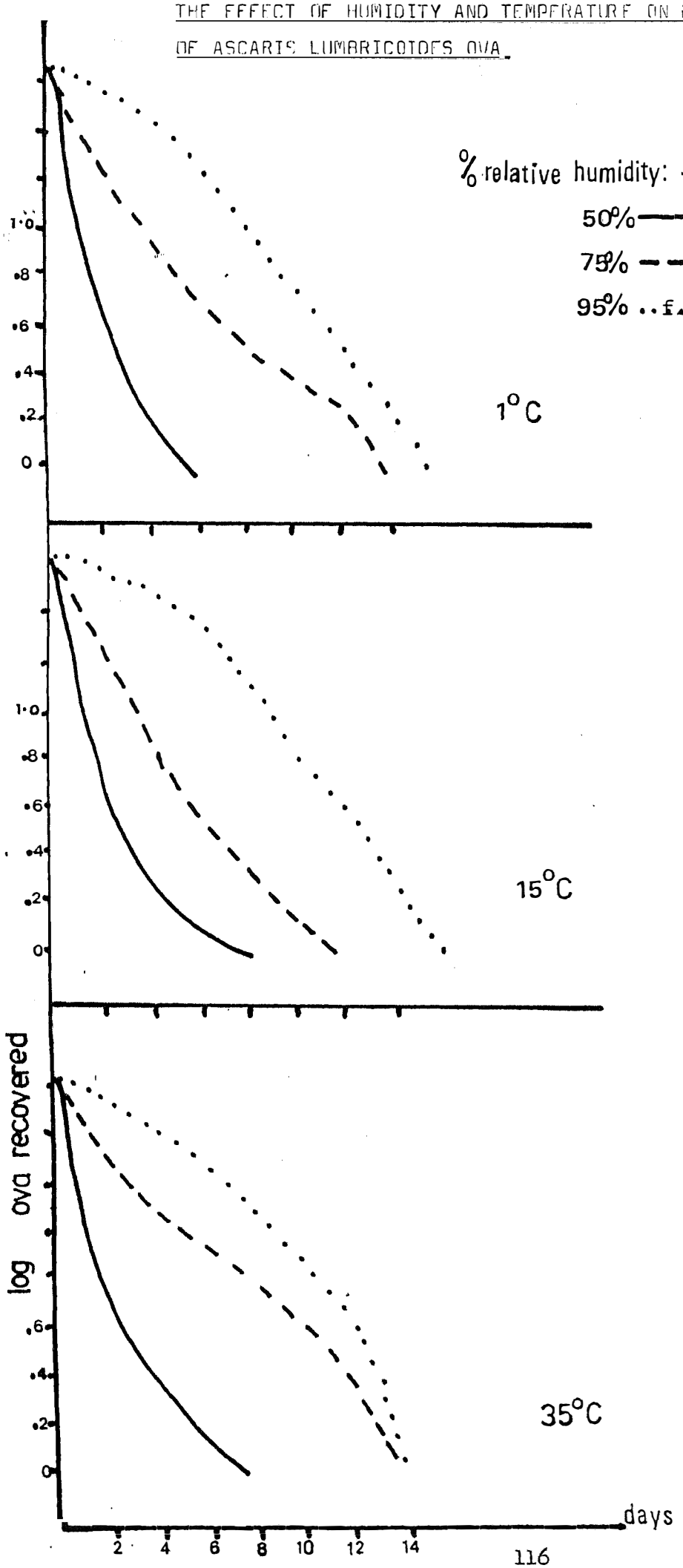


FIG: 12

THE EFFECT OF TEMPERATURE AND HUMIDITY ON THE RECOVERY OF ASCARIS LUMBRICOIDES OVA

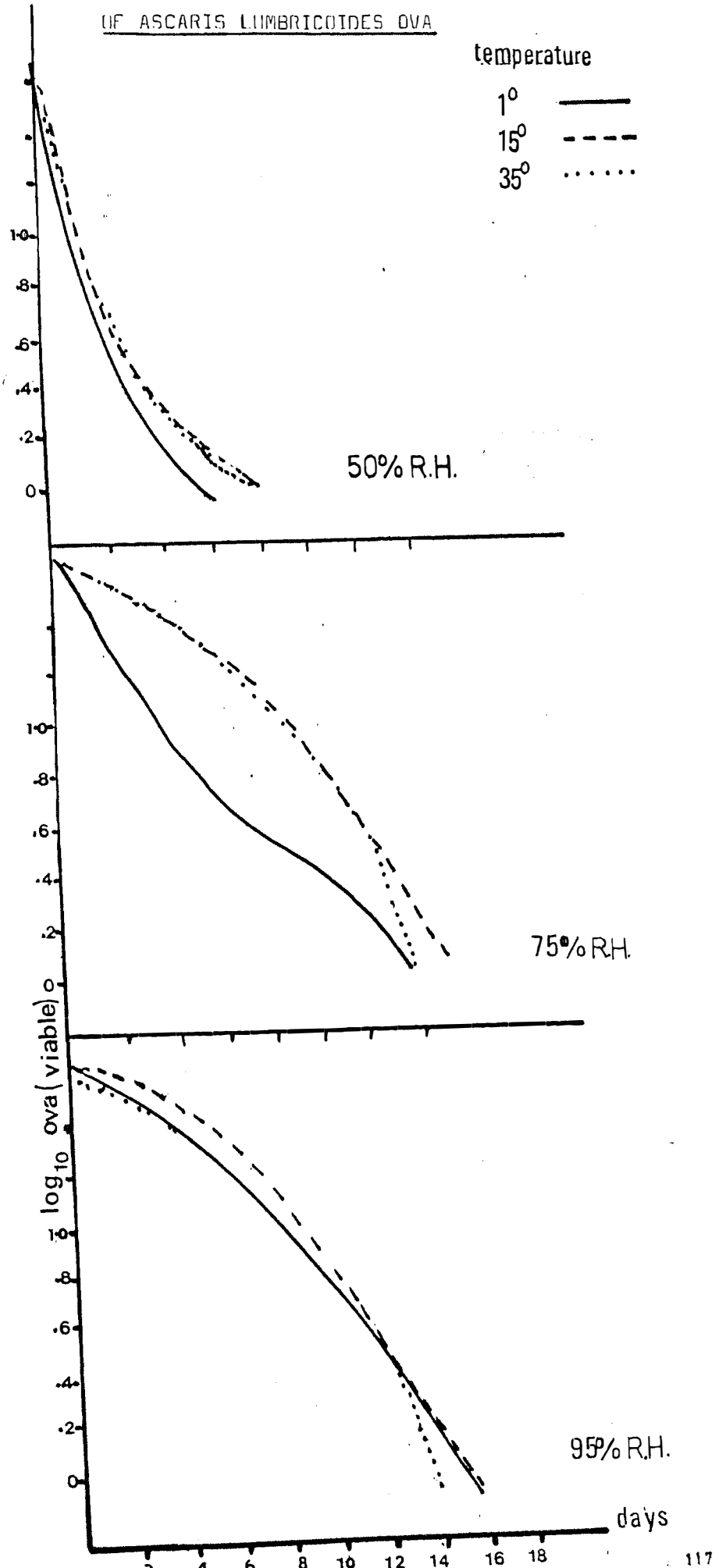


FIG: 13

THE EFFECT OF TEMPERATURE AND HUMIDITY ON TAENIID OVA

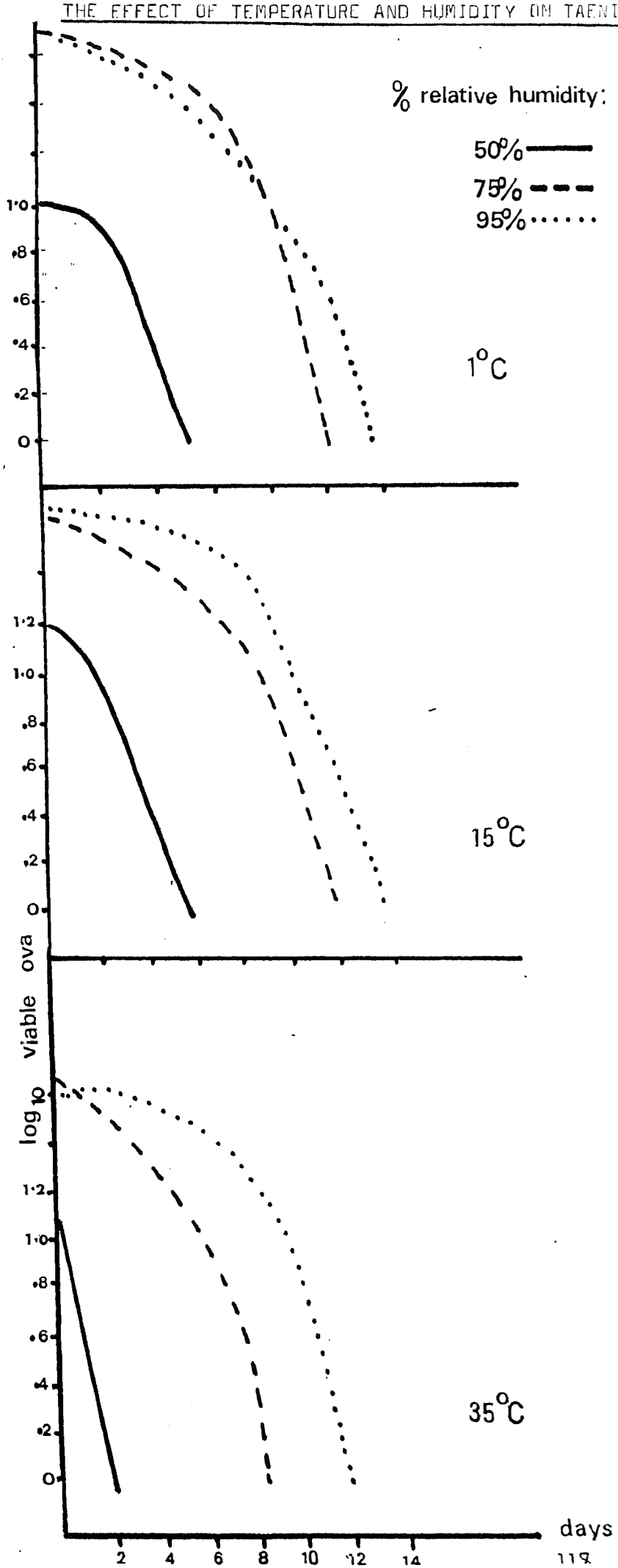
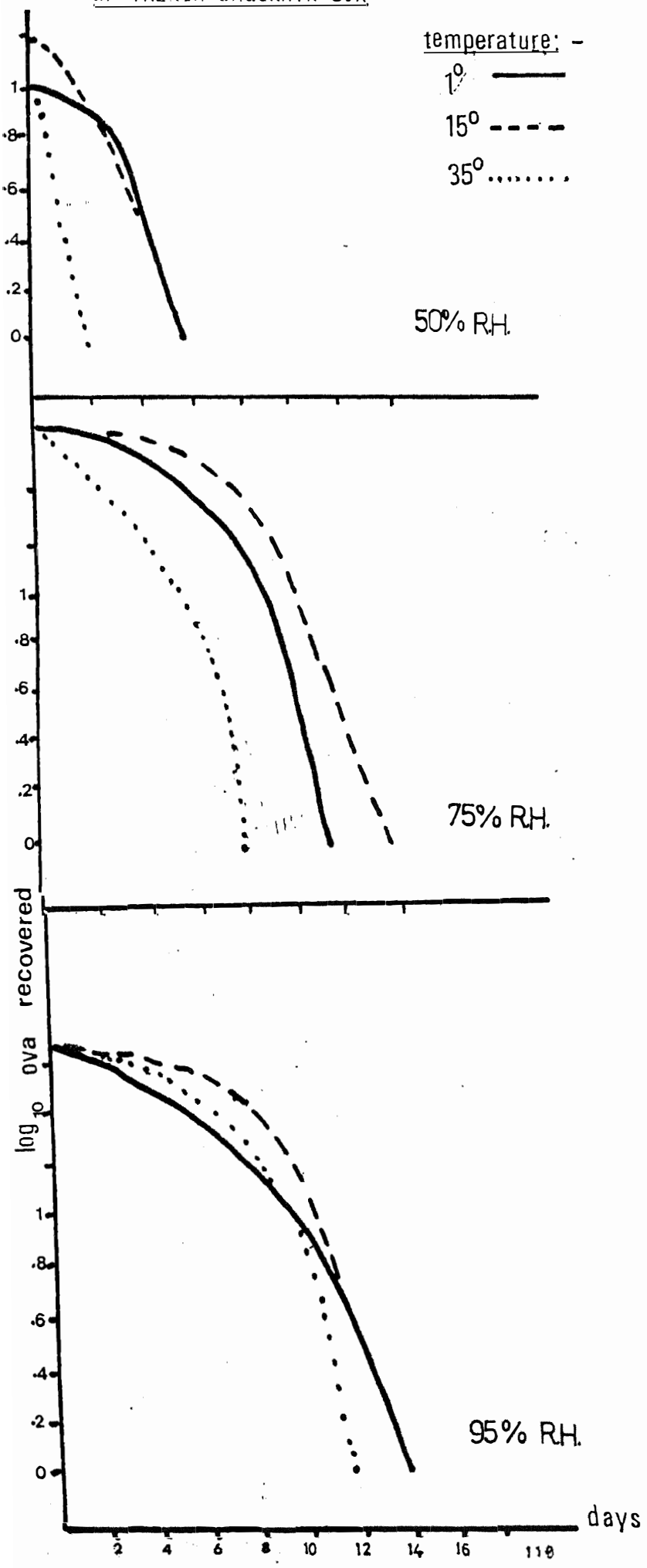


FIG: 14

THE EFFECT OF TEMPERATURE AND HUMIDITY ON THE RECOVERY OF TAENIA SAGINATA OVA



considered optimal for embryonation, viz. 30°C/100% relative humidity.

In the dark series of experiment (i.e. without U.V. light, Ascaris ova survived longer than Taenia ova and at lower relative humiditiese It might have been expected that differences in survival between Ascaris and Taenia ova would have been greater than those observed as Ascaris has a thicker shell which should effectively protect against desiccation.

At 1°C Taenia ova survived up to 10 days at 95% relative humidity (R.H.) but for only 4 days at 50% R.H. At 15°C Taenia ova survived up to 12 days at 95% and 4 days at 50% R.H. At 35°C Taenia ova failed to survive 50% R.H, but a few ova survived up to 6 days at 75% R.H. and for 10 days at 95% R.H.

At all temperatures Ascaris ova survived up to 16 days at 95% R.H. At 1°C and 15°C they survived up to 12 days at 75% R.H. and at all temperatures, up to 6 days at 50% R.H.

D. Discussion

It is clear from the results of these experiments that relative humidity has a strong influence on survival of helminth ova. At all temperatures in the range of 1°C - 35°C the survival of ova was adversely affected when humidity was low (50 percent humidity). Results cited in the literature indicate a differential susceptibility to humidity between different species; (e.g.) Laws (1967)e Studies of this aspect were outside the scope of this experimente

The results obtained are in broad agreement with those of Nolf (1932), but Ascaris ova were found to survive longer than the

8 days reported by Seamster under the same humidity conditions and only slightly different temperature conditions (1.5°C lower than the studies of Seamster)s it is difficult to make direct comparisons with much of the work in the literature however, since a two factor experiment is involved.

As already mentioned, many workers use LD50's or LD90's, but whilst this gives a realistic indication of the average susceptiblity of a population of ova to an external factor, it was of paramount importance here to establish the time and conditions to remove even the most resistant ova, since only then would the potential infection risk be eliminated.

Reference	
a) Effect of Humidity:	
Penfold et al (1937)	Taenia ova survived 102 days "dry" on grass
Jepsen and Roth (1952)	Taenia ova survived 159 days "dry" on grass
Laws (1967)	Taenia ova showed differential susceptibility to humidity viz. text
Otto (1929)	Ascaris ova did not develop below 80% relative humidity (R.H.)
Spindler (1929)	Ascaris and Trichuris ova survived 4 days at 40% - 50% R.H.s 15 - 20 days at 77% R.H.
Cram (1943)	Ascaris ova survived 81 days in sludge with moisture control $\geq$ 5%
Seamster (1948)	Ascaris ova desiccated in 8 days at 80% - 95% R.H.
b) Effect of Temperature:	
Silverman (1956)	Taenia ova killed by 10 min/59°C
Silverman and Guiver (1960)	Taenia ova killed by 20 days/35°C
Froyd (1962)	Taenia ova survived 4°C - 5°C/168 days
Suvarov (1965)	Taenia ova survived 62 - 64 days/-4°C 16 -19 days/-30°C
Brown (1927)	Ascaris ova developed 2½ x faster at 30°C than at 22°C
Otto (1929)	Ascaris ova killed by -30°C

Nolf (1932)	Ascaris ova development prevented by 5 min/50°C or an instant at 56°C
Seamster (1950)	Ascaris ova development optima 31.1°C No development < 14.5°C

c) Effect of U.V. irradiation

Laws (1967)	Taenia ova unaffected after 8 days exposure at 10 cm/20°C
Dognon and Tsang (1925)	10% - 80% Ascaris ova killed by 1 minute exposure at 1 mm/16°C, 20°C
Brown (1927)	Ascaris ova killed by 3 weeks exposure to sunlight
Nolf (1932)	Ascaris ova inactivated by U.V. irradiation at 180 um - 365 um λ
W.H.O. (1964)	Ascaris ova killed by 3 - 10 min exposure on 12 - 19 occasions over 10 -15 days

RECOVERY OF ASCARIS AND TAENIA OVA FROM IN SITU LYSIMETERS

A. Introduction

In situ lysimeters are constructed units containing soil through which water can percolate freely and be collected. The purpose of using lysimeters in this investigation was to study the persistance of ova in sludge applied to agricultural land, and the extent to which such ova might leach through soil, thereby constituting a hazard in soil drainage water. The advantage of using lysimeters is that all drainage water can be collected and monitored. Regional water authorities have aquifer protection policies which restrict the land application of sludge in the vicinity of boreholes or other water sources. The Severn-Trent authority for example, prohibit sludge application within a 2 kilometre radius of boreholes.

Previous workers investigating the survival of helminth ova under field conditions have found that Taeniaeova survive as long as 159 days on grass in Denmark (Jepsen and Roth 1952). Viable Taenia were recovered after more than 4 months on soil in New Zealand by Sweatman and Williams (1963). In Australia, Coman (1975) found that Taenia ova survived for 2 weeks on pasture in summer and for several months in winter.

Forstner (1968) found that 90% of Ascaris ova were still viable after 6 months in soil in Germany. Yoshida (1920) in Japan found that Ascaris ova were still viable after 5 - 6 months in soil. Longer survival times have been recorded, for example the World

Health Organisation report no. 277 (1964) quotes survival times of 2 years under field conditions. Oganov (1977) reported survival times for Ascaris ova of 4 years in sandy soil and Kozlova (1968) recorded 7 years survival of Ascaris ova in drying sludge.

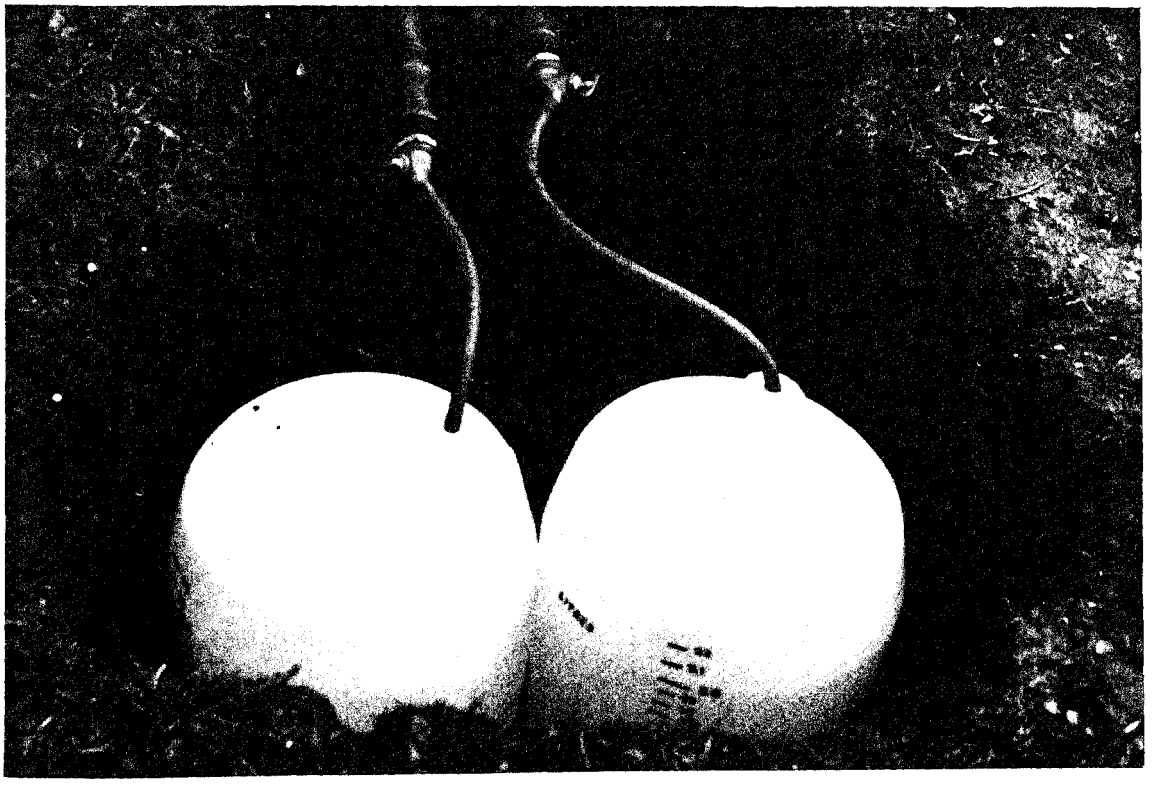
If viability is affected by ultraviolet irradiation it would be expected that survivalotimes would be shorter during summer months, when more ultraviolet irradiation reaches soil and when higher temperatures could also increase egg losses from desiccation.

To test this hypothesis survival trials were conducted in late winter - early spring and summer. It was hoped that the information gained from these trials would give a better indication of the risks presented by land application of sludge, since it is during these periods that cropping and grazing would be most likely to follow sludge application.

B. Methods

(1) Lysimeters:- The four in situ lysimeters used for these experiments were divided into half, each with an independent drainage system (shown in figure 15). The area of each lysimeter was 15 metre^2 or 3.7066×10^{-3} acreo The lysimeters consisted of tanks constructed with plywood sides lined with 1000 gauge polythene sheeting. The concrete base was lined with gravel before being filled with a 0.66 metre layer of a local clay soil (pH value 7.6) Drainage outlets were fitted with taps, under which 25 litre polythene containers were placed to collect leachate. The lysimeter soil was levelled and raked before being hand sown using a commercial





lawn seed mixture at the recommended rate of application (50 gram per metre^2). The lysimeter sward was cropped regularly by hand using hedge clippers.

(2) Sludge Application:- The maximum recommended application of nitrogen suggested by the Department of the Environment (MAFF Bulletin no. 209) gives an application rate (in kilogram of nitrogen per hectare per year) calculated by the following formula;

$$\frac{525 \times 1000}{\text{available nitrogen}} + 50\%$$

It is assumed that 40% of the nitrogen in raw sludge becomes available in the first year (Coker, personal communication). The sludge used in this investigation was analysed for total solids and nitrogen. The total solids value of the sludge was 5.8 percent and the nitrogen value was 2247 p.p.m.

Raw sludge was passed through an 8mm geological sieve to remove rags and coarse debris before inoculating with Ascaris and Taenia ova (6×10^3 ova per litre and 1×10^4 ova per litre respectively). The seeded sludge was spread over the lysimeters at a rate of 8000 gallons per acre, this being the average rate used by a major company dealing with sludge to land application (Bellingham, personal communication). This rate of application was equivalent to 8.987×10^4 litre per hectare, or 30 gallons per lysimeter. The area surrounding the lysimeters was fenced and the top and sides covered with 1.0cm garden mesh to isolate ova from possible contact with wildlife.

(3) Sampling Regime:- In order to compare persistance of Ascaris and Taenia ova during different seasons, two trials were conducted. The first, over late winter/early spring up to April 1979 and the second, over summer up to July 1979. As will be discussed the stage of development of Ascaris ova recovered during winter trials was more advanced than would be normally expected so it was suspected that these ova had been in a developed stage when inoculated into the sludge. The winter trials were therefore repeated using ova freshly dissected from adult female worms.

Initially only sludge was sampled from the lysimeters, but as weathering caused the gradual breakdown of the sludge the samples inevitably contained a mixture of sludge and soil. Replicate 10 gram samples were taken from the lysimeters daily to a depth of 3cm. Herbage samples were clipped using hand scissors, avoiding grass obviously coated with sludge. Leachate samples were collected from the drainage outlets whenever available, depending on rainfall. The required number of soil and sludge replicates to give a decimal accuracy of 0.05 with the extraction technique used, was determined by a method of Southwood (1966) to be 16 replicates. Replicate 5 gram herbage samples were taken; washed as described previously (p.59) and the washings subjected to continuous flow centrifugation. The leachate samples were subjected to continuous flow centrifugation to recover ova which might have passed through the soil profile. The number of leachate samples required for a decimal accuracy of 0.05 was determined by Southwood's formula to be 10 replicates.

C. Results

(1) Climatic Conditions:- During the late winter/early spring trials the air temperature (and therefore soil temperature) did not fall below 0°C. Weather conditions during the summer trials were not markedly different from those of the winter/spring trials, and air temperatures did not rise above 25°C in any 24 hour period. The effects of air temperature on a soil depend on its moisture, a wet soil showing a smaller temperature change than a dry soil. Rainfall during the summer trial period was negligible but rainfall averaged 2.4mm per day during winter/spring trials; ranging from 0mm to 8.5mm in any 24 hour period.

(2) Persistance of Ova:- No ova were isolated from the soil leachate collected during either winter/spring or summer trials. The soil profile was of considerable depth (0.66 metre) and ova evidently did not percolate through the soil to this depth. A later section (p.137) describes a more detailed investigation of the movement of ova through the soil profile, which showed a vertical movement of 5cm in soil was possible.

It was not possible to obtain sludge-free herbage samples for several weeks after application of sludge, and samples taken after completion of the trials yielded no Ascaris or Taenia ova.

The recovery data for Ascaris ova is presented in a semi-log form (figures (16a) and (16b)). From an initial value of 6 ova per ml of sludge the numbers of Ascaris ova recovered from mixed

FIG: 16a

RECOVERY OF ASCARID OVA FROM FIELD LYSIMETERS

SUMMER 1979

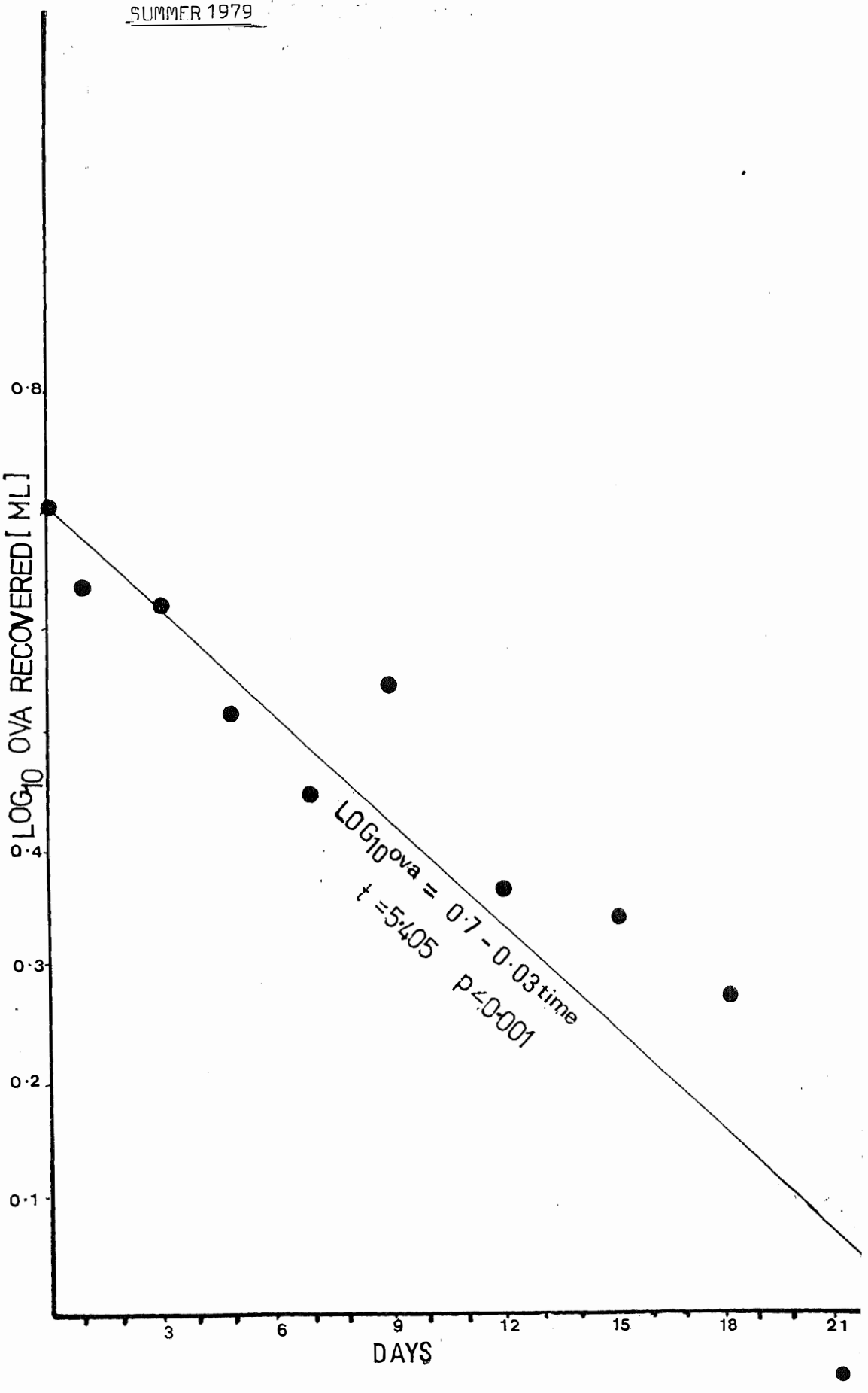
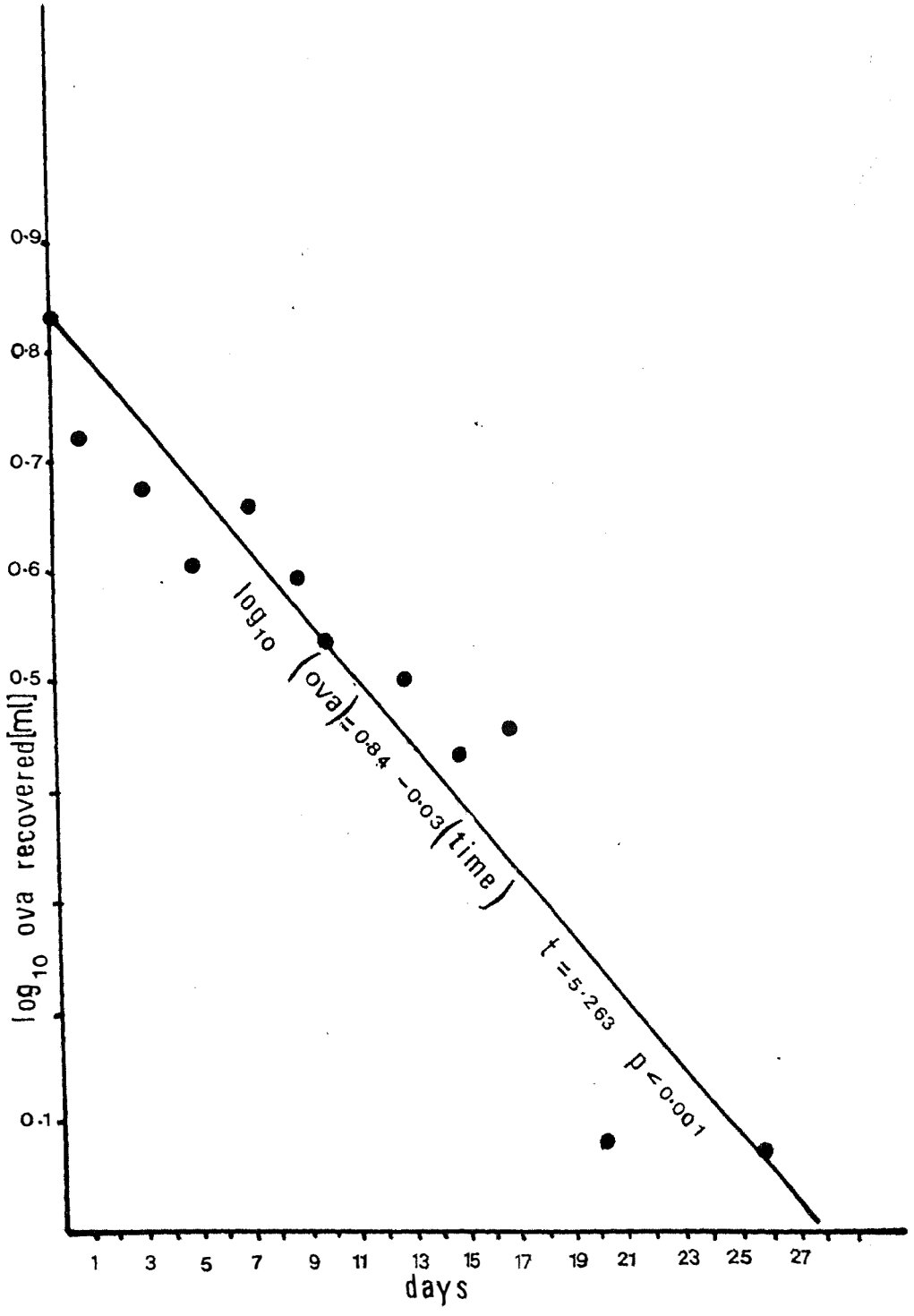


FIG: 16b

RECOVERY OF ASCARID OVA FROM FIELD LYSIMETERS, WINTER 1979

(REPEAT)



sludge and soil taken from the surface of the lysimeters fell rapidly in summer and no ova were recovered after 21 days. In the repeated winter/spring trials the number of recovered Ascaris ova declined at the same rate as in summer, but ova were recovered for 30 days in this instance. The graphs for recovery of Ascaris ova have the same gradients for winter and summer trials indicating that the rate of decline in numbers of ova was equal, although ova persisted a week longer during the winter/spring trials. Figure (17a) and (17b) show the recovery data for Taenia ova presented in a semi-log form. From an initial value of 10 ova per ml of sludge the numbers of recovered ova diminished rapidly in both winter/spring and summer trials as shown by the large negative gradient (-0.07 for both trials). Taenia ova were recovered for 12 days in both trials.

The relative persistance of ova in soil and sludge samples was compared by calculated parameter termed the biological half life ($t_{\frac{1}{2}}$) which is a measure of the time taken for a 50 percent reduction in ova to occur. The $t_{\frac{1}{2}}$ values, and regression data, are summarised in table (15).

Taenia ova had a low $t_{\frac{1}{2}}$ value (4.3 days) by comparison with Ascaris ova which had a $t_{\frac{1}{2}}$ of 10 days. These values applied to both winter/spring and summer trials, so that although Taenia had a lower $t_{\frac{1}{2}}$ than Ascaris ova, neither species showed a difference in $t_{\frac{1}{2}}$ values between winter/spring and summer trials.

FIG: 17a

RECOVERY OF TAENIID OVA FROM FIELD LYSIMETERS

SUMMER 1979

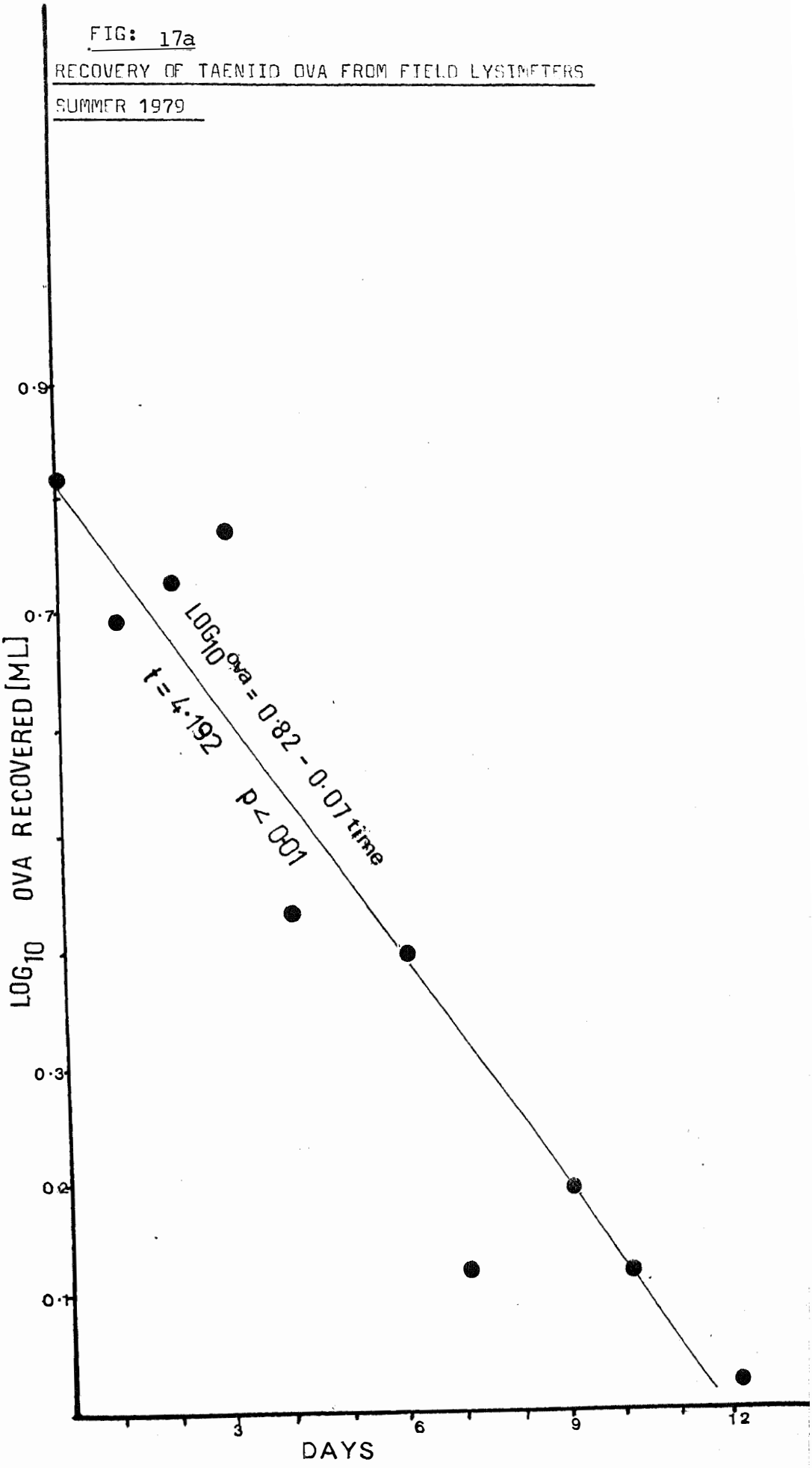


FIG: 17b

RECOVERY OF TAENIID OVA FROM FIELD LYSIMETERS

WINTER

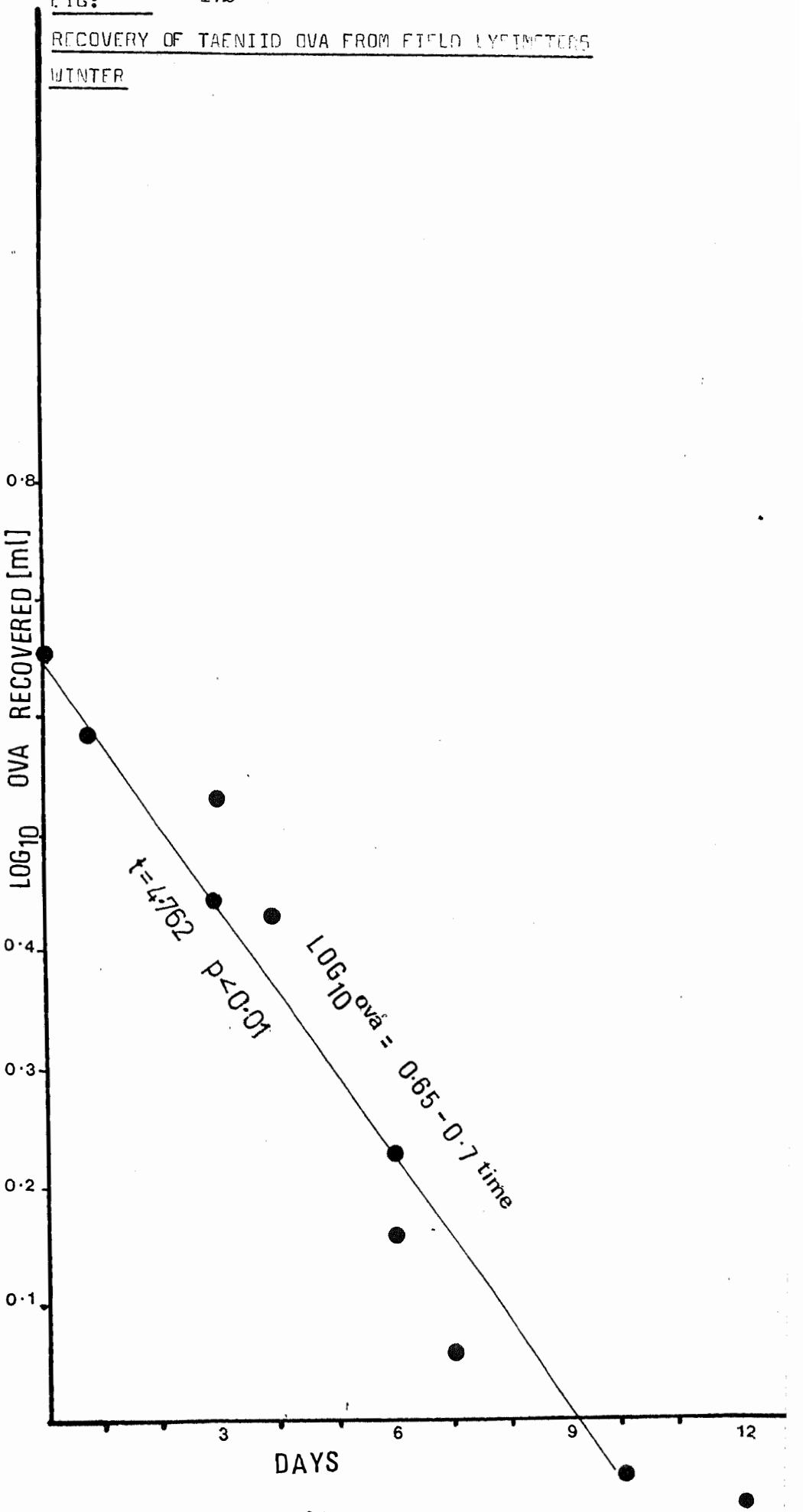


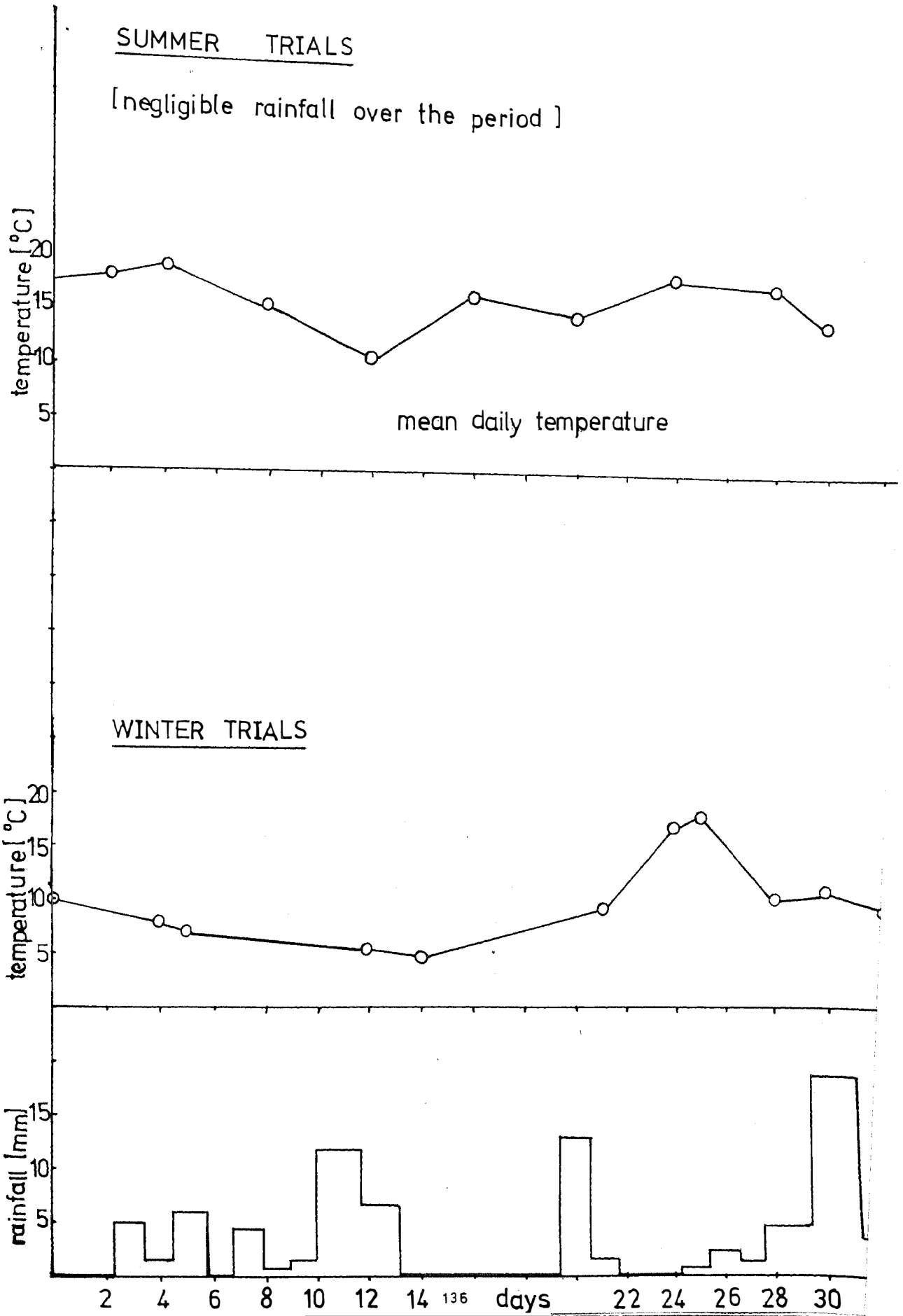
Table 15

Recovery of Ova from *in situ* Lysimeters

	Recovered for	$t_{\frac{1}{2}}$	Gradient	Regression Equation
Winter Trials *Taenia*	12 days	4.3 days	m = -0.07 t = 4.762 p < 0.01	$\log_{10}$ (ova) = 0.65 - 0.07 (time
Summer Trials *Taenia*	12 days	4.3 days	m = -0.07 t = 4.192 p < 0.01	$\log_{10}$ (ova) = 0.82 - 0.07 (time)
Winter Trials *Ascaris*	30 days	10.0 days	m = -0.032 t = 5.263 p < 0.001	$\log_{10}$ (ova) = 0.84 - 0.03 (time)
" Summer Trials	21 days	10.0 days	m = -0.03	$\log_{10}$ (ova) = 0.70 - 0.03 (time) t = 5.405 p < 0.001

FIG: 18

CLIMATIC CONDITIONS DURING LYSIMETER TRIALS



D. Movement ofeOva through Soil

1. Introduction

During the lysimeter trials no ova were recovered from soil leachate, yet ova disappeared quickly from the surface of the lysimeters, suggesting that ova had moved a distance greater than 3cm (but less than 63cm, since no ova were recovered from soil leachate). To investigate whether ova had been moved through soil by percolating water, an experiment was set up in which inoculated soil cores were subjected to a known rate of watering. Both soil leachate and soil samples from different depths could then be examined for the presence of ova.

2. Methods

The soil cores were set up in vertically positioned glass cylinders. These were made from 1cm diameter glass tubing cut into 10cm lengthse each lined with nine 1cm rings cut from propylene tubing. The top 1cm of each core was inoculated with approximately 50 Taenia or Ascaris ova. Cores were watered for 24 hours at a fixed rate controlled by screw clips constricting the watering tubes. Gauze was used to support the cores.

Collected leachate was centrifuged at 2000 rpm for 10 minutes and the sediment examined microscopicallye The soil cores were removed sequentially by pushing the tube rings from the supporting glass cylinder. Each layer of soil was then examined, for ova using the previously described antiformin-sucrose technique.

3. Results

Recoveries of ova from the soil cores and leachate are summarised in table (16). No ova were recovered from leachate samples. Soil from the first two layers (0 - 2cm depth) were pooled in case ova had been inadvertantly mixed into the deeper layer during inoculation. Ova were recovered down to a maximum of 5cm depth but not from any deeper layer. It is possible that a slight settling of watered soil could account for some of the downwards displacement of ova, for example, ova at 2cm depth could then have been recovered in the 2cm - 3cm layer of soil after 24 hours.

Table 16

Recovery of ova from soil cores and from leachate

Ova	Watering rate ml/min	Ova present at depth;								
		0.2cm	3cm	4cm	5cm	6cm	7cm	8cm	9cm	Leachate
Ascaris	0.33	+	+	+	-	-	-	-	-	-
Ascaris	0.33	+	+	-	-	-	-	-	-	-
Taenia	0.33	+	+	+	-	-	-	-	-	-
Taenia	0.33	+	+	+	-	-	-	-	-	-
Ascaris	0.67	+	+	+	-	-	-	-	-	-
Taenia	0.67	+	+	+	+	-	-	-	-	-

(+) indicates ova present

(-) indicates no ova recovered

D. Discussion

Although no Ascaris ova were recovered after 21 days in summer or 30 days in winter/spring trials, and no Taenia ova were recovered after 12 days in either trial, this need not imply that no ova were present. Ova would probably not disintegrate so rapidly and it is considered that two factors could account for the observed drop in numbers of ova recovered. Firstly, ova could have passed into the 63cm of soil beneath the 3cm sampled. Secondly as will be demonstrated, the probability of recovering ova declines rapidly with decline in numbers of ova per sample, thus compounding the observed reduction in recovered ova.

The probability of detection of ova in a sample is directly related to extraction efficiency and from an analysis of the level of infestation the problems associated with recovery of ova can be demonstrated. Recovery of ova from a mixed bulk sample should fit the Poisson distribution and it follows from this, that the accuracy of each count will depend on its size. The difficulties inherent in recovering ova from samples containing different densities of ova are demonstrated in table (17). This summary shows that virtual certainty of detection is not reached until density of ova in soil approaches 4×10^5 ova per hectare. Below this level of infestation the probability of detecting ova declines until, at the level of 1×10^5 ova per hectare there is only a 46.3 percent chance of detection. At a level of 1×10^4 ova per hectare, which is still a significant burden in the soil, the chance of detecting ova in a 10 gram sample is only 8.3 percent. Many samples which are

8 days reported by Seamster under the same humidity conditions and only slightly different temperature conditions (1.5°C lower than the studies of Seamster)s it is difficult to make direct comparisons with much of the work in the literature however, since a two factor experiment is involved.

As already mentioned, many workers use LD50's or LD90's, but whilst this gives a realistic indication of the average susceptiblity of a population of ova to an external factor, it was of paramount importance here to establish the time and conditions to remove even the most resistant ova, since only then would the potential infection risk be eliminated.

Reference	
a) Effect of Humidity:	
Penfold et al (1937)	Taenia ova survived 102 days "dry" on grass
Jepsen and Roth (1952)	Taenia ova survived 159 days "dry" on grass
Laws (1967)	Taenia ova showed differential susceptibility to humidity viz. text
Otto (1929)	Ascaris ova did not develop below 80% relative humidity (R.H.)
Spindler (1929)	Ascaris and Trichuris ova survived 4 days at 40% - 50% R.H.s 15 - 20 days at 77% R.H.
Cram (1943)	Ascaris ova survived 81 days in sludge with moisture control $\geq$ 5%
Seamster (1948)	Ascaris ova desiccated in 8 days at 80% - 95% R.H.
b) Effect of Temperature:	
Silverman (1956)	Taenia ova killed by 10 min/59°C
Silverman and Guiver (1960)	Taenia ova killed by 20 days/35°C
Froyd (1962)	Taenia ova survived 4°C - 5°C/168 days
Suvarov (1965)	Taenia ova survived 62 - 64 days/-4°C 16 -19 days/-30°C
Brown (1927)	Ascaris ova developed 2½ x faster at 30°C than at 22°C
Otto (1929)	Ascaris ova killed by -30°C

Nolf (1932)	Ascaris ova development prevented by 5 min/50°C or an instant at 56°C
Seamster (1950)	Ascaris ova development optima 31.1°C No development < 14.5°C

c) Effect of U.V. irradiation

Laws (1967)	Taenia ova unaffected after 8 days exposure at 10 cm/20°C
Dognon and Tsang (1925)	10% - 80% Ascaris ova killed by 1 minute exposure at 1 mm/16°C, 20°C
Brown (1927)	Ascaris ova killed by 3 weeks exposure to sunlight
Nolf (1932)	Ascaris ova inactivated by U.V. irradiation at 180 um - 365 um λ
W.H.O. (1964)	Ascaris ova killed by 3 - 10 min exposure on 12 - 19 occasions over 10 -15 days

RECOVERY OF ASCARIS AND TAENIA OVA FROM IN SITU LYSIMETERS

A. Introduction

In situ lysimeters are constructed units containing soil through which water can percolate freely and be collected. The purpose of using lysimeters in this investigation was to study the persistance of ova in sludge applied to agricultural land, and the extent to which such ova might leach through soil, thereby constituting a hazard in soil drainage water. The advantage of using lysimeters is that all drainage water can be collected and monitored. Regional water authorities have aquifer protection policies which restrict the land application of sludge in the vicinity of boreholes or other water sources. The Severn-Trent authority for example, prohibit sludge application within a 2 kilometre radius of boreholes.

Previous workers investigating the survival of helminth ova under field conditions have found that Taeniaeova survive as long as 159 days on grass in Denmark (Jepsen and Roth 1952). Viable Taenia were recovered after more than 4 months on soil in New Zealand by Sweatman and Williams (1963). In Australia, Coman (1975) found that Taenia ova survived for 2 weeks on pasture in summer and for several months in winter.

Forstner (1968) found that 90% of Ascaris ova were still viable after 6 months in soil in Germany. Yoshida (1920) in Japan found that Ascaris ova were still viable after 5 - 6 months in soil. Longer survival times have been recorded, for example the World

Health Organisation report no. 277 (1964) quotes survival times of 2 years under field conditions. Oganov (1977) reported survival times for Ascaris ova of 4 years in sandy soil and Kozlova (1968) recorded 7 years survival of Ascaris ova in drying sludge.

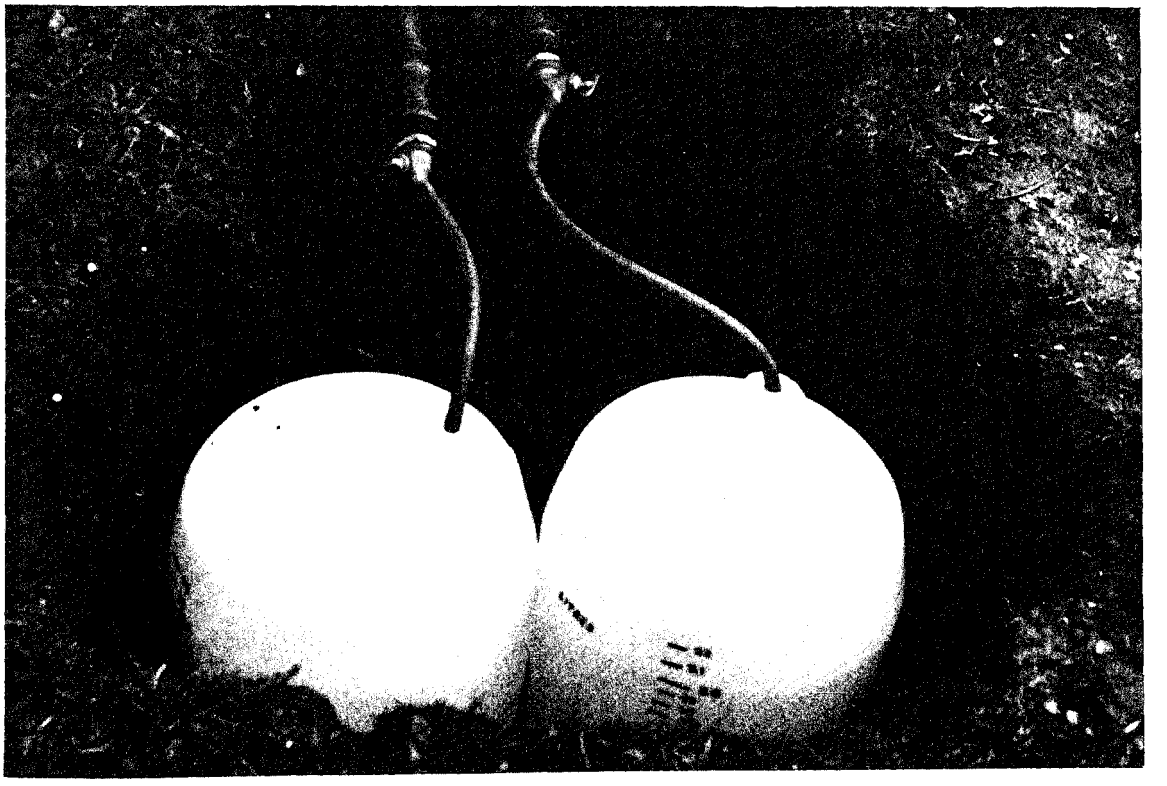
If viability is affected by ultraviolet irradiation it would be expected that survivalotimes would be shorter during summer months, when more ultraviolet irradiation reaches soil and when higher temperatures could also increase egg losses from desiccation.

To test this hypothesis survival trials were conducted in late winter - early spring and summer. It was hoped that the information gained from these trials would give a better indication of the risks presented by land application of sludge, since it is during these periods that cropping and grazing would be most likely to follow sludge application.

B. Methods

(1) Lysimeters:- The four in situ lysimeters used for these experiments were divided into half, each with an independent drainage system (shown in figure 15). The area of each lysimeter was 15 metre^2 or 3.7066×10^{-3} acreo The lysimeters consisted of tanks constructed with plywood sides lined with 1000 gauge polythene sheeting. The concrete base was lined with gravel before being filled with a 0.66 metre layer of a local clay soil (pH value 7.6) Drainage outlets were fitted with taps, under which 25 litre polythene containers were placed to collect leachate. The lysimeter soil was levelled and raked before being hand sown using a commercial





lawn seed mixture at the recommended rate of application (50 gram per metre^2). The lysimeter sward was cropped regularly by hand using hedge clippers.

(2) Sludge Application:- The maximum recommended application of nitrogen suggested by the Department of the Environment (MAFF Bulletin no. 209) gives an application rate (in kilogram of nitrogen per hectare per year) calculated by the following formula;

$$\frac{525 \times 1000}{\text{available nitrogen}} + 50\%$$

It is assumed that 40% of the nitrogen in raw sludge becomes available in the first year (Coker, personal communication). The sludge used in this investigation was analysed for total solids and nitrogen. The total solids value of the sludge was 5.8 percent and the nitrogen value was 2247 p.p.m.

Raw sludge was passed through an 8mm geological sieve to remove rags and coarse debris before inoculating with Ascaris and Taenia ova (6×10^3 ova per litre and 1×10^4 ova per litre respectively). The seeded sludge was spread over the lysimeters at a rate of 8000 gallons per acre, this being the average rate used by a major company dealing with sludge to land application (Bellingham, personal communication). This rate of application was equivalent to 8.987×10^4 litre per hectare, or 30 gallons per lysimeter. The area surrounding the lysimeters was fenced and the top and sides covered with 1.0cm garden mesh to isolate ova from possible contact with wildlife.

(3) Sampling Regime:- In order to compare persistance of Ascaris and Taenia ova during different seasons, two trials were conducted. The first, over late winter/early spring up to April 1979 and the second, over summer up to July 1979. As will be discussed the stage of development of Ascaris ova recovered during winter trials was more advanced than would be normally expected so it was suspected that these ova had been in a developed stage when inoculated into the sludge. The winter trials were therefore repeated using ova freshly dissected from adult female worms.

Initially only sludge was sampled from the lysimeters, but as weathering caused the gradual breakdown of the sludge the samples inevitably contained a mixture of sludge and soil. Replicate 10 gram samples were taken from the lysimeters daily to a depth of 3cm. Herbage samples were clipped using hand scissors, avoiding grass obviously coated with sludge. Leachate samples were collected from the drainage outlets whenever available, depending on rainfall. The required number of soil and sludge replicates to give a decimal accuracy of 0.05 with the extraction technique used, was determined by a method of Southwood (1966) to be 16 replicates. Replicate 5 gram herbage samples were taken; washed as described previously (p.59) and the washings subjected to continuous flow centrifugation. The leachate samples were subjected to continuous flow centrifugation to recover ova which might have passed through the soil profile. The number of leachate samples required for a decimal accuracy of 0.05 was determined by Southwood's formula to be 10 replicates.

C. Results

(1) Climatic Conditions:- During the late winter/early spring trials the air temperature (and therefore soil temperature) did not fall below 0°C. Weather conditions during the summer trials were not markedly different from those of the winter/spring trials, and air temperatures did not rise above 25°C in any 24 hour period. The effects of air temperature on a soil depend on its moisture, a wet soil showing a smaller temperature change than a dry soil. Rainfall during the summer trial period was negligible but rainfall averaged 2.4mm per day during winter/spring trials; ranging from 0mm to 8.5mm in any 24 hour period.

(2) Persistance of Ova:- No ova were isolated from the soil leachate collected during either winter/spring or summer trials. The soil profile was of considerable depth (0.66 metre) and ova evidently did not percolate through the soil to this depth. A later section (p.137) describes a more detailed investigation of the movement of ova through the soil profile, which showed a vertical movement of 5cm in soil was possible.

It was not possible to obtain sludge-free herbage samples for several weeks after application of sludge, and samples taken after completion of the trials yielded no Ascaris or Taenia ova.

The recovery data for Ascaris ova is presented in a semi-log form (figures (16a) and (16b)). From an initial value of 6 ova per ml of sludge the numbers of Ascaris ova recovered from mixed

FIG: 16a

RECOVERY OF ASCARID OVA FROM FIELD LYSIMETERS

SUMMER 1979

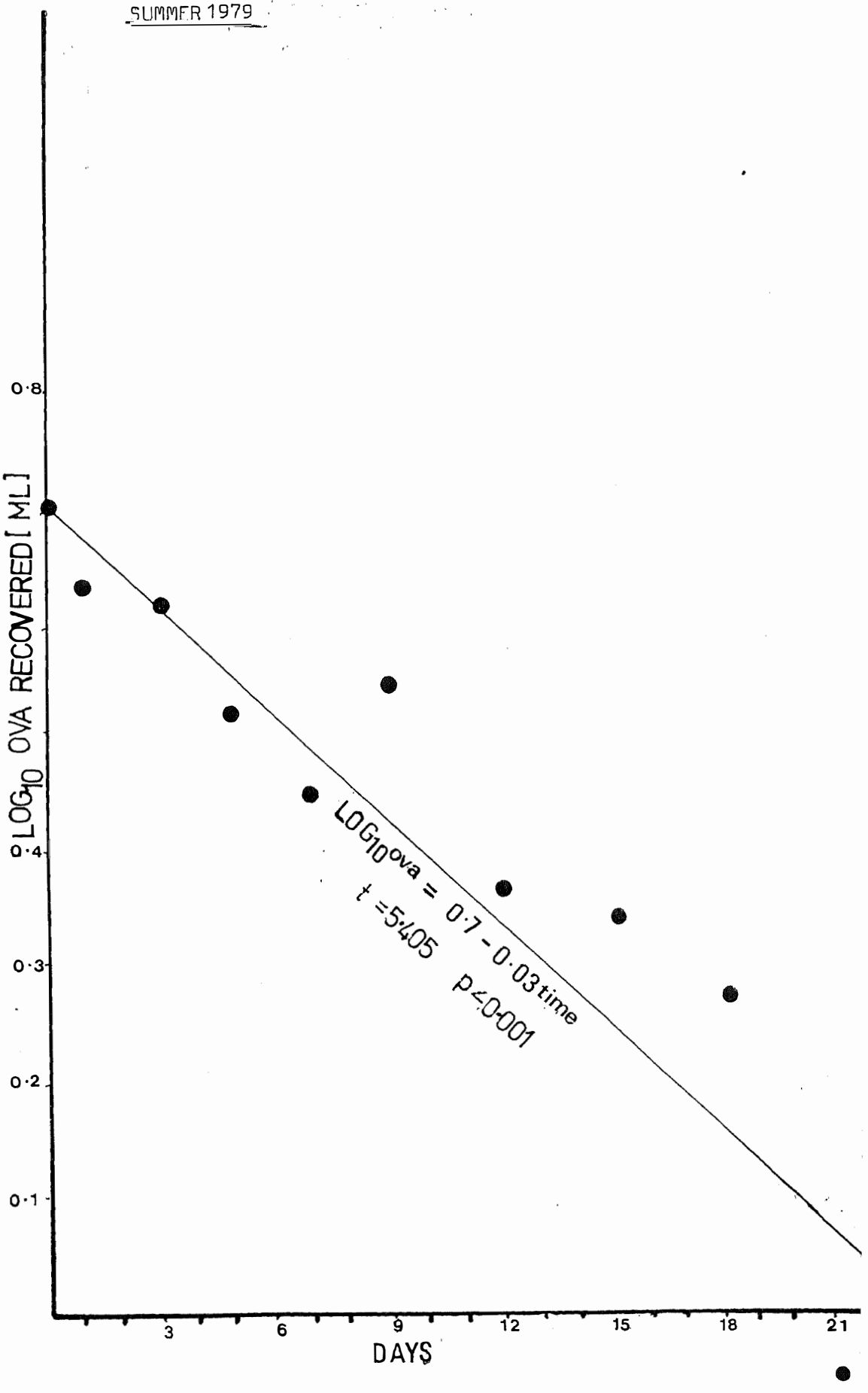
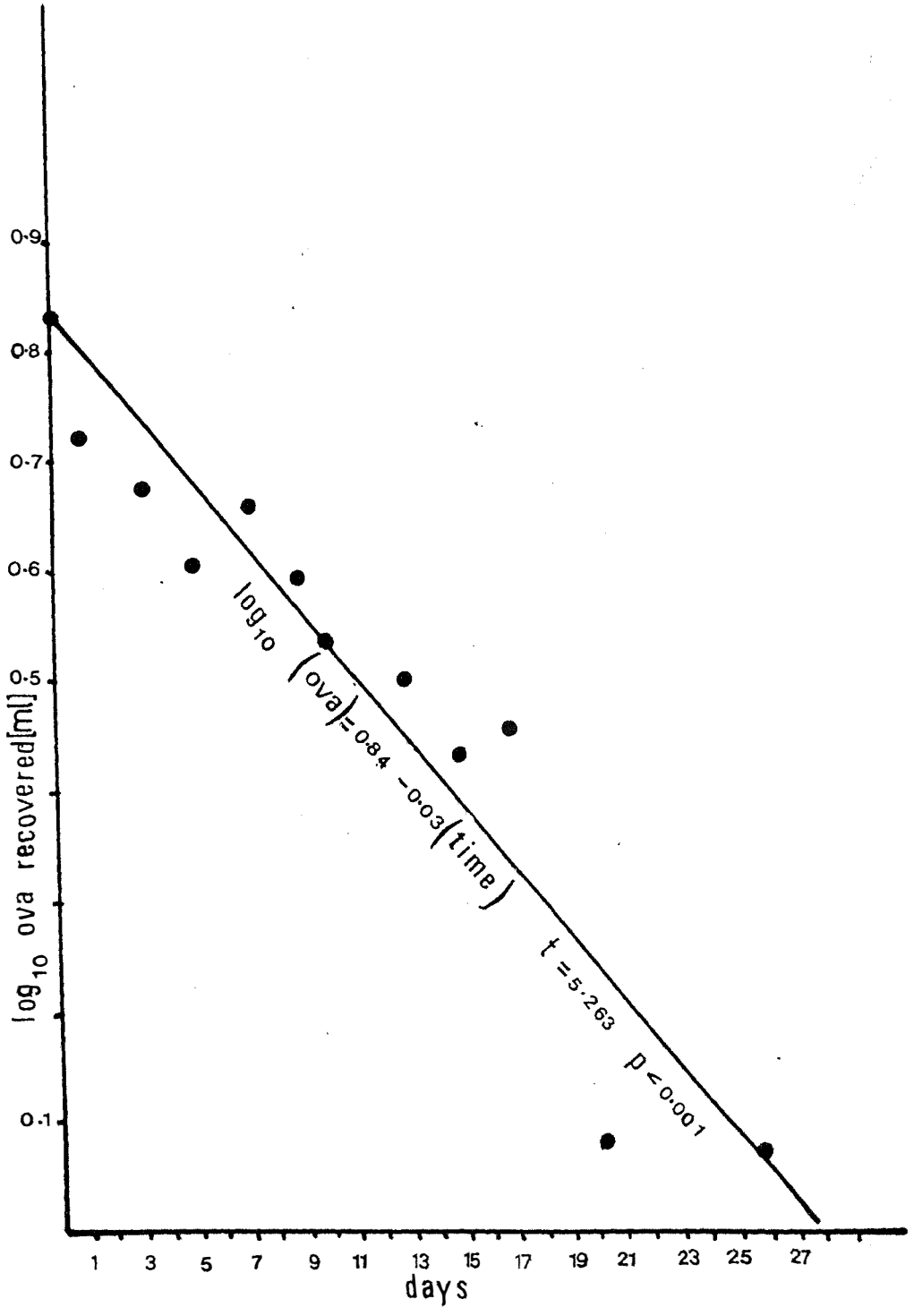


FIG: 16b

RECOVERY OF ASCARID OVA FROM FIELD LYSIMETERS, WINTER 1979

(REPEAT)



sludge and soil taken from the surface of the lysimeters fell rapidly in summer and no ova were recovered after 21 days. In the repeated winter/spring trials the number of recovered Ascaris ova declined at the same rate as in summer, but ova were recovered for 30 days in this instance. The graphs for recovery of Ascaris ova have the same gradients for winter and summer trials indicating that the rate of decline in numbers of ova was equal, although ova persisted a week longer during the winter/spring trials. Figure (17a) and (17b) show the recovery data for Taenia ova presented in a semi-log form. From an initial value of 10 ova per ml of sludge the numbers of recovered ova diminished rapidly in both winter/spring and summer trials as shown by the large negative gradient (-0.07 for both trials). Taenia ova were recovered for 12 days in both trials.

The relative persistance of ova in soil and sludge samples was compared by calculated parameter termed the biological half life ($t_{\frac{1}{2}}$) which is a measure of the time taken for a 50 percent reduction in ova to occur. The $t_{\frac{1}{2}}$ values, and regression data, are summarised in table (15).

Taenia ova had a low $t_{\frac{1}{2}}$ value (4.3 days) by comparison with Ascaris ova which had a $t_{\frac{1}{2}}$ of 10 days. These values applied to both winter/spring and summer trials, so that although Taenia had a lower $t_{\frac{1}{2}}$ than Ascaris ova, neither species showed a difference in $t_{\frac{1}{2}}$ values between winter/spring and summer trials.

FIG: 17a

RECOVERY OF TAENIID OVA FROM FIELD LYSIMETERS

SUMMER 1979

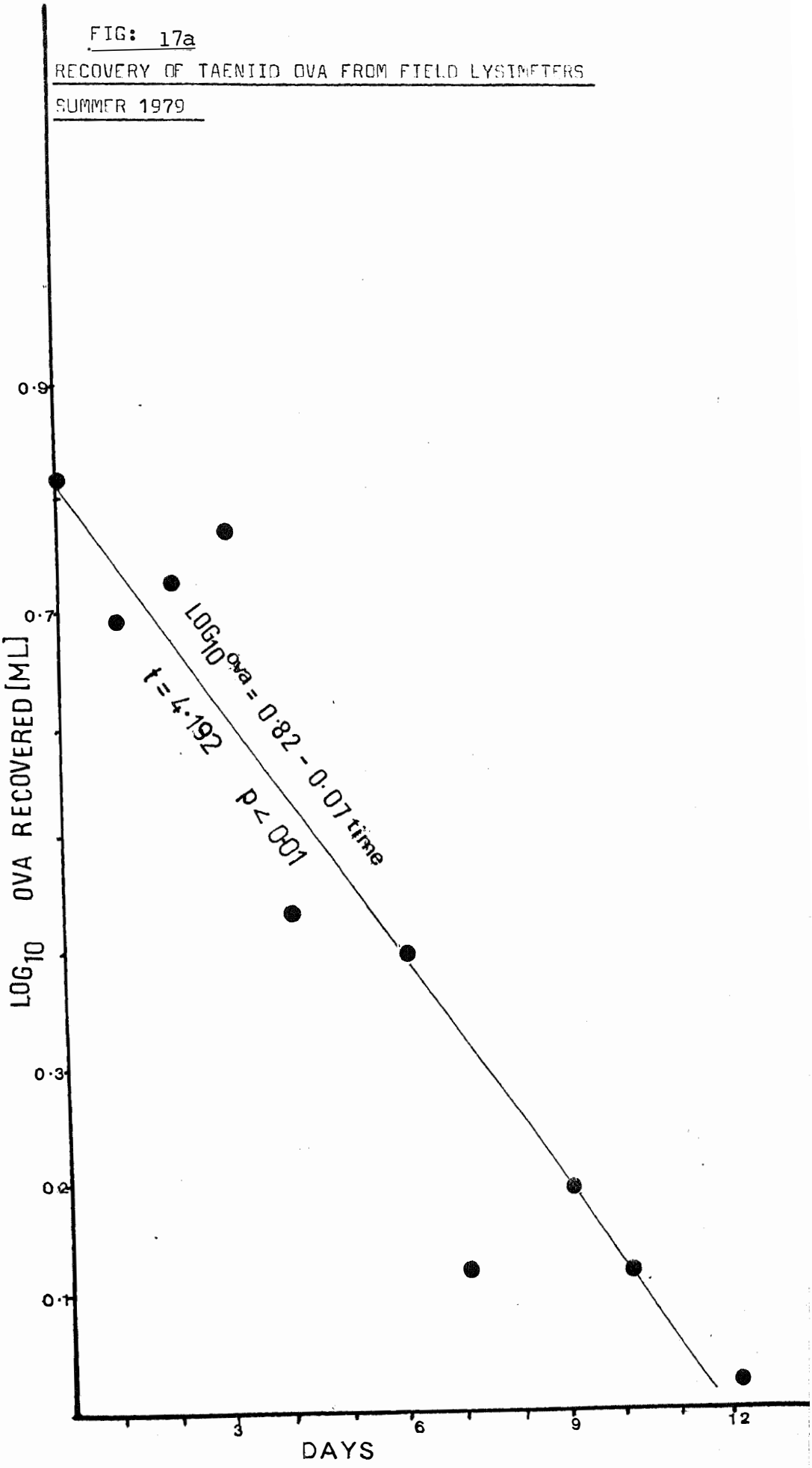


FIG: 17b

RECOVERY OF TAENIID OVA FROM FIELD LYSIMETERS

WINTER

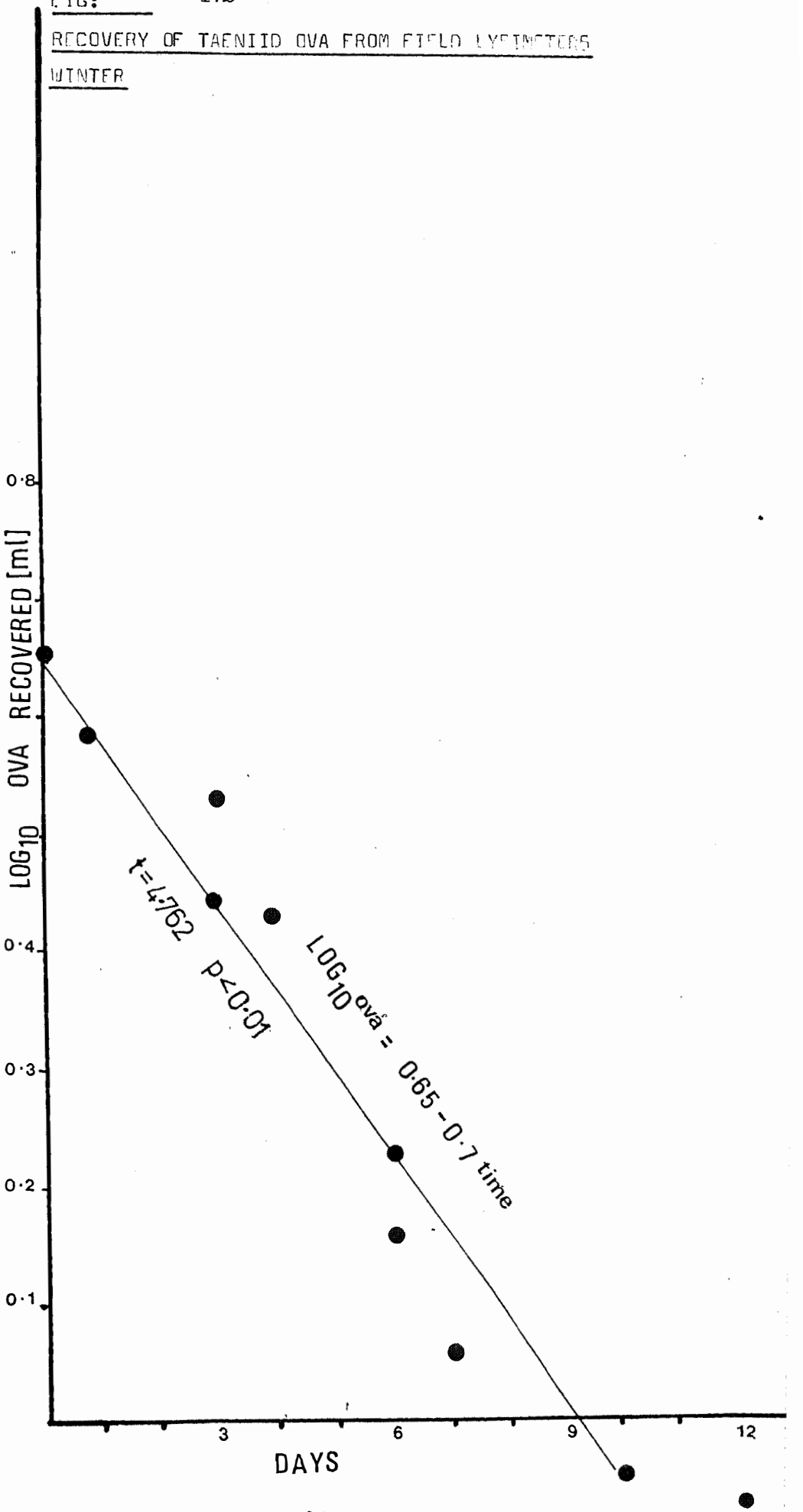


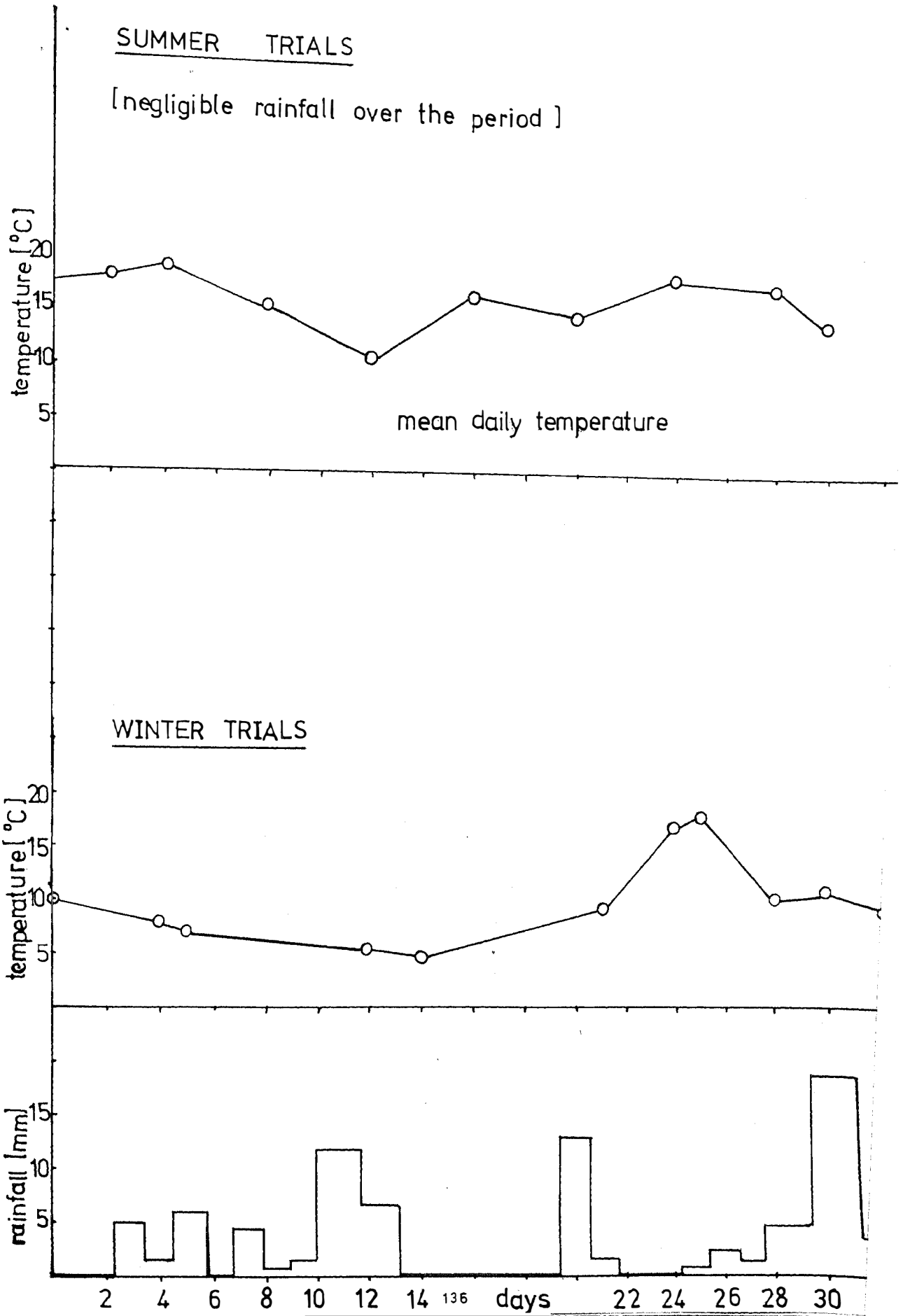
Table 15

Recovery of Ova from in situ Lysimeters

	Recovered for	$t_{\frac{1}{2}}$	Gradient	Regression Equation
Winter Trials Taenia	12 days	4.3 days	m = -0.07 t = 4.762 p < 0.01	$\log_{10}$ (ova) = 0.65 - 0.07 (time
Summer Trials Taenia	12 days	4.3 days	m = -0.07 t = 4.192 p < 0.01	$\log_{10}$ (ova) = 0.82 - 0.07 (time)
Winter Trials Ascaris	30 days	10.0 days	m = -0.032 t = 5.263 p < 0.001	$\log_{10}$ (ova) = 0.84 - 0.03 (time)
" Summer Trials	21 days	10.0 days	m = -0.03	$\log_{10}$ (ova) = 0.70 - 0.03 (time) t = 5.405 p < 0.001

FIG: 18

CLIMATIC CONDITIONS DURING LYSIMETER TRIALS



D. Movement ofeOva through Soil

1. Introduction

During the lysimeter trials no ova were recovered from soil leachate, yet ova disappeared quickly from the surface of the lysimeters, suggesting that ova had moved a distance greater than 3cm (but less than 63cm, since no ova were recovered from soil leachate). To investigate whether ova had been moved through soil by percolating water, an experiment was set up in which inoculated soil cores were subjected to a known rate of watering. Both soil leachate and soil samples from different depths could then be examined for the presence of ova.

2. Methods

The soil cores were set up in vertically positioned glass cylinders. These were made from 1cm diameter glass tubing cut into 10cm lengthse each lined with nine 1cm rings cut from propylene tubing. The top 1cm of each core was inoculated with approximately 50 Taenia or Ascaris ova. Cores were watered for 24 hours at a fixed rate controlled by screw clips constricting the watering tubes. Gauze was used to support the cores.

Collected leachate was centrifuged at 2000 rpm for 10 minutes and the sediment examined microscopicallye The soil cores were removed sequentially by pushing the tube rings from the supporting glass cylinder. Each layer of soil was then examined, for ova using the previously described antiformin-sucrose technique.

3. Results

Recoveries of ova from the soil cores and leachate are summarised in table (16). No ova were recovered from leachate samples. Soil from the first two layers (0 - 2cm depth) were pooled in case ova had been inadvertantly mixed into the deeper layer during inoculation. Ova were recovered down to a maximum of 5cm depth but not from any deeper layer. It is possible that a slight settling of watered soil could account for some of the downwards displacement of ova, for example, ova at 2cm depth could then have been recovered in the 2cm - 3cm layer of soil after 24 hours.

Table 16

Recovery of ova from soil cores and from leachate

Ova	Watering rate ml/min	Ova present at depth;								
		0.2cm	3cm	4cm	5cm	6cm	7cm	8cm	9cm	Leachate
Ascaris	0.33	+	+	+	-	-	-	-	-	-
Ascaris	0.33	+	+	-	-	-	-	-	-	-
Taenia	0.33	+	+	+	-	-	-	-	-	-
Taenia	0.33	+	+	+	-	-	-	-	-	-
Ascaris	0.67	+	+	+	-	-	-	-	-	-
Taenia	0.67	+	+	+	+	-	-	-	-	-

(+) indicates ova present

(-) indicates no ova recovered

D. Discussion

Although no Ascaris ova were recovered after 21 days in summer or 30 days in winter/spring trials, and no Taenia ova were recovered after 12 days in either trial, this need not imply that no ova were present. Ova would probably not disintegrate so rapidly and it is considered that two factors could account for the observed drop in numbers of ova recovered. Firstly, ova could have passed into the 63cm of soil beneath the 3cm sampled. Secondly as will be demonstrated, the probability of recovering ova declines rapidly with decline in numbers of ova per sample, thus compounding the observed reduction in recovered ova.

The probability of detection of ova in a sample is directly related to extraction efficiency and from an analysis of the level of infestation the problems associated with recovery of ova can be demonstrated. Recovery of ova from a mixed bulk sample should fit the Poisson distribution and it follows from this, that the accuracy of each count will depend on its size. The difficulties inherent in recovering ova from samples containing different densities of ova are demonstrated in table (17). This summary shows that virtual certainty of detection is not reached until density of ova in soil approaches 4×10^5 ova per hectare. Below this level of infestation the probability of detecting ova declines until, at the level of 1×10^5 ova per hectare there is only a 46.3 percent chance of detection. At a level of 1×10^4 ova per hectare, which is still a significant burden in the soil, the chance of detecting ova in a 10 gram sample is only 8.3 percent. Many samples which are