

METHODS AND TECHNIQUES FOR NEMATODOLOGY

By J. van Bezooijen

Revised version 2006

PREFACE

This Manual 'Methods and Techniques for Nematology' is based on the 'Manual for Practical Work in Nematology' (s'Jacob & van Bezooijen 1984), which has been thoroughly revised. The 'Practicumhandleiding Nematologie' (van Bezooijen & Ettema 1996) was the basis for even further revision. The final product, which you will be using now, does not pretend to be complete, but is an augmented and in many parts corrected version of the earlier manuals.

The author is greatly indebted to A. Raeymaekers for preparing the final layout and for her useful proposals to increase the readability. Her impressive computer skills contributed substantially to a higher quality of the book.

Additional improvements and corrections may remain necessary and the author welcomes any comments and suggestions. In spite of this, hopefully this manual will be useful to you

Wageningen, December 2006

CONTENT

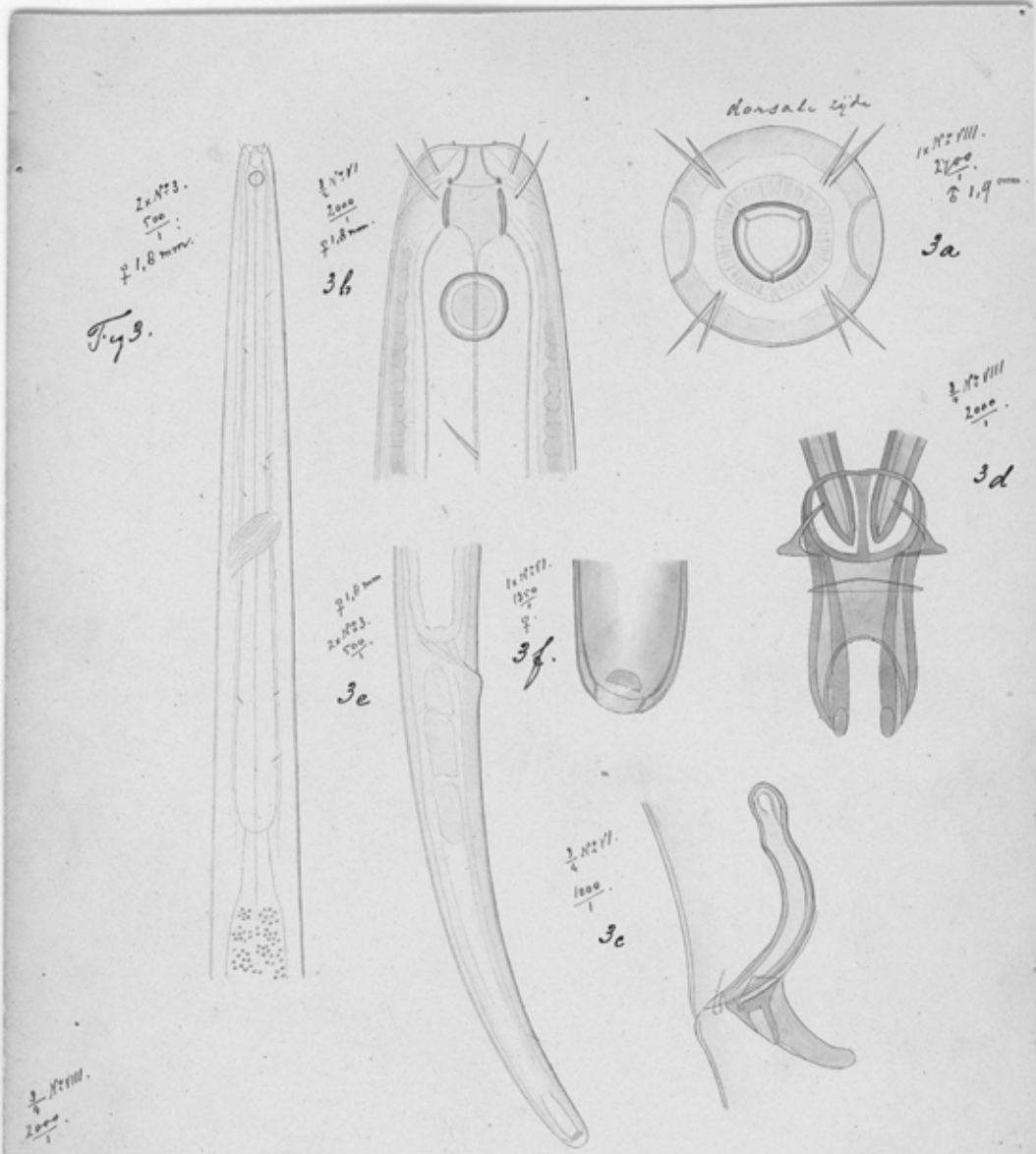
PREFACE

CONTENT

CHAPTER 1. SAMPLING	2
1.1. PRACTICAL AND THEORETICAL CONSIDERATIONS	2
1.1.1. Purpose and accuracy of sampling	2
1.1.2. Variation in space: horizontal and vertical	2
1.1.3. Variation in time: seasonal fluctuations and life cycles	3
1.1.4. Statistics	3
1.2. THE SAMPLING PROCESS	4
1.2.1. Tools	4
1.2.2. Sampling patterns	5
1.2.3. Examples of sampling methods	5
1.3. TRANSPORT AND STORAGE	8
1.3.1. Fixing samples in the field	8
1.4. PRE-PROCESSING OF THE SAMPLE	8
1.4.1. Measuring: volume or weight?	8
1.4.2. Taking sub-samples; Mixing	9
1.4.3. Pre soaking of soil samples	10
1.4.4. Drying	10
CHAPTER 2. EXTRACTION	11
2.1. INTRODUCTION	11
2.1.1. Principles	11
2.1.2. Extraction from plant material	11
2.1.3. Extraction from soil and other substrates	12
2.1.4. Extraction of cysts	12
2.1.5. Selection of an extraction method	14
2.2. EXTRACTION FROM PLANT MATERIAL	18
2.2.1. Dissection	18
2.2.2. Baermann funnel	19
2.2.3. Funnel spray method	21
2.2.4. Blender nematode filter method	23
2.2.5. Blender centrifugal flotation method	25
2.3. EXTRACTION FROM SOIL AND OTHER SUBSTRATES	27
2.3.1. Nematode cotton wool filter method	27
2.3.2. Decanting and sieving: Cobb's method	30
2.3.3. Erlenmeyer- or (milk) bottle method	34
2.3.4. Oostenbrink elutriator	37
2.3.4.1. Standard use	38

2.3.4.2.	Modification for the extraction of large nematodes	42
2.3.4.3.	Modification for the extraction of stem nematodes	43
2.3.5.	Centrifugal flotation method	46
2.3.5.1.	Modification for the extraction of fixed samples	51
2.4.	EXTRACTION OF CYSTS	52
2.4.1.	Baunacke method	52
2.4.2.	Fenwick can	54
2.4.3.	Kort's cyst extraction elutriator	56
2.4.4.	Seinhorst cyst extraction elutriator	58
2.4.5.	Isolating cysts from the debris	60
2.4.5.1.	Isolating cysts with organic solvents	60
2.4.5.2.	Isolating cysts with the centrifugal flotation method	62
2.5.	ANALYSIS OF EXTRACTS	64
2.5.1.	Counting of nematodes in the suspension	64
2.5.2.	Estimating and counting the cyst content	65
2.5.3.	Presentation of numbers	66
	CHAPTER 3. PROCESSING	68
3.1.	PICKING NEMATODES	68
3.2.	CONCENTRATING NEMATODE SUSPENSIONS	68
3.3.	ANAESTHETIZING	69
3.4.	KILLING AND FIXING	69
3.4.1.	Mass fixation of suspensions	70
3.4.2.	Recipes of fixatives	71
3.5.	TRANSFERRING TO GLYCERIN	72
3.5.1.	Glycerin-ethanol method	72
3.5.2.	Glycerin-formalin method	73
3.6.	MOUNTING	74
3.6.1.	Materials	74
3.6.2.	Temporary mounts	76
3.6.2.1.	Mounts in water	76
3.6.2.2.	Mounts in lacto phenol	76
3.6.3.	Permanent mounts	77
3.6.3.1.	Mounts in glycerine	77
3.6.3.2.	Mass mount	78
3.6.3.3.	Mount of perineal patterns	79
3.6.3.4.	Mount of the vulval cone	80
3.6.3.5.	Mount of the head and cross sections	81
3.6.3.6.	Perspex slides for mounting cysts	83
3.7.	STAINING OF ROOT MATERIAL	84
3.7.1.	Staining in lacto phenol-cotton blue or lacto phenol-acid fuchsine	84

3.7.2.	Staining in Lugol's solution	85
3.8	HISTOLOGY	86
3.8.1.	Preparing the plant material for making microtome sections	86
3.8.2.	Cutting sections with the microtome	88
3.8.3.	Staining of microtome sections	89
3.8.4.	Recipes for histology	92
CHAPTER 4. IN VITRO CULTURING OF NEMATODES		93
4.1.	THE VINEGAR EELWORM <i>Turbatrix aceti</i>	93
4.2.	THE FREE-LIVING NEMATODE <i>Panagrellus redivivus</i>	93
4.3.	RHABDITIS	93
4.4.	THE CHRYSANTHEMUM FOLIAR NEMATODE <i>Aphelenchoides ritzemabosi</i>	94
4.5.	POTATO CYST NEMATODES <i>Globodera rostochiensis</i> or <i>G. pallida</i>	94
4.6.	THE ROOT-KNOT NEMATODE <i>Meloidogyne</i> spp.	95
CHAPTER 5. MICROSCOPY		97
5.1.	INTRODUCTION	97
5.2.	THE DISSECTING MICROSCOPE	98
5.3.	THE COMPOUND MICROSCOPE	100
5.4.	MAINTENANCE OF MICROSCOPES	102
5.5.	MEASURING WITH THE MICROSCOPE	102
5.6.	NEMATODE MEASUREMENTS (Ratios)	103
CHAPTER 6. OTHER TECHNIQUES		104
6.1.	CONCENTRATING AND SURFACE STERILIZING OF NEMATODE SUSPENSIONS	104
6.2.	QUANTITATIVE EXTRACTION OF RESTING SPORES OF NEMATODE PARASITIC FUNGI BY MEANS OF A CENTRIFUGAL FLOTATION METHOD	105
6.3.	PHYTOTOXICITY TEST	107
LITERATURE		108



Monohystera stenosoma n. sp.

J. G. de Man del.

III

Original drawing by J.G. de Man (1850-1930)

CHAPTER 1. SAMPLING

1.1. PRACTICAL AND THEORETICAL CONSIDERATIONS

When making a sampling plan for estimating nematode numbers, biomass and species composition, a number of practical and theoretical considerations must be observed. The design of the sampling plan will partly depend on the purpose of the study, the required accuracy, the time frame, as well as the costs involved. Besides, when making the plan, knowledge about the variation in space and time of nematode populations, their biology, and the influence of (a)biotic factors has to be considered, to arrive at a correct choice regarding timing and depth of sampling, tools, number of samples and the sampling pattern.

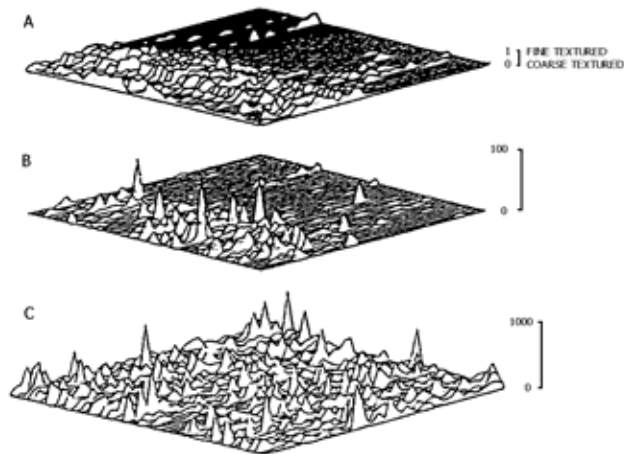
1.1.1. Purpose and accuracy of sampling

The degree of required accuracy depends on the sampling purpose. In case of qualitative studies, such as diagnosis of diseased plants or taxonomic studies, the required accuracy is relatively low and a simple sampling plan is sufficient. In case of an ecological study to establish the species composition of a nematode population, sampling needs to be done more accurately, as rare species need to be included in the study. The required level of accuracy further increases if the sampling purpose is to advise on the need for control measures or crop choice, and for the determination of biomass. A very high level of accuracy is needed if sampling is done to issue a phytosanitary certificate as part of legal requirements and inspection regulations, which mandate that plants for multiplication or export purpose are free of infection (so-called 'zero tolerance'). A higher accuracy is generally more expensive and time consuming because more samples need to be taken (see § 1.1.4).

1.1.2. Variation in space: horizontal and vertical

One of the biggest problems while sampling soil nematodes is their distribution, which is not at random, but clustered. Many physical, biological and agronomic factors may play a role in nematode distribution patterns. Examples are: concentration of *Helicotylenchus digonicus* in fine-textured soil (Fig. 1), high numbers of bacterivorous nematodes in a dung heap, and a localised infestation of plant parasitic nematodes caused by planting of an infected tuber. If factors such as soil texture or cultivation history are known, sampling different field patches separately will reduce the variation per patch (Fig. 1).

Spatial variation refers not only to horizontal but also to vertical distribution of nematodes. Depending on root growth, soil texture and nematode species, nematodes can be found up to a few metres of depth. Generally the major share of nematodes can be found in the upper 0 - 25 cm of the soil, i.e. in the area where the bulk of plant roots are located, or at ploughing depth (furrow). For that reason, the sampling depth on cultivated land is mostly the furrow depth. With trees, sampling is done at greater depths, e.g. for *Xiphinema* or *Longidorus* the sampling depth may be up to 40 cm.



Three-dimensional projections show distribution of (A) soil texture, (B) *Helicotylenchus digonicus*, and (C) *Meloidogyne arenaria* in a 17-acre alfalfa field.

Figure 1.
(From Goodell & Ferris, 1980).

1.1.3. Variation in time: seasonal fluctuations and life cycles

Sampling results are influenced not only by spatial variations but also by large temporal variations of nematode populations. These can be seasonal fluctuations caused by different temperature, moisture, and food conditions. For example, *Rhabditidae* has a population peak in autumn, when decomposition of crop residues and fallen leaves starts. Other species have high population numbers in summer, when temperatures are high and food is abundant as a result of root development. Knowledge of the life cycle of the nematodes to be sampled – especially if the nematodes are plant parasites – is required to choose the ‘optimum’ time of sampling. For example, for the purpose of a population study it is not useful to carry out soil sampling in a standing crop. At that time, ectoparasites are concentrated in the rhizosphere of the crop, while endoparasites are not present in the soil but in the roots. A population study based on soil samples only results in a bias away from the endoparasitic nematodes.

The number of endoparasites plant parasites in the soil is highest late in the growing cycle or immediately after harvest (this applies for temperate regions). In more tropical areas minimum and maximum population levels may occur at other times. In general, the drier and warmer the climate, the fewer nematodes are present. Also, the type of crop and cultivation practices influence population development.

1.1.4. Statistics

The aim of sampling is to *estimate* the nematode number or biomass in a particular field plot as accurately as possible. The level of inaccuracy of this process is called ‘sampling error’, which can be expressed in terms of variance and standard deviation. The different phases in the sampling process (taking cores, mixing individual soil samples, taking sub-samples, washing and analysing sub-samples of the suspension) all add a component of variance to the total sampling error (Alkemade 1993).

The errors introduced in each phase can be differentiated into systematic and random errors. Systematic errors (e.g. losses caused by using a soil sampler or auger that is

too narrow) can be partly avoided by working *accurately*, but cannot be totally excluded because the methods are never 100% efficient (e.g. extraction methods). Random errors can be reduced by taking *a higher number of (sub)samples or larger (sub)samples* and could in theory be prevented by digging off the whole area, mixing and stirring all the collected soil and analysing the whole suspension. Apart from the fact that this is not practical and too expensive, it would not exclude systematic errors and might even increase them.

By balancing the shares that each of the phases of the sampling process contribute to the total sampling error it is possible to search for an optimum, characterised by a combination of highest possible accuracy and lowest possible costs (Alkemade 1993). For example, due to the fact that the variance between samples is always greater than within a single sample, it is preferable to dedicate more time on taking many samples (replicates) than on analysing many sub-samples of a particular sample. Taking more samples does not only have statistical advantages; it also provides more information about location or distribution of an infestation in the field plot (also see § 1.2.2).

1.2. THE SAMPLING PROCESS

1.2.1. Tools

Sampling can be carried out using a variety of tools, ranging from a cigar tin or an ordinary spade to special soil samplers or augers (fig.2). The type of sampling device depends on the purpose of sampling. Usually half-open or closed soil samplers of varying length and diameter are used. One sample consists of several cores. Since the level of accuracy increases with the number of cores, it makes sense to take out only a small amount of soil per core, thus limiting total sample size. However, the diameter of the soil sampler or auger should not be too small, since too much friction harms the nematodes. A 17 mm auger is generally used. If vulnerable nematode genera of the *Trichodoridae* and *Longidoridae* families are to be sampled, it is recommended to use a sampling tube with a very wide diameter, e.g. a preserving can with both lids removed (see 'Stretcher'-sampling, p. 7). The soil sampler must be inserted at a straight angle and be given a quarter or half turn before retracting, so that the soil will stick in the tube. Equipment always needs to be clean to avoid contamination from a previously sampled plot, and to prevent a 'second degree error' (wrongly conclude that nematode species A attached to a dirty tool is present in the plot). Apart from the described sampling tools, sketchpaper and a pencil are used to record other details (the condition of the crop, the weather etc.).

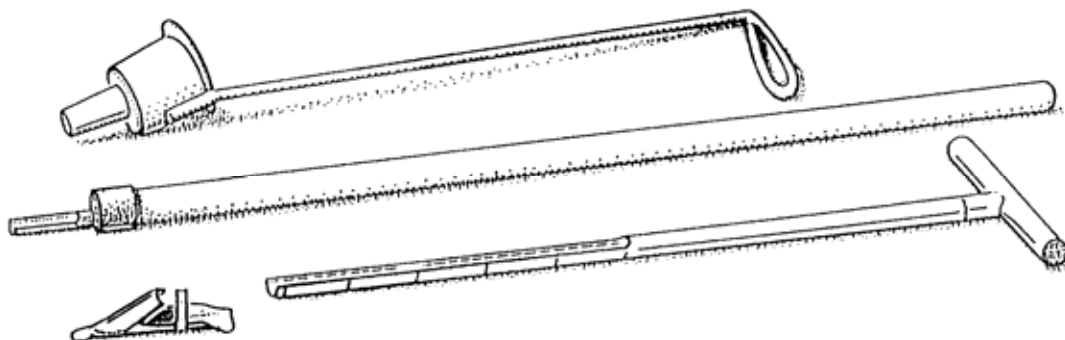


Figure 2.
Soil sampling tubes.

Soil sampler for pastureland, for chemical research
Sampler for researching potato cyst nematodes, 5 cm depth auger
Soil sampler suitable for arable land, different applications with a soil scraper

1.2.2. Sampling patterns

Sampling can be carried out at random or systematically. Theoretically, random sampling (if really carried out in a random fashion!) is best as it excludes bias; by sampling in a haphazard and criss-cross way samples can be considered independent observations. In practice, however, sampling is usually carried out in a systematic way because it requires much less time (R. Alkemade, pers. comm.). In this case the samples are taken along a line or at points on a grid, at a fixed distance from each other. Prior to sampling a field plot may be divided into strips or blocks (e.g. based on knowledge about divergent soil texture; see § 1.1.2.) and after that the sampling can be carried out either randomly or systematically.

1.2.3. Examples of sampling methods

- 1) **Diagnosis:** When carrying out a diagnosis of diseased plants, three types of samples need to be taken: diseased plants in the middle of an infested field patch, the surrounding plants showing poor development, and healthy plants outside the patch. Root, stem, and leaf material, and cores from the surrounding soil are collected for each sample. All symptoms and signs of disease have to be described and all available information about factors influencing crop growth needs to be collected.
- 2) **Quadrant sampling** (amongst others applied for ecological studies in natural systems): a 10 x 10 metre area is marked out and a sample, consisting of 50 cores, is taken from this area. An auger of 10 cm length and a diameter of 1.7 cm, yields a sample of approximately 1 litre (> 1 kg soil). Replications within a quadrant are obtained by taking, for example, five samples consisting of 10 cores each.
- 3) **Sampling for damage prevention advice:** the intensity, size, and depth of sampling depends on the nematode species, the crop (Fig. 3), and on national rules (Table 2.2 from McSorley 1987). Sampling carried out for advisory purposes by BLGG (“Bedrijfslaboratorium voor Grond en Gewasonderzoek”, Oosterbeek, the Netherlands) is done on fallow land 2-3 weeks after harvest. The samples consist of 60-70 cores taken at a fixed distance from each other, following a grid pattern. Density of the grid and number of samples per hectare increase if the research has to be done more thoroughly (e.g. in case of an expensive crop where economic damage is expected to be higher). Fig. 4 shows an example of advisory sampling on arable land; a sample consists of 70 cores.

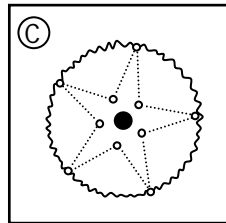
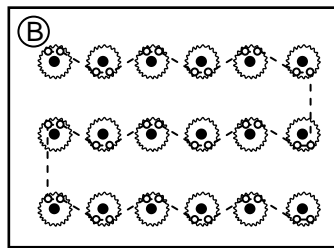
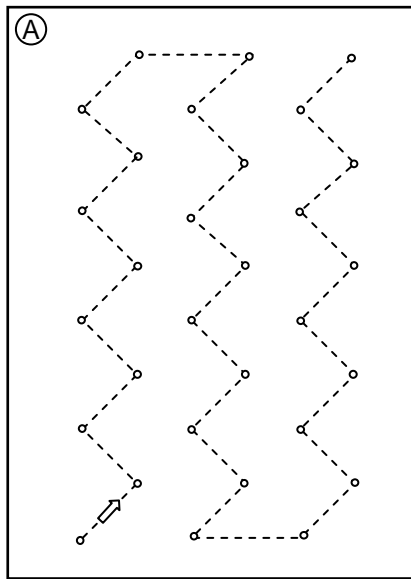


Figure 3.

*Different patterns of sampling.
A) on fallow land B) in an orchard C) for a single plant/tree
(from Barker et al., 1978)*

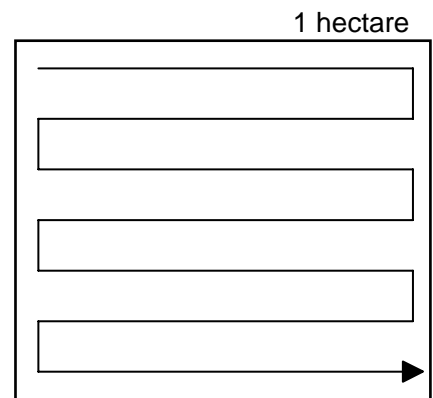


Figure 4.

*Advisory sampling on arable land,
taking one sample of 70 cores per
hectare.*

- 4) Sampling for issuing a phytosanitary certificate: the intensity, size, and depth of sampling depend on the nematode species, the crop and national rules. Fig. 5, for example, illustrates how sampling can be carried out as part of intensive research on potato cyst nematode (“potato sickness”). The grid pattern is very dense and a total of 12 samples of 70 cores each are taken, dividing the field plot into blocks with a maximum length-width relation of 3:1. For this research it is important to discover and localise an infestation in an early stage, so certificate rejection or soil fumigation can be limited to just one section of the field plot.
If the purpose is to obtain a growers contract, sampling for other nematodes, e.g. *Ditylenchus*, is also carried out in an intensive way.

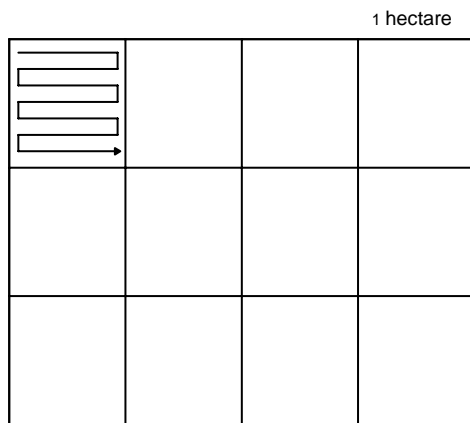


Figure 5.

Sampling as part of intensive research, in which 12 samples per hectare are taken.

- 5) ‘Stretcher’ sampling: this method is done for sampling nematodes which are extremely vulnerable to friction and other types of mechanical damage, such as Trichodoridae and Longidoridae. To carry out this technique, a wide tube is used for sampling (§ 1.2.1) (Fig.6).
 When sampling a plant suspected to harbour these nematode species, a wide tube is placed over the plant and pushed into the soil. The tube, together with the plant and soil are carefully withdrawn and placed in a plastic bag, which is carefully transported to the laboratory, trying to avoid heavy shaking of the sample. In the laboratory the entire sample is washed.

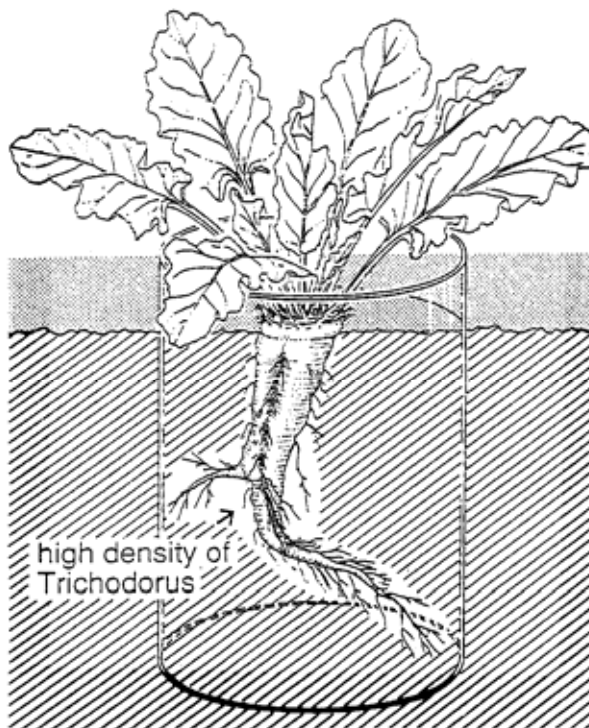


Figure 6.

Sampling of Trichodorus using a wide tube.

1.3. TRANSPORT AND STORAGE

To avoid dehydration, samples are preferably collected and stored in plastic bags or paper bags coated with paraffin, the latter having the advantage that they can be written on, without the need of separate labels. Use a pencil or a permanent waterproof marker for writing on the label or bag, to avoid decoloration and bleaching. Avoid exposure of samples to high temperatures (e.g. leaving them in the boot of a car in the sun); a cooler box is preferred. The samples must be handled with care because nematodes may die from shock and friction. The samples must be processed as soon as possible, because storage may cause changes in nematode numbers and species composition. Data on storage effects mostly refer to plant parasites; increases and decreases in numbers have been observed. Bacterivorous *Rhabditidae* multiply at temperatures as low as 4°C. For temperate regions, a general guideline is to store samples at 4°C and to keep storage time as short as possible. Samples can be stored up to half a year without considerable effects on nematode numbers.

1.3.1. Fixing samples in the field

Fixation of samples in the field is an option if samples cannot be processed directly. In general, formalin (4-10%) is added until the sample is just submerged and the mixture is then stirred or shaken (Elmiligy & de Grisse 1970; Freckman *et al.* 1977). In wet samples, especially samples of sediments, the concentration of formalin sharply decreases due to dilution by the water present in the sample. Fixation of the sample is incomplete if the final concentration drops below 4%.

1.4. PRE-PROCESSING OF THE SAMPLE

1.4.1. Measuring: volume or weight?

The size of a sample can be described in terms of volume or weight, both having advantages and disadvantages. In research for advisory purpose or phytosanitary certification, sample size is generally given as volume (100 or 200 ml soil), while in other studies sample size is usually based on weight (100 or 200 g). Sampling and mixing destroy the natural structure of the soil, altering the degree of soil compaction. For that reason it is difficult to find the amount of soil that would correspond with a particular volume of soil under undisturbed field conditions. Using weight to express sample size has the disadvantage that large differences in the amount of soil per unit of weight occur, due to variations of soil moisture content. This problem, however, can be solved by making a simple measurement of dry matter content (see § 2.5. Analysis of extracts).

Determination of the dry matter content: Take a small sub-sample (± 20 g is sufficient) of the moist soil and weigh. Dry the sub-sample at 105°C until the weight does not decrease any further (Southey 1986) (drying for 24 hours should be sufficient at this high temperature. If a stove reaching this temperature is not available and drying is done at lower temperatures, more time will be required). The weight after drying, divided by the weight before drying gives the dry matter content.

In ecological studies, sample size is sometimes expressed in units of area. Based on nematode numbers encountered, a calculation can be made to estimate nematode density per square metre. For example, x cores with an auger, with width d , results in a sampled area of $x * (\pi * (\frac{1}{2}d)^2)$. A problem when using a half-open (guts) soil sampler is that when taking a core, soil may fall out, so less soil is collected than expected from the value of d . By consequence the formula results in underestimating of nematode density. Nematode numbers of samples taken at different depths cannot be compared because nematode densities vary with depth.

1.4.2. Taking sub-samples; Mixing

To achieve sufficient accuracy, a relatively large sample size is often needed. Usually, these cannot be processed completely, making it necessary to take sub-samples (Note: this represents an extra source of variance!). Before taking a representative sub-sample, the sample needs to be homogenised by mixing. If homogenisation is carried out thoroughly, analysis of one sub-sample is sufficient (Carbonell & Angulo 1979). Mixing the sample can be done mechanically or manually. The sample should be mixed with care as each treatment causes mortality of nematodes.

Mixing by hand causes less damage to the nematodes and is therefore preferred to mechanical mixing. Manual mixing is a useful method for most samples, except for heavy clay soil. Disadvantages of manual mixing are its labour intensity and difficulty to standardise. A procedure suitable for soil or other fine materials (e.g. seed) is to pass the sample through a coarse sieve (mesh size of 0.5-1 cm) to remove stones, roots, etc. Mix the sample on a small sheet ($\pm 1 \times 1$ m) by lifting one sheet corner making the soil roll over to the opposite corner. Do the same with all corners and repeat a fixed number (generally six) of times. Take a sub-sample of the desired volume or weight, using small scoops from different parts of the sample (*in principle* it is possible to obtain a representative sub-sample by taking small scoops from different parts of the sample without previous mixing! This may be preferred when vulnerable nematodes are expected, e.g. *Trichodorus*).

Mechanical mixing is generally not used for taking sub-samples, but for homogenising or making a suspension. For plant material, a blender can be used, whereas soil samples are usually mixed with a mixing machine such as the Hobart dough mixer, used at bakeries, which consists of a large batter bowl with a dough blade. Prior to mixing, water is added to the sample until it is just submerged. For mixing heavy clay or large soil samples, sodium hexametaphosphate or other appropriate chemical is added to the sample (a dose of 1% of the soil weight) to break bonds between (clay) particles. Sodium hexametaphosphate is not harmful to most of the Tylenchid nematodes, but its effect on other nematodes is not known.

1.4.3. Pre soaking of soil samples

Usually samples of rock wool, manure, litter and other uncommon substances can be washed without any problem using one of the extraction methods for plant material or soil (§ 2.2 and § 2.3). However, these samples need to be soaked before extraction, to ease the extraction process and increase extraction yield. To soak the sample, it is placed in a bowl, submerged in water and stirred with care. Soaking time depends on the substrate but is at least one hour. The sample can be homogenised with a mixer after soaking (e.g. see Schouten & Arp (1991) referring to litter).

1.4.4. Drying

If extraction requires dried samples, as is the case for some of the cyst extraction methods for cysts, it is best to take a sub-sample before drying, rather than drying the whole sample. The methods described below can also be used for drying debris that remains after a first cyst extraction.

- *Drying at room temperature*: Spread out the (sub) sample out and allow it to dry uncovered. Be aware of draught: dry cysts are easily blown away and lost for the analysis. The airborne cysts may contaminate other samples.
- *Drying in a drying chamber/ drying cabinet*: Keep the (sub) samples in porous (paper) bags. Heat and air circulation (air fans) in the drying chamber accelerate the drying process.
- *Quick drying in a stove*: Spread the sample on a tray or drawer. Drying occurs at a temperature of 40-100°C.

Please note: Drying – especially when carried out at high temperatures, or not gradually – is extremely damaging to the vitality of the cysts contents, notably those of *Heterodera* and *Punctodera* (see § 2.1.4.).

Literature

Proctor and Marks 1975, Barker *et al.* 1978, Elliott 1979, Barker & Campbell 1981, Goodell 1982, McSorley & Parrado 1982, Ferris 1984, McSorley 1987, Prot & Ferris 1992, Alkemade 1993.

2.1. INTRODUCTION

2.1.1. Principles

The easiest way to isolate nematodes from their host material is by submerging the sample in water and select the nematodes under a microscope. However, this is a tedious and laborious job and it can only be done with very small samples. For that reason most of the extraction methods are indirect, making use of a number of properties to separate nematodes from the surrounding medium:

a) *Weight and rate of settling:*

In water, nematodes are separated from particles that settle faster and can subsequently be poured off (decanted). This principle is the basis of a number of applications, such as the use of an undercurrent that keeps nematodes afloat while other particles settle (elutriation) and use of a liquid with higher specific gravity than nematodes, which keeps them afloat while other particles (with a higher specific gravity than the liquid) sink to the bottom. This is applied in centrifuge floatation techniques. Dried cysts contain air bubbles, making them to float on the water surface, which separates them from sinking particles.

b) *Size and shape:*

Because of their size and elongated shape, nematodes can be separated from other (soil) particles by using a set of sieves with different mesh size.

c) *Mobility:*

Because of their mobility, living nematodes can be separated from other particles. When a sample is placed on a sieve with a moist filter paper, positioned in an shallow water-filled tray, nematodes will crawl from the sample into the water where they can be collected as a clear suspension.

Many extraction methods are based on a combination of these principles (see Table 2.1).

2.1.2. Extraction from plant material

Nematodes are present in many types of plant material. Plant parasites are not only found in roots but also in tubers, bulbs, stems, leaves, and seeds. Substrates such as litter, moss, or compost also often contain high numbers of non-parasitic (saprophagous) nematodes. Extraction methods for plant material (Fig. 7) are usually based on nematode mobility. These methods vary depending on whether or not the samples have been cut in smaller pieces to speed up crawling out of nematodes. The blender centrifugal flotation method is the only method, apart from picking nematodes under a microscope, which does not make use of nematode mobility, making it suitable for the extraction of swollen endo-parasitic stages and eggs.

2.1.3. Extraction from soil and other substrates

Extraction methods for soil (Fig. 7) are also frequently used for sediments, rock wool, manure, and other substrates which may harbour nematodes and can be washed into suspension (also refer to § 1.4.3). Most methods use a combination of different principles. There are major differences in terms of extraction efficiency, size of the sample that can be handled, and costs (Table 2.1). The centrifugal flotation method is the only method, apart from picking nematodes under a microscope, that allows isolation of active as well as slow-moving and inactive nematodes.

<u>PLANT</u>							
Direct	Sort out under a microscope after dissection (if desired apply a staining technique first)						
Indirect	<ul style="list-style-type: none"> - <i>Active nematodes</i> <ul style="list-style-type: none"> ▪ Baermann funnel ▪ Funnel spray method ▪ Blender nematode filter method - <i>Inactive + active nematodes</i> <ul style="list-style-type: none"> ▪ Blender centrifugal flotation method 						
<u>SOIL etc.</u>							
Direct	Sorting out in water using a microscope						
Indirect	<table style="border: none; width: 100%;"> <tr> <td style="border: none; vertical-align: top;"> <ul style="list-style-type: none"> - Decanting method - Cobb's method - Erlenmeyer method - Oostenbrink funnel (elutriator) </td> <td style="border: none; vertical-align: middle; font-size: 2em;">}</td> <td style="border: none; vertical-align: middle;">cleaning of the suspension</td> </tr> </table>	<ul style="list-style-type: none"> - Decanting method - Cobb's method - Erlenmeyer method - Oostenbrink funnel (elutriator) 	}	cleaning of the suspension			
<ul style="list-style-type: none"> - Decanting method - Cobb's method - Erlenmeyer method - Oostenbrink funnel (elutriator) 	}	cleaning of the suspension					
Cleaning of the suspension	<table style="border: none; width: 100%;"> <tr> <td style="border: none; vertical-align: top;"> <ul style="list-style-type: none"> - Active nematodes - Inactive + active nematodes </td> <td style="border: none; vertical-align: middle; padding: 0 10px;">→</td> <td style="border: none; vertical-align: middle;">Nematode filters</td> </tr> <tr> <td style="border: none; vertical-align: top;"> <ul style="list-style-type: none"> - Inactive + active nematodes </td> <td style="border: none; vertical-align: middle; padding: 0 10px;">→</td> <td style="border: none; vertical-align: middle;">Centrifugal flotation method</td> </tr> </table>	<ul style="list-style-type: none"> - Active nematodes - Inactive + active nematodes 	→	Nematode filters	<ul style="list-style-type: none"> - Inactive + active nematodes 	→	Centrifugal flotation method
<ul style="list-style-type: none"> - Active nematodes - Inactive + active nematodes 	→	Nematode filters					
<ul style="list-style-type: none"> - Inactive + active nematodes 	→	Centrifugal flotation method					

Figure 7
Extraction methods for plant and soil samples

2.1.4. Extraction of cysts

Special methods are developed for extracting cysts, because their size, shape, and weight differ a lot from other nematode stages. Distinction can be made between extractions from wet or dry soil, also referred to as 'wet' or 'dry' extraction in short (Fig. 8). 'Dry' extraction is based on the fact that dried cysts (usually) float on water because they contain an air bubble. For a successful extraction, the sample must be completely dry. *Globodera* resists drying, but in *Heterodera* and *Punctodera*, the vitality of the cyst content (eggs and larvae) strongly decreases, especially when the

drying happened too fast. When isolated cysts of these vulnerable species are to be used for further multiplication or inoculation, a 'wet' extraction method is needed. Another disadvantage of the 'dry' extraction method is that not all young (without the definite colouration) full cysts are isolated, because they do not float well. As a consequence, (half) empty cysts are detected more frequently, which results in an underestimation of the population. In 'wet' extraction, an undercurrent keeps the cysts afloat in the suspension, while soil particles settle (elutriation). Even young full cysts can easily be isolated by this method. After extraction, the remainder of the sample often needs to be further cleaned, because it still consists of high amounts of organic matter (see § 2.4.5). Again 'dry' or 'wet' methods can be used to clean the remainder of the sample, depending on the vulnerability of the nematodes and the need of viable cyst contents (Fig 8).

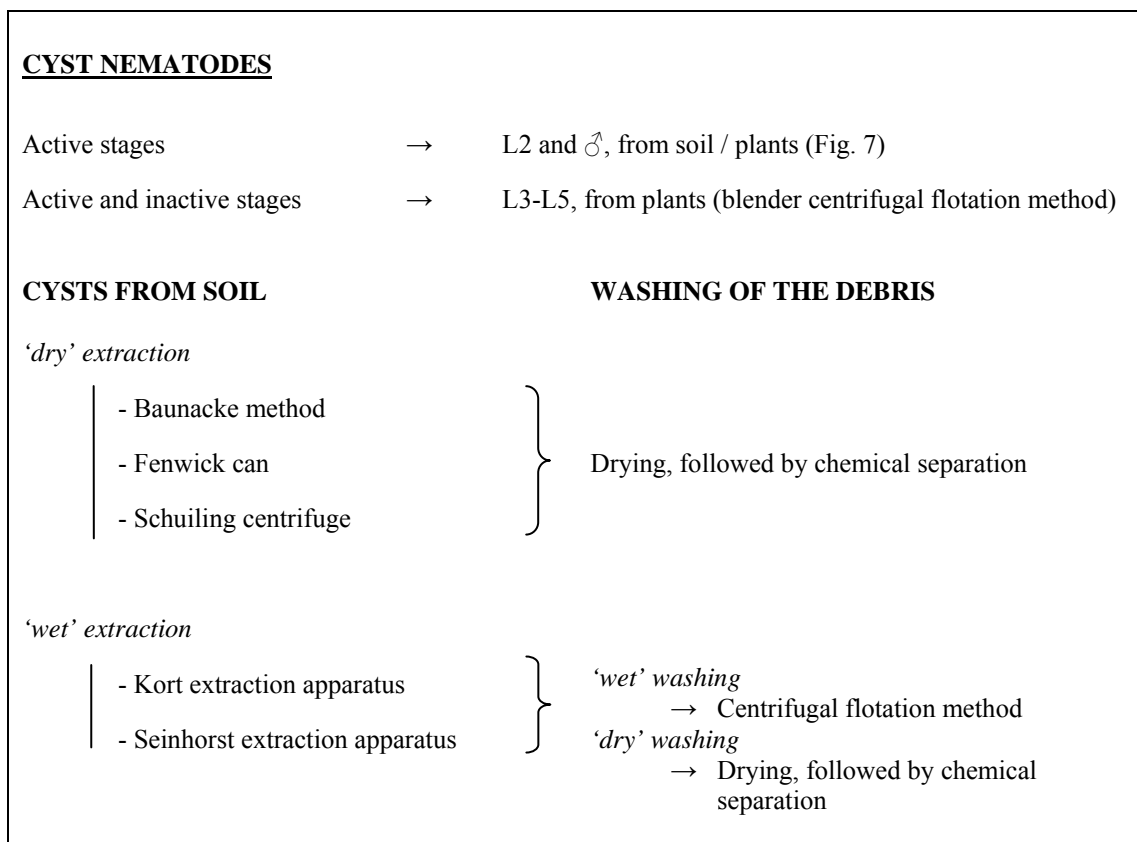


Figure 8
Extraction methods for cyst nematodes

2.1.5. Selection of an extraction method

An ideal extraction method would make it possible to extract all stages of all nematode species at 100% efficiency, irrespective of temperature and soil type, and at low costs (labour, equipment, water) (McSorley 1987). Unfortunately, none of the existing methods comply with this ideal, making it necessary to select the most appropriate method for each and every situation. A problem when selecting a method is that it is difficult to compare different methods. Not only does published data deal with a great variety of nematode species and soil types, the *exact* implementation of a particular method also varies considerably, at local as well as international level. Based on the principles from the first paragraph, it is possible to indicate some trends. Table 2.1, and Table 2.2 (p. 16 and 17) (McSorley 1987) provide information about a number of methods (and about specific species of nematodes):

- For methods based on differences in *weight* and *rate of sedimentation*, the critical moment is when sufficient dirt has settled, but most nematodes are still afloat. Methods that apply an undercurrent (Oostenbrink, Seinhorst, and Kort funnels) are better to control than methods without an undercurrent (decanting, Baunacke, Fenwick), although the first ones require more expensive equipment and larger volumes of water. While the sedimentation rate is not a problem with sandy soils, it is for clay or organic soils. Fine clay particles settle almost as slowly as the nematodes, making it necessary to further treat the decanted or drained suspension (e.g. sieving). The centrifugal flotation methods, using differences in *specific gravity*, are the only suitable methods to isolate slow and inactive nematodes. But these methods are selective, because not all nematodes stay afloat in a fluid of particular density and not all nematodes are resistant to the extraction fluid (dorylaimids are relatively sensitive).
- When using methods based on differences in *size* and *shape* between nematodes and other particles, sieves may clog if the mesh is too small (especially when the soil contains high levels of silt) and nematodes will be washed away and lost if the mesh is too wide. According to Byrd *et al.* (1976) and McSorley & Parrado (1981) a mesh size of 45 µm is too large for capturing small larvae of *Meloidogyne*, *Tylenchus*, or *Rotylenchus*. They recommend 38 µm sieves. In the Netherlands, sets of four sieves of 45 µm are generally used. Small larvae, washed through the first sieve, most probably stay behind on the second or subsequent sieve (also refer to Oostenbrink 1954 and Seinhorst 1956).
- Methods (partly) based on *nematode mobility* do not capture slow and inactive nematodes or eggs. The number of nematodes moving out of a sample depends on extraction duration (see § 2.3.1. Nematode filter method) and sample type. The efficiency usually increases when the debris layer (sludge, root material etc) on the filter or funnel is thin and, for plant material, when the sample is cut prior to extraction, e.g. by using a blender. The ambient laboratory and water temperature may also influence nematode mobility in the sample and therefore numbers in the final suspension. Optimal temperatures for mobility may differ between nematode species (McSorley 1987). Even the prevailing seasonal temperature at the time of collection can have an effect. Barker *et al.* (1969) suggest that the extraction yield is lower in winter because of the inactivity of nematodes at low temperatures. Finally,

a number of nematode species prefers moving up instead of crawling down (so-called negative geotaxis), so they will never be found in the extraction disk (*Turbatrix aceti*, the vinegar eelworm, *Rhadinaphelenchus*, the redring nematode, and a number of insect parasites).

Apart from these considerations, extraction yield also depends on the type of soil and properties of nematode species. Generally, for many methods applies that isolating nematodes out of clay or organic soils is harder than from sandy soils. Like nematodes, clay and organic particles float, clog the sieves, and contaminate the final suspension. Extraction efficiency diminishes with increasing sample size. Table 2.2. provides some extraction guidelines for a number of nematode species.

Finally, there are big differences between methods in terms of costs (equipment, labour, water) (Table 2.1). Generally speaking, methods only using the mobility of nematodes are cheapest, whereas methods involving an undercurrent (elutriation) are more expensive. These methods, on the other hand, often have much higher extraction efficiency, even for larger samples.

Literature

Oostenbrink 1970, Ayoub 1980, McSorlay 1987

Table 2.1
Features of different extraction methods

EXTRACTION METHOD	Principle (*)	Maximum sample size (g)	Extraction efficiency	Cost of equipment	Labour cost	Water consumption
(for PLANTS)						
Dissection	mb	5	-	-	-	-
Baermann funnel	mb	20	+	+	+	+
Funnel spray apparatus	mb, rs	50	+++	++	+	+++
Blender filter method	mb	50	+++	++	++	+
Blender centrifugal flotation method	sg	50	+++	+++	+++	+
(for SOIL etc.)						
Nematode filter method	rs, mb	50	+	+	+	+
Cobb's method	rs, ss, mb	100	+++	++	+++	+
Erlenmeyer method	rs, ss, mb	100	++	+	+++	+
Oostenbrink elutriator	rs, ss, mb	250	+++	+++	++	++
Centrifugal flotation method without pre-extraction	sg	50	+	+++	++	+
Centrifugal flotation method with pre-extraction	rs, (ss), sg	250	+++	+++	+++	+ / +++
(For CYSTS)						
Baunacke method	ss, da	50	+	-	+	+
Fenwick can	ss, da, rs	300	++	++	++	+++
Schuling centrifuge	sg, da	200	++	+++	++	+
Kort extraction apparatus	rs, ss	300	+++	+++	++	+++
Seinhorst extraction apparatus (cysts)	rs, ss	300	+++	+++	++	+++

(*): mb: mobility (nematode filters, Baermann versions)
rs: rate of settling (decanting, elutriation)
da: dry cysts float because of air bubble
ss: size and shape (sieve)
sg: specific gravity (flotation methods, Schuling centrifuge)

- Not relevant (or very small/low)
+ Small (low)
+++ Big (high)

Please note: gradations have been roughly estimated

Table 2.2

Some extraction methods for a number of genera and groups of nematodes (based on Table 2.4, McSorley 1987)

NEMATODE GENUS OR GROUP	EXTRACTION PROBLEM	POSSIBLE SOLUTION
<i>Aphelenchoides</i> <i>Ditylenchus</i>	Present in uncommon substrates	Funnel spray apparatus
Please note: <i>Rhadinaphelenchus</i>	They 'swim' and are therefore lost in a funnel spray apparatus	
Criconematidae	Slow-moving nematodes, low extraction rate when using methods based on mobility	Centrifugal flotation method Longer extraction period
<i>Meloidogyne</i> <i>Rotylenchulus</i> <i>Tylenchulus</i>	Small larvae may be lost during sieving; other stages are swollen	Use an additional 45 µm sieve or an 38 µm sieve at the end
Cysts	Deviating shape, size and weight	See extraction methods for cysts (Fig. 8)
<i>Heterodera</i> <i>Punctodera</i>	Vitality of the cyst content dramatically decreases during drying	If cyst content has to stay alive, use a 'wet' extraction
Trichodoridae	Very susceptible to damage	After carrying out 'Stretcher' sampling (§ 1.2.3), bring the whole sample into suspension, and sieve with care (e.g. top sieve Oostenbrink elutriator)
<i>Longidorus</i> <i>Xiphinema</i> Other large-sized Dorylaimida	Heavy and big, settle quickly	Modification of the Oostenbrink elutriator method (§ 2.3.4.2)
Dorylaimida in general	Susceptible to heavy metals	Avoid sieving and using dishes containing heavy metals (e.g. copper)
	Susceptible to extraction liquid (especially sugar) in (centrifugal) floatation methods	Avoid using sugar and limit the time spent in the extraction liquid; rinse the nematodes thoroughly in water
Rhabditida in general	Fast recoreuction, even at low temperatures; hatching of eggs and dauer larvae	Avoid long extraction periods (> 2 days), if using methods depending on mobility. For studying population structures, the sample must be fixed first.
Some insect parasites (e.g. <i>Fungiotonchium</i>)	Crawl upward in stead of downward, so that they will not end up in the extraction dish if extraction methods depending on mobility are used	Centrifugal flotation method (if desired, fix the samples first)

2.2. EXTRACTION FROM PLANT MATERIAL

2.2.1. Dissection

Area of application

The method is suitable for diagnostic purpose. (Infected) plant material is analysed under a microscope for the presence of nematodes.

Requirements

- Dissecting microscope
- Petri dish
- Dissecting needles, pair of forceps
- Handling ('fishing') needle, painting brush (no. 1)
- Clean water

Procedure

Carefully wash the plant material and place it in a water-filled petri dish. Dissect the sample with dissecting needles and forceps, using a 15-50x magnification. The emerging nematodes, egg masses, etc. can be picked from the suspension with a handling needle (see § 3.1) or painting brush. After 2-3 hours, the sample should be observed again, because active stages of nematodes may have crawled out of the material.

Please note: Detection can be simplified by staining the material, but the advantage of nematode mobility is lost. See § 3.7. 'Staining'

2.2.2. Baermann funnel

Area of application

The Baermann funnel is used for extraction of active nematodes from plant material and soil (see § 2.3.1). The sample size depends on the funnel diameter and the type of material (Fig. 9). If extraction is from soil, the final suspension is dirty.

Principle

The method makes use of nematode mobility. If (infected) plant material is placed in water, nematodes crawl out of the material and sink.

Requirements

Pair of scissors or a knife

Funnel (slope of the sides should be 45°) with a piece of soft silicone tube attached to the stem, closed with a squeezer clip

Piece of cheesecloth

Clean water

Procedure

1. Carefully wash the sample and cut it into ± 1 cm-sized pieces. Take a sub-sample of particular size (e.g. 10 g) and wrap it in a piece of cheesecloth, forming a loose ball.
2. Make sure the funnel is clean. Place the funnel in a stand and fill it with water until it reaches up to ± 1 cm below the rim. Be careful to avoid formation of air bubbles. Make sure that the clip closes well and that the rubber tube does not leak. Tap some water when an air bubble has formed.
3. Hang the cheesecloth with the sample in the funnel so that the sample is totally submerged, without touching the bottom of the funnel. Nematodes will crawl out of the material into the water and settle.
4. After a period of 16-72 hours (Please note: samples obtained using different extraction times cannot be compared, see § 2.3.1) the nematode suspension can be tapped by opening the squeezer clip. Regularly tapping and adding water increases nematode vitality.

Instead of wrapping the sample in cheesecloth, it can also be spread out on a sieve, which is just touching the water level. The extraction surface is larger compared to the cheesecloth and extraction efficiency a little higher.

Make sure the sample never becomes dry.

Advantages and disadvantages

The method is simple and cheap and when extracting nematodes from plant material, the final suspension is virtually clean. The extraction efficiency is rather high for small samples, but for larger samples efficiency decreases exponentially. Many nematodes die due to accumulation of metabolic coreucts and microorganisms, and lack of oxygen at the bottom of the funnel. To inhibit bacterial growth add a few ml of methyl-p-hydroxybenzoate (0.15%) to the water and to avoid lack of oxygen use a 0.15% solution of hydrogen peroxide instead of water.

Literature: Baermann 1917, Southey 1986 (overview)

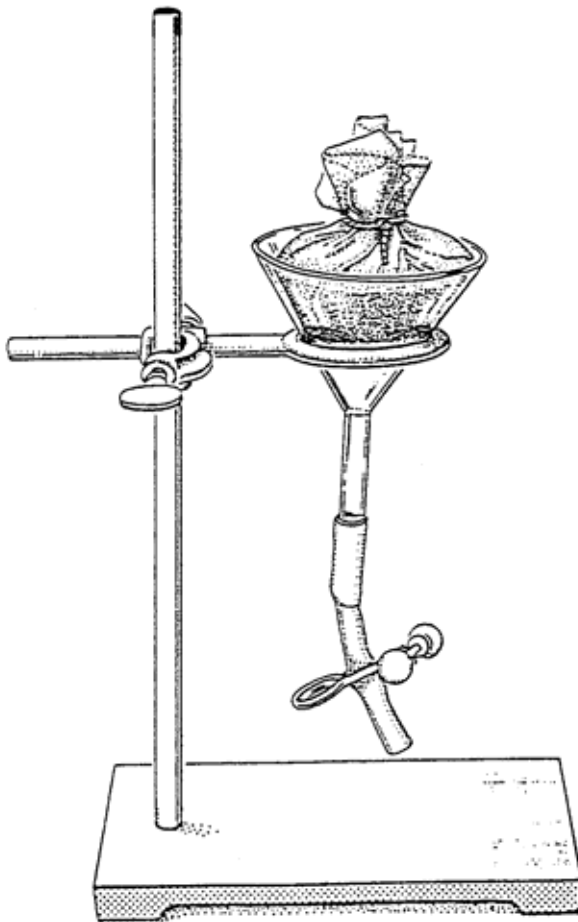


Figure 9
Baermann funnel arrangement for a single sample

2.2.3. Funnel spray method

Area of application

The funnel spray apparatus (mist chamber or mistifier) is used to extract active nematodes from plant material. Sample size depends on the funnel diameter and on the sampled material.

Principle

The method makes use of nematode mobility and sedimentation rate. When infected plant material is moistened in water, nematodes crawl out and sink.

The funnel spray apparatus consists of a set of 16 Baermann funnels, with a diameter of 10 cm, placed in a supporting frame, under a spray nozzle. A constant mist of fine water droplets on the samples avoids oxygen depletion. Plant juices, decomposition coreucts and microorganisms are drained together with the overflowing water, avoiding accumulation (Fig. 10).

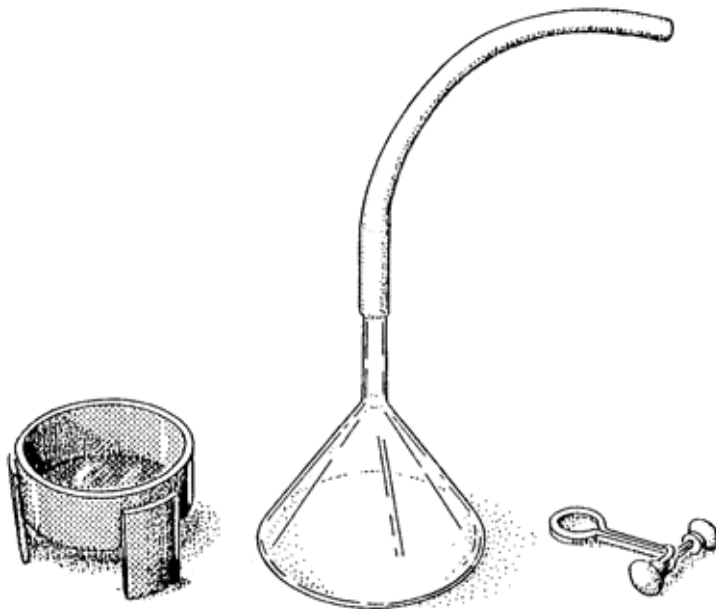


Figure 10.

Sieve, funnel with soft rubber tube and clip to be used for the funnel spray apparatus.

Requirements

Funnel spray apparatus (van Bezooijen and Stemerding, 1971, Catalogue, No. 700)

A pair of scissors or a knife

Clean water

0.385-mm sieves (Ø 8 cm) with three legs

The legs prevent the sieve touching the water level. Oostenbrink's idea was that in this way, nematodes would fall into the funnel giving them a falling speed, which would make them sink. They would not get lost if the funnel overflows due to the constant water supply. The much lighter fungal and bacterial spores, however, would not settle easily and be carried off together with the water overflowing the funnel. Viglierchio & Schmitt (1983), however, did not find any significant nematode yield differences when using sieves with or without legs.

Procedure

1. Carefully wash the plant material and cut it into pieces of ± 1 cm. Take a sub-sample of measured size (e.g. 10 g) and spread it on the sieve. First place a filter paper on top of the gauze.
2. Wash the funnel. Place the funnel in the apparatus and fill it with water. Be careful to avoid formation of air bubbles. Make sure that the clip closes well and that the rubber tube does not leak. If there is an air bubble, some of the water must be tapped.
3. Place the sieve together with sample on the funnel. The sieve must not touch the water level.
4. Turn the water for the mistifier on.
5. After completion of a certain extraction period* the nematode suspension is drained by opening the clip attached at the rubber hose. To preserve nematode vitality, the suspension should not only be tapped at the end of the extraction period, but also at intermediate intervals.

*: Duration of extraction depends, among others, on the type of material and the nematode species. For *Pratylenchus*, the extraction time is 5-7 days, whereas for isolating *Ditylenchus* from stem material an extraction time of a few days is sufficient. Please note: It is not recommended to use this method for extracting *Rhadinaphelenchus*. Due to its high mobility this nematode does not settle and would therefore be carried off with the overflowing water.

Advantages and disadvantages

Compared to the Baermann funnel, the funnel spray method yields nematodes in better condition, because accumulation of decomposition coreucts and microorganisms, and depletion of oxygen are avoided. Extraction efficiency is also higher. Experiments have demonstrated that nematodes keep crawling out of roots after several weeks, probably because of continuing recoreuction and hatching of eggs. The method requires a lot of water and it is difficult to keep the apparatus free of algae and fungi. The air humidity in the room accommodating the equipment may become high, so it is preferable to keep computers, microscopes etc. in a different room.

Literature: Oostenbrink 1960

Further reading: Viglierchio & Schmitt (1983). Rather than a constant water supply Viglierchio and Schmitt prefer an interrupted water flow: every ten minutes the sprinkler is switched on for 1.5 minutes. Fewer nematodes are lost because of decreased turbulence. They also provide data on sedimentation rates of different nematode species and extraction yields obtained with different types of (filter) paper.

2.2.4. Blender nematode filter method

Area of application

The blender nematode filter method is used to extract active nematodes from plant material. The maximum sample size depends on the type of material; often only a few tens of grams can be extracted at once.

Principle

The method makes use of nematode mobility. The sample is placed in water, homogenised with a domestic blender and poured on a nematode filter. The filter with sample is placed on a sieve in a shallow water-filled tray. The nematodes move out of the material, through the filter into the water. After a certain extraction period they are poured off as a clear suspension.

Requirements

- Pair of scissors or a knife
- Domestic blender
- Decanting tray with cross piece
- Extraction sieve (Ø 16 cm) with supporting wires
- Clamping ring for securing the nematode filters
- Nematode filters
- Watch glass (Ø 6 cm)
- Extraction dish
- Area free of vibrations to keep the tray (at constant, non-extreme temperature; about 20°C)
- Clean water

Procedure

1. Carefully wash the plant material from soil and adhering dirt and cut it into pieces of ± 1 cm. Introduce a sub-sample of a particular size (e.g. 10 g) in a domestic blender, submerge the sample with water and mix for approximately 5 seconds ($\pm 10,000$ rpm).
 - The optimal 'blending' time depends on the material (hard or soft roots) and needs to be established experimentally. The time must be sufficient to free nematodes from the material (or facilitate the crawling out), but not so long that nematodes get damaged or killed.
2. Place 2 nematode filters in an extraction sieve and secure them with a clamping ring. Moisten them with a sprayer to remove air bubbles between the filters. Place the sieve in a water-filled decanting tray with cross piece and place a watch glass on the filter papers (*for more explanations about points 2-5, refer to § 2.3.1*).
3. Carefully pour the suspension from the blender beaker onto the watch glass. Remove the watch glass and when all the water has passed through the sieve, remove the clamping ring. Add a sample label to the filter.
4. Place the extraction dish in an area free of vibrations. The dish should contain sufficient water to keep filter and sample moist (± 100 ml). Carefully place the sieve in the dish.

5. After an extraction period of 16-48 hours (Please note: samples obtained using different times of extraction cannot be compared, see § 2.3.1) the sieve is removed from the dish and the suspension is poured into a 100 ml beaker for analysis.

Advantages and disadvantages

Compared to ‘incubation methods’ such as the Baermann funnel technique, this method is generally faster (as the sample is homogenised, nematodes crawl out more easily) and more efficient (because of the larger surface of the extraction dish). Optimal ‘blending’ times have to be established experimentally for each sample type and for each nematode species. When handling soft plant material a disadvantage is the rapid decomposition of the plant parts, releasing substances toxic to nematodes. Regular tapping during the extraction period may prevent nematodes from dying. This is more labour intensive, but strongly enhances the vitality of nematodes. Especially if nematodes are to be used in inoculation experiments, this might be important

Literature: Stemerding 1964

Further reading: For example Schouten & Arp 1991: they apply the blender nematode filter method for extractions from litter.

2.2.5. Blender centrifugal flotation method

Area of application

The blender centrifugal flotation method is used to extract active and inactive nematodes (and eggs) from plant material. The maximum sample size depends on the type of material; often only a few tens of grams can be extracted at once.

Principle

The method is based on differences in specific gravity (s.g.) between nematodes and other particles in a sample. In an extraction fluid with higher specific gravity compared to nematodes, nematodes keep afloat. Particles with higher specific gravity compared to the fluid will sink. This separation process is accelerated by centrifugation.

The sample is macerated in water with a domestic blender. The suspension is centrifuged and all particles with a specific gravity higher than 1 (including nematodes) precipitate. The supernatant is poured off and the sediment (pellet) is brought in suspension in the extraction fluid. After centrifugation, the nematodes float in the supernatant and the other particles are precipitated in the pellet. The nematodes are collected by passing the supernatant through a fine sieve. They can be collected and observed instantaneously.

For details about choice and density of the extraction fluid and the centrifugal force (calculation), see § 2.3.5.

Requirements

- Domestic blender
- Centrifuge
- Vibro-mixer
- Balance
- Densimeter
- Pair of scissors or knife
- 1200 μm sieve
- Plastic bowl with a capacity of ± 4 litres
- At least two centrifuge tubes
- 2 pipettes to balance (for water and MgSO_4)
- 5 μm sieve
- Beaker
- Wash-bottle with water
- MgSO_4 solution, s.g. 1.18

Procedure

1. Carefully wash the plant material and cut it in pieces of ± 0.5 cm length. Place a sub-sample of a particular size (e.g. 10 g) in a domestic blender, submerge the sample in water and blend for 5 seconds ($\pm 10,000$ rpm).

2. Pour the suspension through a 1200 μm sieve into a plastic bowl. Rinse the residue on the sieve with water to collect all nematodes and discard it. Divide the suspension in the plastic bowl over two (or another even number of) centrifuge tubes.

Adding kaolin may be required to make the pellet more solid, see discussion in § 2.3.5 (point 3).

3. Balance the centrifuge tubes with water and centrifuge for 4 min. at 1800 g.
4. Carefully decant the supernatant over a 10 μm sieve (see point 7) to collect nematodes which did not precipitate. A 5 μm sieve is required if eggs need to be collected as well.
5. Add MgSO_4 (1.18) solution to the pellet and mix thoroughly with a vibro-mixer (20 sec). Rinse the head of the vibro-mixer above the centrifuge tube with MgSO_4 .
6. Balance the centrifuge tubes with MgSO_4 and centrifuge for 3 min. at 1800 g.
7. Pour the supernatant on the 10 μm sieve. Rinse the sieve with water, using a wash-bottle and collect the nematodes in a beaker. Please note: the MgSO_4 passing through the sieve can be collected and reused. Again, a 5 μm sieve is needed for collecting eggs.

Advantages and disadvantages

With this method even inactive nematodes can be isolated. Within half an hour the extraction of the sample is completed and a relatively clean nematode suspension obtained. However, the extraction fluid may influence the nematode shape (making identification more difficult) and negatively affect their vitality (making them less useful for inoculation purpose). The required equipment is expensive (e.g. centrifuge), although it is usually part of the laboratory equipment.

Literature: after Coolen 1979

Further reading: For example:

- Coolen & D'Herde (1972) supply different routine procedures for swollen stages, eggs and active stages. They also provide analyses of the effect of different blending speeds and 'mixing' time on extraction efficiencies for different nematode species etc.;
- Hussey & Barker (1973) stir tomato roots in bleach (0.53% solution of NaOCl) to isolate eggs from gelatinous egg masses of *Meloidogyne*. McSorley *et al.* (1984) found more eggs using this method compared to the Coolen & D'Herde method;
- Greco & D'Abbaddo (1990) extract *Tylenchulus semipenetrans* from *citrus* roots, using the blender centrifugal flotation method and compare it to yields obtained with sugar, MgSO_4 and colloidal silica.

2.3. EXTRACTION FROM SOIL AND OTHER SUBSTRATES

2.3.1. Decanting method

Area of application

The decanting method is used for extraction of active nematodes from soil and sediment (max. 50 g) and substrates such as rock wool or moss (Also refer to § 1.4.3). A part of the method (cleaning of the suspension with nematode filters) is used in many other methods as a last step in the extraction process.

Principle

The method makes use of difference in specific gravity (s.g.) between nematodes and soil particles, and of nematode mobility.

The sample is stirred in a beaker with water to detach nematodes from soil particles. When heavy particles have settled, the nematode suspension is poured off (decanted). The suspension is cleaned by passing it through a nematode filter, removing water and fine particles without significant nematode loss. The filter is placed in a shallow water-filled tray. The nematodes move through the filters into the water and after a predetermined extraction period they can be poured off as a clear suspension. This method is derived from the Baermann technique. However, because of the relatively large surface of the extraction dish, it does not have the same disadvantages, such as the accumulation of metabolic coreucts, lack of oxygen in the narrow funnel leg, and low efficiency.

Requirements

- 2-litre beaker (high model)
- Wooden stirring rod
- Plastic bowl with a capacity of 4 litres
- Decanting tray with cross piece
- Extraction sieve (Ø 16 cm) with supporting wires
- Clamping ring for securing the nematode filters
- 2 nematode filters or equivalent
- Watch glass (Ø 6 cm)
- Extraction dish
- If necessary, covering material to cover the extraction dish
- Area free of vibrations to store the extraction dish (at constant, non-extreme temperature; about 20°C)
- Clean water

Procedure

Decanting:

1. Take a (sub) sample (max. 50 g) following the guidelines in § 1.4. Put it in a beaker and add \pm 1 litre of water.
2. Stir water and soil until a homogenous suspension is obtained. Leave the suspension for 15 seconds and decant the suspension in the plastic bowl.
3. Stir the remaining sediment again with water. After 15 seconds, decant the suspension in the plastic bowl previously used, and repeat a third time. The

sediment in the beaker can be discarded. Let the suspension in the bowl settle for about 5 minutes.

Proper sedimentation of the suspension improves cleaning with nematode filters. When poured on the filter, the liquid passes through quickly, as the first litres of suspension hardly contain debris. Pour the sediment with a little water on the filter. The dirt remains on the filter and the final suspension is clean.

Cleaning the suspension with nematode filters:

4. Use a clamping ring to attach 2 nematode filters in the extraction sieve. Moisten them with the sprayer to remove air bubbles from between the filters. Place the sieve in a water-filled decanting tray with cross piece and put a watch glass on the filters.

Placing the sieve in water causes counter-pressure when pouring the suspension, preventing the nematodes from being swept through the filter. The debris is also spread more evenly over the filter. The watch glass prevents damage to the filter when pouring the suspension.

5. Carefully pour the settled suspension from the bowl onto the watch glass on the filter. The last 200-300 ml of suspension should be well-stirred and quickly poured. Remove the watch glass, rinse it, and as soon as all the water poured on the filter, lift the sieve and remove the clamping ring. Add a sample label to the filter.

Do not leave the sieve in the water-filled tray longer than necessary. Very active nematodes such as *Ditylenchus* and some rhabditid nematodes are capable of quickly crawling through a filter and might get lost for analysis.

6. Place an extraction dish in an area free of vibrations. The dish should contain sufficient water to keep the filter moist (\pm 100 ml). Carefully place the well-drained sieve in the dish. Do not move the dish with the sieve in it, otherwise the suspension is contaminated with dirt from the filter. The dish may be covered to prevent evaporation and dust falling in.
7. After an extraction period of 16-48* hours, the sieve can be removed and the nematode suspension is poured into a 100 ml beaker for analysis.

*: Samples obtained using different extraction times cannot be compared. Because of differences in activity (e.g. *Ditylenchus* moves much faster through a filter than slow species such as Criconematidae), hatching of eggs and multiplication of nematodes with a short lifecycle, a longer extraction period leads to higher nematode number in the extraction dish. Population increase as a result of egg hatching may be interesting when studying plant parasites, because eggs are potential plant parasites (e.g. egg masses of *Meloidogyne*). Population increase due to fast recoreuction (e.g. in the case of some rhabditides), leads to numbers and species proportions which do not reflect the original situation.

Please note: This method is most suitable for sandy soils. Other soil types contain tiny and light particles which remain in suspension at the time of decanting; leading to a dirty final suspension.

Possible solutions are:

a. (after Oostenbrink 1960) Pour the suspension on a double filter and use an extraction sieve without supporting wires. Put the sieve on a somewhat larger sieve with supporting wires and a single filter and place both sieves in an extraction dish. The dirty bottom of the first sieve is not in direct contact with the water. The final suspension is clean, but probably contains fewer nematodes, as they need to crawl through 3 instead of 2 filters.

b. First pass the decanted suspension over a set of four 45 µm sieves (or four times over one 45 µm sieve, etc.), eliminating tiny particles. The debris can be washed from the sieves and poured on a double filter in an extraction sieve with supporting wires.

Advantages and disadvantages

Nematodes can be isolated using small amounts of water and simple tools. However, yields vary between individuals and the sample size is limited to 50 g only (for larger samples, the debris layer on the sieves becomes too thick, making it difficult for nematodes to crawl through). The final suspension often remains dirty, unless the sample consists of sandy soil.

Literature: Oostenbrink 1960

Further reading: Stolk (1989) Relation between time of extraction and nematode numbers crawling through the filter.

2.3.2. Decanting and sieving: Cobb's method

Area of application

Cobb's method is used for the extraction of active nematodes from soil and sediments (max. 100 g).

Principle

The method makes use of differences in size, shape, and sedimentation rate between nematodes and soil particles, and of nematode mobility.

The sample is stirred in a water-filled beaker, detaching the nematodes from the soil particles. After heavy particles have settled, the nematode suspension is poured off (decanted) and sieved. Nematodes remain on the sieves while tiny soil particles pass through. Sieving is carried out with a series of sieves of decreasing mesh size, so nematodes of different size are collected separately. Soil particles in the suspension stay behind on the subsequent sieves, so the 45 µm sieve at the bottom is not easily clogged and samples up to 100 g can be extracted. The debris collected from the sieves is placed on nematode filters. Nematodes move through the filters into the water and can, after a certain extraction time, be poured off as a clear suspension.

Requirements

2-litre beaker (high model)

Stirring rod

3 plastic bowls, each with a capacity of ±4 litres

A set of 'Cobb's sieves' (Ø 15 or 20 cm):

500-1000 µm, 350-375 µm, 175 µm, 100 µm, 45 µm (other sets are also available)

100 ml beaker

Decanting tray with cross piece

Extraction sieve (Ø 16 cm) with supporting wires

Clamping ring for securing the nematode filters

2 nematode filters or equivalent

Watch glass (Ø 6 cm)

Extraction dish

If necessary, covering material to cover the extraction dish

Area free of vibrations to keep the extraction dish (at constant, non-extreme temperature; about 20°C)

100 ml beaker

Clean water

Procedure

1. Take a (sub) sample following the guidelines in § 1.4. Put it in a beaker and add ± 1 litre of water.
2. Stir water and soil until a homogenous suspension is obtained. Leave the suspension for 15 seconds and decant the supernatant in a bowl.
3. Stir the remaining sediment again with water. After 15 seconds, decant the supernatant in the same plastic bowl (used under point 2). Repeat a third time. The sediment in the beaker can be discarded.
4. Pass the suspension from the plastic bowl through a 500 or 1000 μm sieve into another bowl. Shake the sieve, which is submerged in the suspension, to help nematodes to pass through. The debris remaining on the 500 μm sieve can be discarded.
5. Pass the suspension through a 350 μm sieve into a bowl. Wash the debris from the 350 μm sieve into a beaker.

The suspension in the beaker can be checked for large nematodes such as *Longidorus* and *Xiphinema*, or immediately added to the suspension in the bowl (collecting pan, see point 6).

6. Pass the suspension through a 175 μm sieve into a bowl. Wash the debris from the 175 μm sieve into a third bowl, the 'collecting pan'. The debris from the 100 μm and 45 μm are also added to this pan.

If the objective is to analyse nematodes according to size, the residues washed from the different sieves can be kept separately. In this procedure all residues are mixed.

7. Pass the suspension through a 100 μm sieve into a bowl. Wash the debris from the 100 μm sieve into the 'collecting pan'.
8. Pass the suspension through a 45 μm sieve into a bowl. Wash the debris from the 45 μm sieve into the 'collecting pan'.
9. Repeat point 8, depending on the soil type, 2 to 4 times to capture all small nematodes. The suspension passing through the last sieve can be discarded. Do not discard the suspension in the 'collecting pan'!
10. Attach, with a clamping ring, two nematode filters in the extraction sieve. Moisten them with the sprayer to remove air bubbles from between the filters. Place the sieve in a water-filled decanting tray with cross piece and place a watch glass on the filters (*refer to § 2.3.1 for an explanation about points 10-13*).

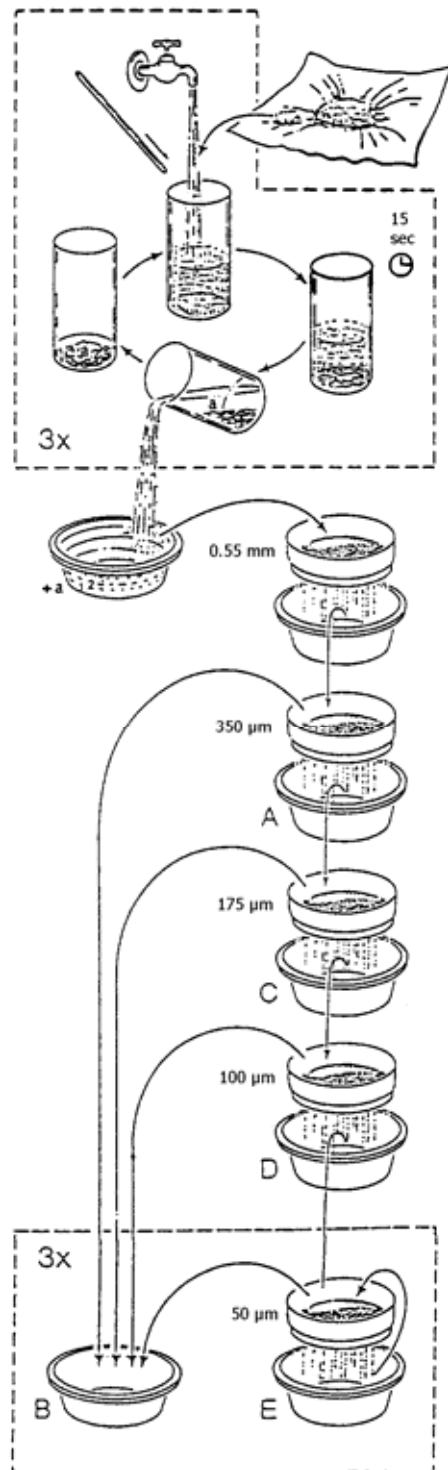


Figure 11

Schematic overview of Cobb's method

Steps 10, 11, 12 and 13: Extraction of active nematodes with Cobb's method

11. Carefully pour the suspension from the 'collecting pan' onto the watch glass on the filter. The last 200-300 ml of suspension should be stirred well and quickly poured. Remove the watch glass and, as soon as all the water is poured on the filter, lift the sieve and remove the clamping ring. Add a sample label to the filter.
12. Place an extraction dish in an area free of vibrations. The dish should contain sufficient water to keep the filter moist (\pm 100 ml). Carefully place the well-drained sieve in the dish. Do not move the dish with the sieve in it, otherwise the suspension is contaminated with dirt from the filter. The dish may be covered to prevent evaporation and dust falling in.
13. After an extraction period of 16-48 hours (Please note: Samples obtained using different extraction times cannot be compared, see § 2.3.1), the sieve is removed and the nematode suspension poured into a 100 ml beaker for analysis.

Advantages and disadvantages

High extraction efficiency is obtained using only a small set of sieves and little water. However, the sieves are relatively expensive and practice is required to carry out the method in a reliable way. Because of the high number of operations, it is a relatively time-consuming method. As no running tap water is required, it can be carried out outside the lab.

Literature: Cobb 1918

Further reading: for example Southey 1986 (p. 12-14)

2.3.3. Erlenmeyer- or (milk) bottle method

Area of application

The Erlenmeyer- or (milk) bottle method is used for extraction of active nematodes from soil (max. 100 g).

Principle

The method makes use of difference in size, shape, and sedimentation rate between nematodes and soil particles and of nematode mobility.

A bottle, filled with a soil suspension is turned upside down and placed on another bottle, which is completely filled with water. Soil particles sink from the upper bottle into the lower, while water from the lower bottle moves up into the upper, replacing the sinking soil particles. In the first couple of minutes, the upward water stream prevents sinking of lighter soil particles and nematodes.

The suspension is passed through sieves and the debris left on the sieves is placed on nematode filters. Nematodes from the debris move through the filters into the water and, after a certain extraction time, they can be poured off as a clear suspension.

Requirements

Stand with ring and clip

3 milk bottles, numbered A, B and C

Originally erlenmeyers were used, but these are expensive and very fragile. Apart from milk bottles all other types of bottles having a wide opening and a wide (funnel-shaped) neck can be used.

Domestic sieve (mesh 2 mm)

Funnel with a cork stopper

'Junction' with cork stopper

4 sieves of Ø 30 cm and 45 µm pore size

Plastic bowl with a capacity of 4 litres

Decanting tray with cross piece

Extraction sieve (Ø 16 cm) with supporting wires

Clamping ring for securing the nematode filters

Watch glass (Ø 6 cm)

2 nematode filters (or equivalent)

Extraction dish

If necessary, covering material to cover the extraction dish

Area free of vibrations to keep the tray (at constant, non-extreme temperature; about 20°C)

100 ml beaker

Clean water

Procedure

1. Take a sub-sample following the guidelines in § 4.1.
2. Place the funnel, closed with the cork stopper, in the ring on the stand, so it hangs over bottle A. Fill the funnel with water, place a domestic sieve in the funnel, and put the sample on the sieve. Shake the sieve sideways, so the sample passes through the sieve into the funnel. (Fig. 12)

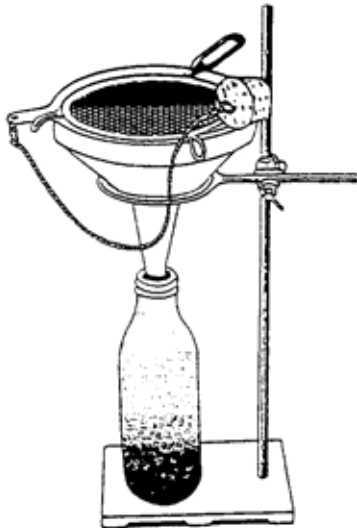


Figure 12
 Bottle method
 Before filling the bottle with a suspension, the soil should be sieved with a domestic sieve.



Figure 13A
 Arrangement for the purpose of sedimentation

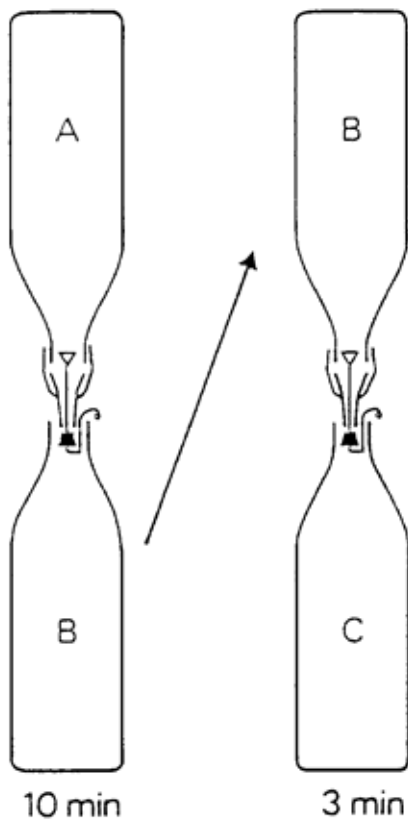


Figure 14
 Sequence of events in the bottle method

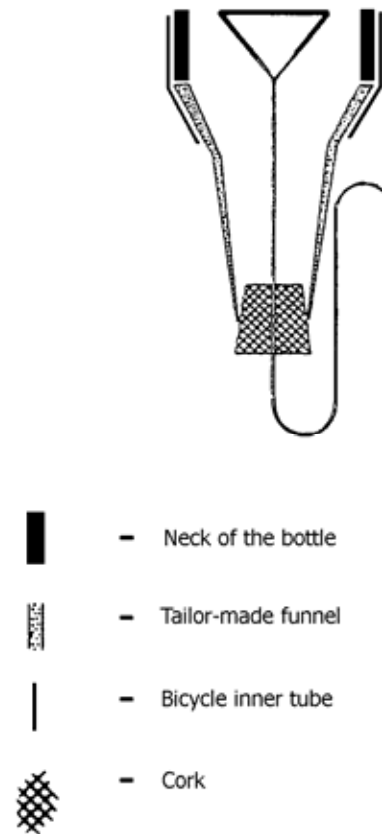


Figure 13B
 Junction (funnel part) used for the bottle method

3. Uncork the funnel; the suspension flows into bottle A. Rinse the funnel and the sieve with water until bottle A is completely filled. Attach a closed junction to bottle A.
4. Fill bottle B with water and place it in a stand. Thoroughly shake bottle A and place it upside down in the ring on the stand, so the closed end of the junction is hanging in bottle B. Quickly open the junction and leave the set up for 10 minutes. Heavy soil particles and big nematodes sink from bottle A into bottle B. (Fig. 13A and 13B)
5. After 10 minutes the junction is closed and bottle A is taken away. Repeat the procedure (point 4) with bottle B, placing it for a 3 minutes period on water-filled bottle C. Heavy soil particles sink from bottle B into bottle C, whereas nematodes keep afloat in bottle B. After this the contents of bottle C can be discarded. (Fig. 14)
6. Moisten the set of four 45 μm sieves and empty bottles A and B on the sieves. Immediately wash the debris from the sieves, into a bowl. Tilt the sieves forward, and wash both sides of the sieves with little water. Let the suspension in the bowl settle for at least 5 minutes.
7. Attach 2 nematode filters with a clamping ring in an extraction sieve. Moisten them with the sprayer to remove air bubbles from between the filters. Place the sieve in a water-filled decanting tray with cross piece and place a watch glass on the filters (*refer to § 2.3.1 for an explanation about points 7-10*).
8. Carefully pour the suspension from the bowl onto the watch glass on the filter. The last 200-300 ml of suspension should be stirred well and quickly poured. Remove the watch glass, rinse it, and as soon as all the water is poured on the filter, remove the clamping ring.
9. Place an extraction dish in a vibration free area. The dish should contain sufficient water to keep the filter moist (± 80 ml). Carefully place the well-drained sieve in the dish. Do not move the dish with the sieve in it, otherwise the suspension is contaminated with dirt from the filter. The dish may be covered to prevent evaporation and dust falling in.
10. After an extraction period of 16-48 hours (Please note: Samples obtained after using different times of extraction cannot be compared, see § 2.3.1) the sieve can be removed and the nematode suspension in the dish poured into a 100 ml beaker for analysis.

Advantages and disadvantages

Relatively high extraction efficiency can be obtained with simple equipment and small amounts of water. However, the method is labour intensive and, when one set of bottles is used, very time consuming.

As the method does not require running tap water, it can be carried out outside the lab.

Literature: Seinhorst 1955, 1962

2.3.4. Oostenbrink elutriator

Area of application

The Oostenbrink elutriator is used for the extraction of nematodes from soil, river silt, manure, litter and other substrates which may harbour nematodes and can be washed into a suspension (Also refer to § 1.4.3). Depending on the type of sample the maximum sample size is 250 ml.

Principle

The method makes use of difference in size, shape and sedimentation rate between nematodes and soil particles. If the suspension is cleaned by passing it through nematode filters the method also makes use of nematode mobility.

In the Oostenbrink elutriator, an upward water stream makes nematodes and fine particles to float in the extraction column, whereas heavier particles settle in the lower part of the apparatus. The suspension in the extraction column is decanted through a side-outlet and sieved to remove the fine particles. The debris that is washed from the sieves can be placed on nematode filters. Nematodes move through the filters into the water and, after a certain extraction time, they can be poured off as a clear suspension.



Figure 15

Oostenbrink elutriator

Set-up of the Oostenbrink elutriator for the extraction of nematodes from soil

2.3.4.1. Standard use

Requirements

- Oostenbrink elutriator, complete (Van Bezooijen and Stemerding, 1971 Catalogue, No 100)
- 4 sieves of \varnothing 30 cm and 45 μ m pore size
- Plastic bowl with a capacity of 4 litres
- Decanting tray with cross piece
- Extraction sieve (\varnothing 16 cm) with supporting wires
- Clamping ring for securing the nematode filters
- Watch glass (\varnothing 6 cm)
- 2 nematode filters (or equivalent)
- Extraction dish
- If necessary, covering material to cover the extraction dish
- Area free of vibrations to keep the tray (at constant, non-extreme temperature; about 20°C)
- 100 ml beaker
- Clean water

Procedure

1. Take a sub-sample, following the guidelines in § 1.4.
2. Wash the funnel with water. Close the side-outlet with a rubber stopper and the lower part with the plug. Fill the funnel with water up to level 1 (the point at which the water level just touches the small funnel) and start the undercurrent water stream at a rate of 1000 ml/min.

When washing large (> 200 g) samples in the funnel, it may be necessary to increase the rate of the upward water stream to avoid nematodes being swept away by heavy particles and carried to the bottom. However, a higher upward water current, apart from keeping more nematodes afloat, also leaves more dirt in the suspension.

3. Wash the sample through the top sieve into the funnel, using the nozzle above. Continue until the water level in the funnel has reached level 2 (2/3 of the column). The sample must be washed through completely! Close the nozzle. For large samples, it is more efficient to wash the sample slowly into the funnel.

For nematodes which are extremely sensitive to mechanical damage or friction (e.g. *Trichodorus teres*), the top sieve is not used. Instead, the sample is suspended before bringing it in the funnel. Carefully pour it on your flat hand, into the funnel upper water layer, to avoid nematodes being swept downward by the force of the water current.

4. Few seconds after closing the sprayer, the undercurrent must be reduced to 600 ml/min. This undercurrent fills the funnel.

5. Moisten the four 45 μm sieves to avoid clogging of the mesh and place them under the side-outlet.

For tiny nematodes such as *Paratylenchus*, it may be necessary to use more than four sieves. For large nematodes, three, or sometimes two sieves are sufficient (see Oostenbrink 1954 and Seinhorst 1956). Please note: always check whether the sieves are undamaged, especially along the border, because the gauze may detach from the sides. Keep the sieve against the light to spot damage.

6. As soon as the water has reached level 3, the side-outlet is unplugged and the suspension flows on the sieves. Incline the sieves a little (by placing a piece of wood underneath or by slightly lifting them up) and tap on the side of the sieves, helping the suspension to pass through more easily.

The funnel can be rinsed if nematodes are expected to be stuck on the side. However, observations demonstrated that nematode numbers remaining in the funnel are negligible. Using too much water while rinsing may cause loss of nematodes.

7. Immediately wash the debris off the sieves into a bowl. Tilt the sieves forward, and wash both sides with small amounts of water. Let the suspension in the bowl settle for at least 5 minutes. In the meantime all parts of funnel and sieves should be carefully cleaned.
8. Attach 2 nematode filters with a clamping ring in an extraction sieve. Moisten them with the sprayer to remove air bubbles from between the filters. Place the sieve in a water-filled decanting tray with cross piece and place a watch glass on the filters (*refer to § 2.3.1 for an explanation about points 8-11*).
9. Carefully pour the suspension from the bowl onto the watch glass on the filter. The last 200-300 ml of suspension should be stirred well and quickly poured. Remove the watch glass, rinse it, and as soon as all the water is poured on the filter, lift the sieve and remove the clamping ring. Add a sample label to the filter.
10. Place an extraction dish in an area free of vibrations. The dish should contain sufficient water to keep the filter moist (± 100 ml). Carefully place the well-drained sieve in the dish. Do not move the dish with the sieve in it, otherwise the suspension is contaminated with dirt from the sieve. The dish may be covered to prevent evaporation and dust falling in.
11. After an extraction period of 16-48 hours (Please note: Samples obtained after using different times of extraction cannot be compared, see § 2.3.1) the sieve can be removed and the nematode suspension in the dish poured in a 100 ml beaker for analysis.

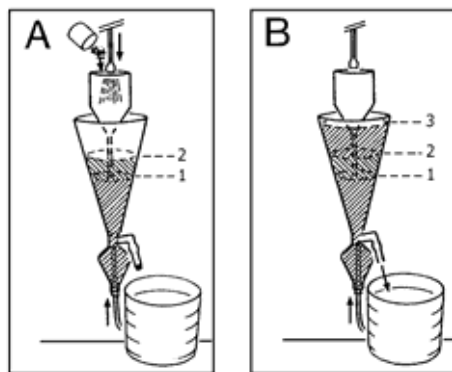
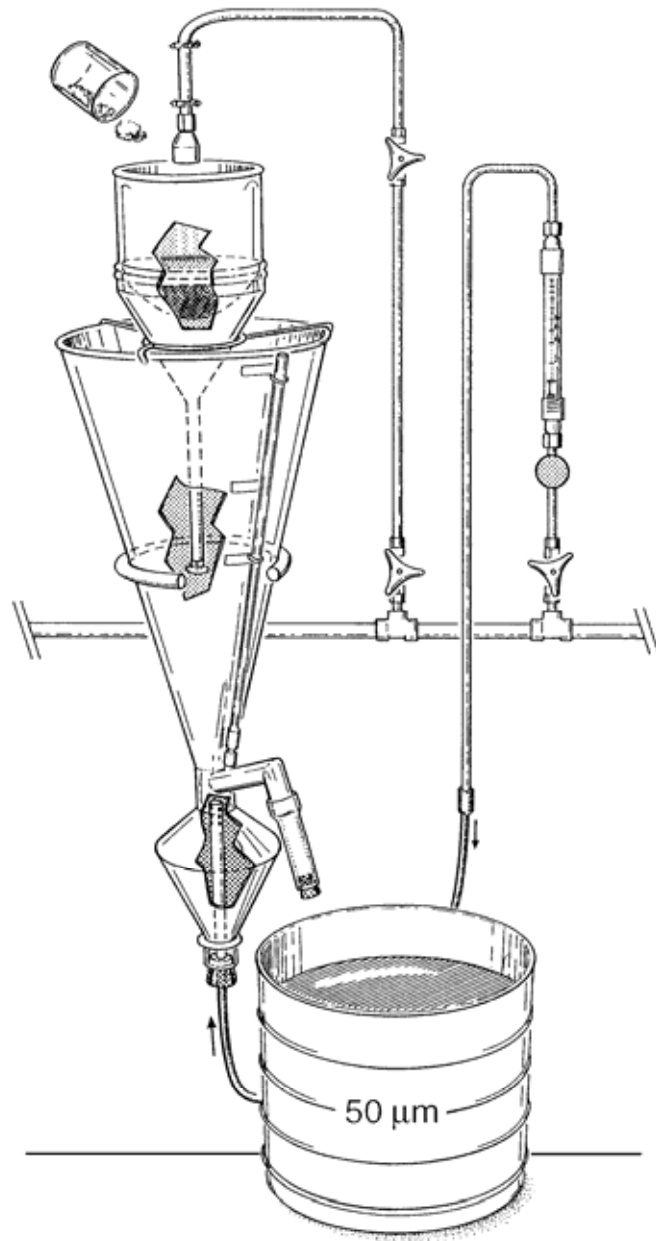


Figure 16
Oostenbrink elutriator diagram

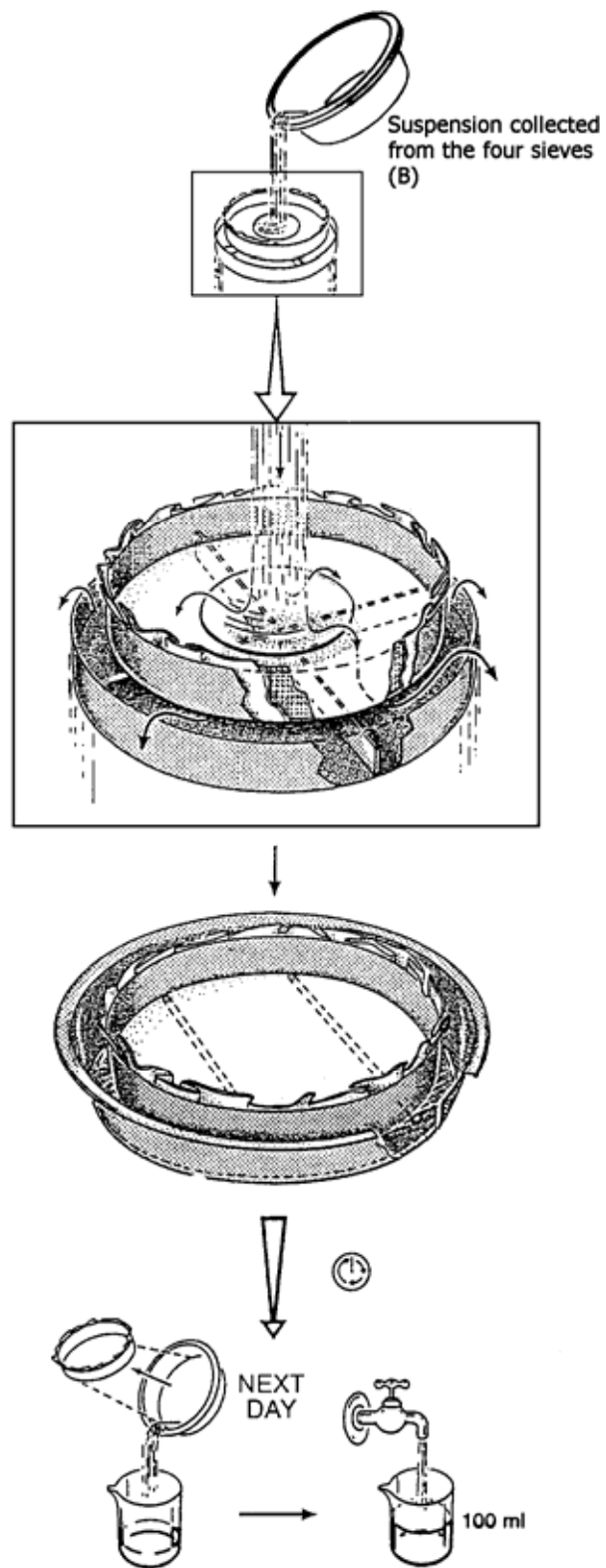


Figure 17
Steps 8, 9, 10, and 11: extraction of active nematodes with the Oostenbrink elutriator.

Advantages and disadvantages

The method is efficient and easy to standardize. Compared to the decanting method, the sedimentation of heavy soil particles is easier to control. Because of this, larger samples can be managed (up to 250 ml), without extra contamination of the final suspension. The equipment, however, is expensive and the method requires relatively large amounts of water.

Literature: Oostenbrink 1954, 1960

Further reading: Schouten & Arp 1991: an application of the Oostenbrink elutriator for nematode extraction from litter. First the litter sample is soaked in water for at least 1 hour. Then it is homogenized with a domestic blender (for 10 sec) and finally it is washed following the above-described procedure.

2.3.4.2. Modification for the extraction of large nematodes

The standard set-up of the Oostenbrink elutriator gives satisfactory yields of small and middle-sized nematodes, but yields of larger and heavier nematodes such as *Longidorus* and *Xiphinema*, are insufficient. Isolating these nematodes requires an increased upward water stream rate and nematode filters cannot be used for isolating nematodes from the suspension, as large nematodes can not easily pass through the filters.

Requirements

See under § 2.3.4.1 (standard use), and also:

- 2 sieves of Ø 20 cm and 175-180 µm pore size
- '*Longidorus*' extraction sieve (125 µm)
- Wash-bottle with water

Procedure

1. Take a sub-sample, following the guidelines in § 1.4. (The standard sample size for checking presence of *Longidorus/Xiphinema* under EU guidelines is 200 ml). Put the sample in a plastic bowl and add water until the soil is just submerged. Let it soak for half an hour.
2. Wash the funnel with water. Close the side-outlet with a rubber stopper and the lower part with the plug. Fill the funnel with water up to level 1 and start the undercurrent at a rate of **1300 ml/min**.

Due to the increased undercurrent more large nematodes keep afloat, but more dirt stays in suspension. The water moves also faster from level 1 to level 2. This is the time available to wash the sample into the funnel and should be considered when selecting the sample size.

3. Bring the content of the plastic bowl in the apparatus by pouring it via your flat hand in the upper water layer (not via the top funnel and top sieve). Be careful to avoid nematodes being swept downward by the soil.
4. After washing the sample into the funnel (and as soon as the water reaches level 2), the undercurrent must be reduced to **800 ml/min**.

5. As soon as the water level reaches level 3, the side-outlet is unplugged and the suspension passes over the two 180 µm sieves.
6. Wash the debris from the 180 µm sieves into a bowl and pour this suspension over a 45 µm sieve to further concentrate it.
7. Wash the debris on the 45 µm sieve to one side with the wash-bottle. Put a small water layer in a dish, and place a 125 µm extraction sieve in the water. Carefully wash the debris from the 45 µm sieve on the 125 µm extraction sieve.
8. Carefully transfer the extraction sieve to an extraction dish, filled with clean water (\pm 100 ml). The minimum extraction period is 24 hours (EU standard: 2 days)

Please note: It is possible to capture large and middle-sized nematodes in one single routine. Use, at point 5 of the procedure, the set of four 45 µm sieves instead of the 180 µm sieves, so all sizes of nematodes are caught. Wash the debris from the 45 µm sieves and pass it through two 180 µm sieves into a second plastic bowl. The small and middle-sized nematodes end up in the second bowl and the large nematodes are retained on the 180 µm sieves. Extraction of nematodes from the suspension in the bowl is done according to the standard method (§ 2.3.4.1, points 8-11), while extraction of nematodes from the debris on the 180 µm sieves is performed according to the guidelines above (§ 2.3.4.2, points 6-8).

Literature: after D’Herde & Van den Brande 1964

2.3.4.3. Modification for the extraction of stem nematodes

In the case of *Ditylenchus dipsaci* (stem nematode), economic damage to a crop can be expected at a population density of *only one* nematode per kilogram of soil. Therefore one kilogram of soil is washed and the entire nematode suspension is checked.

Requirements

See under § 2.3.4.1 (standard use), and also:

- Instead of 4 sieves of 45 µm, 2 sieves (Ø 30 cm) of 75 µm pore size
- 10 g of sodium hexametaphosphate (1% of the sample size)
- If necessary: dough mixer
- 1% solution of Halamid (100 ml)

Procedure

1. Put the sample (1 kg) in a bowl and add water until the soil is almost submerged. Add 10 g of sodium hexametaphosphate and let it soak for half an hour (see § 1.4). If necessary a dough mixer can be used to make a homogenous suspension.
2. Clean the apparatus with water and fill the funnel up to level 1. Activate the undercurrent at a rate of 1000 ml/min.

Carefully clean the equipment to be certain that if *Ditylenchus* is found, it is not derived from previously extracted samples.

3. Wash the suspended sample through the top sieve into the funnel with the spray nozzle and stop spraying as soon as level 2 is reached (the sample must be washed through completely). Reduce the undercurrent to 600 ml/min.
4. Place the set of two 75 µm sieves under the side-outlet.

The increased pore size is sufficient to capture *Ditylenchus*, but smaller nematode species pass through. The final suspension contains few small nematodes, making it easier to detect *Ditylenchus*.

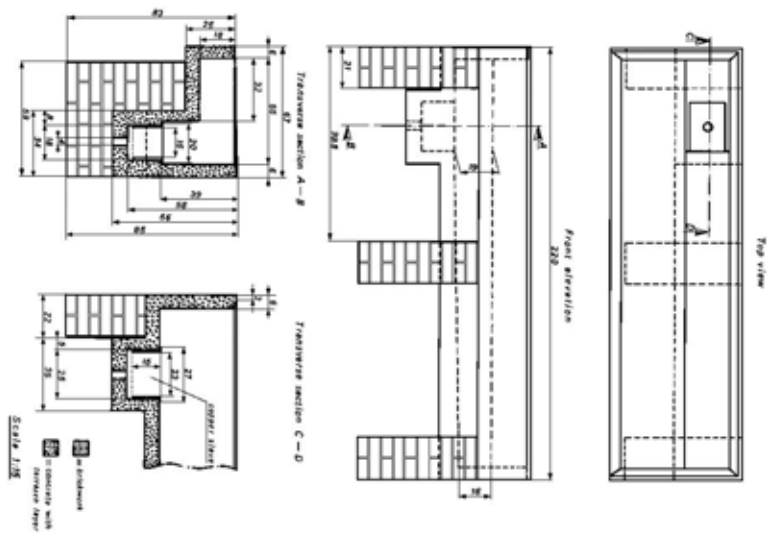
5. As soon as the funnel is filled up to level 3, the side-outlet is unplugged and the suspension passes through the sieves.
6. Immediately wash the debris from the sieves into a bowl. Tilt the sieve forward and wash both sides of the sieves with little water. Let the suspension in the bowl settle for at least 5 minutes. In the meantime all parts of the funnel and sieves should be carefully cleaned.
7. Attach 2 nematode filters in an extraction sieve with a clamping ring. Moisten them with the sprayer. Place the sieve in a water-filled decanting tray with cross piece and place a watch glass on the filters (*refer to § 2.3.1 for an explanation on points 7-10*).
8. Carefully pour the suspension from the plastic bowl onto the watch glass on the filter. The last 200-300 ml of suspension should be stirred well and quickly poured. Remove the watch glass and as soon as all the water is poured on the filter, lift the sieve and remove the clamping ring. Add a sample label to the filter.
9. Fill an extraction dish with ± 100 ml Halamid 1% solution and carefully place the extraction sieve in the dish.

Halamid restricts movement and may cause mortality among many nematode species, but it does not affect *Ditylenchus dipsaci*. The final suspension contains almost exclusively *Ditylenchus*, which can be detected more easily.

10. After an extraction period of 18-24 hours the nematode suspension in the extraction dish can be poured into a 100 ml beaker for analysis.

Literature: Kleijburg 1960, Oostenbrink 1960

DESIGN FOR A WASH-BASIN TO BE USED WITH THE OOSTENBRINK ELUTRIATOR
 scale 1 : 15
 dimensions in centimetres



The length of the basin depends on the number of elutriators used.
 In general 100 cm per elutriator is sufficient.
 The bottom of the gutter has a slope of 0.5 % in order to facilitate the water and debris to flow away into the removable coarse copper sieve over the sink-hole.

ARRANGEMENT FOR THE OOSTENBRINK ELUTRIATOR
 A — arrangement with constant pressure tank
 B — arrangement with Brooks flowmeter

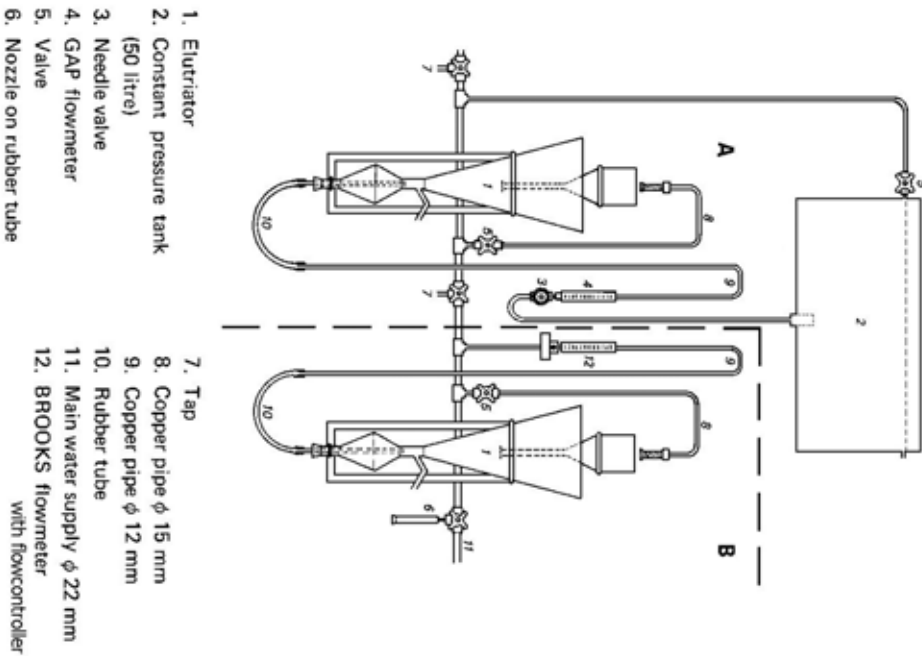


Figure 18
 Design for the Oostenbrink elutriator arrangement

2.3.5. Centrifugal flotation method

Area of application

The centrifugal flotation method is used for the extraction of active as well as inactive nematodes (or nematode stages) from soil, sediments, and substrates such as manure (also refer to § 1.4.3). Pre-extraction of the sample is often carried out to obtain a concentrated suspension, which is centrifuged. This makes it possible to handle large samples with a small table centrifuge. Direct centrifugation of the whole sample limits the sample size and decreases the extraction efficiency (Dickerson 1977).

Principle

The centrifugal flotation method makes use of difference in specific gravity (s.g.) between nematodes and other fractions of a sample. If the sample is suspended in an extraction fluid with a higher specific gravity compared to nematodes, the nematodes float. Particles with a higher specific gravity compared to the extraction fluid, sink. This separation process is accelerated with a centrifuge. The method involves two steps. In the first step, an aqueous nematode suspension, still containing soil particles (obtained with one of the extraction methods described earlier), is centrifuged. Particles with a specific gravity higher than 1 (including nematodes) precipitate. The supernatant can be poured off. In the second step, the sediment (pellet) is brought in suspension in the extraction fluid. After centrifugation, the nematodes float in the supernatant, whereas most other particles are precipitated in the pellet. The supernatant is passed through a fine sieve, after which the nematodes can be collected and observed instantaneously. Not only active, but also slow-moving species (such as *Criconeematidae*) and inactive nematodes can be isolated. Inactive nematodes can be inactive stages of root-knot nematodes and cyst nematodes, eggs, and parasitized and fixed nematodes (see to § 2.3.5.1.).

Choice of the extraction fluid. Solutions of sugar, $MgSO_4$ or $ZnSO_4$ are often used. Sugar seems to be a cheap option, but cannot be reused. It also makes the solution very sticky, and the osmotic value is so high that many nematodes do not survive (especially Dorylaimid species are sensitive). $ZnSO_4$ has a lower osmotic value than sugar or $MgSO_4$, but is acid and toxic. $MgSO_4$ is slightly more expensive than sugar, but can be reused. Sometimes colloidal silica such as Ludox, Percoll and Ficoll are used, as their osmotic effect is negligible, but they are very expensive. In the following procedure $MgSO_4$ is used.

Specific gravity (s.g.) of the extraction fluid. To keep nematodes afloat, the specific gravity of the fluid has to be at least equal to 1.084, the specific gravity of (living) nematodes as calculated by Andrassy (1956). Usually extraction fluids with a specific gravity of 1.15 or 1.18 are used. Increasing the s.g. of the fluid not necessarily leads to increased extraction efficiency; because of the increasing osmotic tension more nematodes get damaged, and more particles keep afloat, resulting in a dirty suspension.

Required quantity of a particular substance (in grams per litre water), to obtain solutions with the indicated specific gravity (Southey 1986) (specific gravity should be checked with a densimeter):

Specific gravity (20°C)	1.15	1.18	1.22	1.28
Sugar	401	484	588	?
MgSO ₄ (pure)	166	200	245	?
MgSO ₄ ·7 H ₂ O	339	409	503	?
ZnSO ₄ (pure)	156	187	229	?
ZnSO ₄ ·7 H ₂ O	279	335	410	?

Centrifugal force. The relative centrifugal force (RCF) is a function of the distance between the centre of the centrifuge and the bottom of the centrifuge tube (R, in cm), and the number of revolutions per minute (N):

$$RCF = 0.0000118 \times R \times N^2$$

RCF's of 700 to 2900 can be used, but 1800 g is generally used. The two Centaur centrifuges at the Department of Nematology need to be adjusted at ± 3000 revolutions per minute (R = 16.3). The used RCF is not critical, as long as a stable pellet is achieved (which depends, among others, of the sample type). Extraction results can only be compared if the same RCF is used.

Requirements

- Centrifuge
- Vibro-mixer
- Balance
- Densimeter
- At least 2 centrifuge tubes
- 2 pipettes for taring (for water and MgSO₄)
- A 10 μ m sieve
- Beaker
- Wash-bottle with water
- MgSO₄ solution, s.g. of 1.18

Procedure

1. Extract the nematodes from the sample, following one of the previously described methods. As the content of the centrifuge tubes is limited, the nematodes should be concentrated in only a small amount of suspension.

For example, for the Oostenbrink elutriator, it is important to rinse the four sieves with as little water as possible. This can be done by washing the debris on the sieve to one side with a little water, keeping the sieve above the other three sieves, so washed off nematodes are intercepted. Wash the collected debris with a little water into a plastic bowl, and repeat this procedure with the other three sieves.

2. Transfer the suspension to two (or another even number of) centrifuge tubes and equalize for weight (with water) on a balance. Centrifuge for 4 minutes at 1800 g.
3. Pour the supernatant off on a 10 μm sieve.

For samples containing a low fraction of silt or some organic material, the pellet (containing the nematodes) may whirl up when the supernatant is poured. Pouring off the supernatant through a sieve intercepts the nematodes that have whirled up or did not precipitate. Coolen and D'Herde (1972) use kaolin, a silt mineral, to make the pellet firmer (before the first centrifugation, add 1 ml of kaolin per 100 ml suspension and stir well). The disadvantage is that the kaolin will precipitate again during the second centrifugation, and possibly carries nematodes, which should stay afloat in the extraction fluid, along with it.

4. Add MgSO_4 (1.18) to the pellet and mix thoroughly with a vibro-mixer (for 15 sec). Rinse the head of the mixer with MgSO_4 above the centrifuge tube.

Please note: The high osmotic value of the extraction fluid causes plasmolysis in the nematodes, altering their shape or even killing them (depending on the species). Therefore it is essential to limit the time nematodes being in the fluid, and to thoroughly rinse them afterwards with water (point 6).

5. Equalize the tubes for weight on a balance, using MgSO_4 and centrifuge for 3 minutes at 1800 g.
6. Pour off the supernatant into the 10 μm sieve. Carefully rinse it with water and collect the nematodes on the sieve in a beaker. The MgSO_4 that passes through the sieve can be collected and reused.

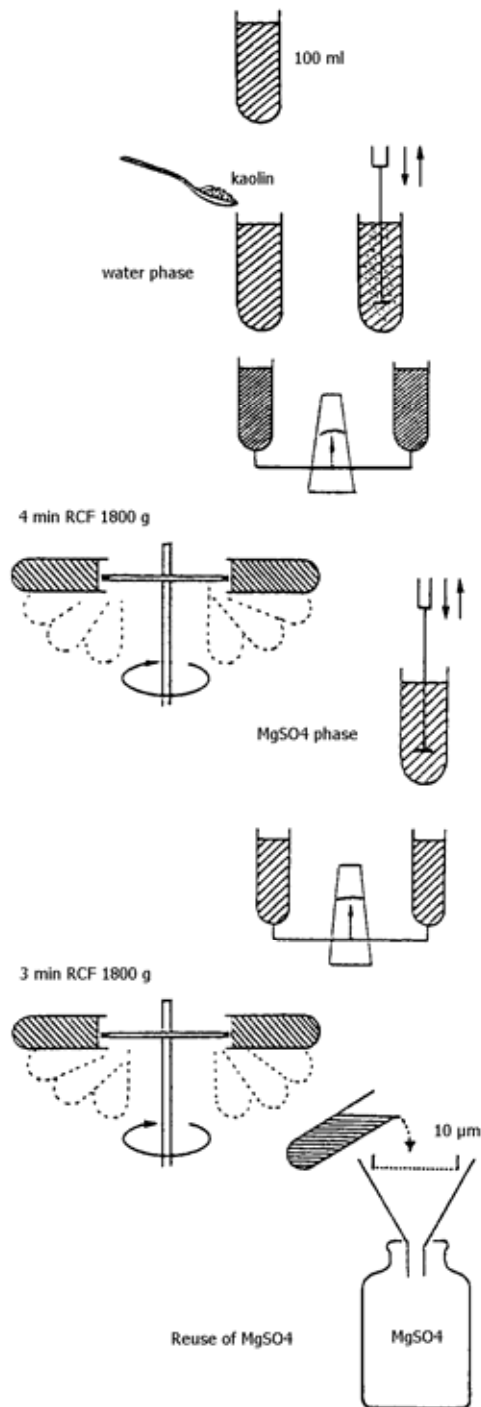


Figure 19
Schematic overview of the centrifugal method

Advantages and disadvantages

The great advantage of the centrifugal flotation method is that it is also suitable for isolating inactive nematodes. The sample can be extracted within an hour and the result is a relatively clean nematode suspension, but the extraction fluid can have negative effects on the nematodes. It may slightly change their shape, making identification harder. If nematodes are used for taxonomical purpose, the problem can be solved by fixing the samples prior to extraction (see § 1.3.1. and § 2.3.5.1.). The nematode vitality can also be affected (species dependent), which may be a problem if nematodes are used for infection experiments. The required equipment is expensive (e.g. centrifuge), although it is usually part of the normal laboratory equipment.

Literature: Gooris & D'Herde 1972

Further reading: for example:

- Southey 1986: overview
- Byrd *et al.* 1966 describe a flotation method without need of centrifuging: by using a strong sugar solution nematodes keep afloat whereas soil particles settle because of adding so-called 'flocculating chemicals' (e.g. Separan). This method is mainly used in the USA.
- Viglierchio & Yamashita 1983 compare the extraction yields obtained with different concentrations of sugar, MgSO₄, ZnSO₄, Percoll and Ficoll, and the effect of those fluids on four nematode species.
- Coolen & D'Herde 1977, Griffiths *et al.* 1990 use Ludox (colloidal silica).

2.3.5.1. Modification for the extraction of fixed samples

Samples of (anaerobic) freshwater sediments or marine sediments are usually immediately fixed to record the situation at the time of sampling (also refer to § 2.3.1). The fixative makes the nematode skin permeable, hampering flotation in dense fluid during the second centrifugation; the fluid freely penetrates the nematode body, making it heavier and causing it to sink. The time the nematodes spend in the fluid is therefore even more critical than for living nematodes. Especially in the case of large marine nematodes a specific gravity value of 1.18 is insufficient to keep them afloat. Therefore a specific gravity of 1.28 is needed.

Requirements

Refer to § 2.3.5 (Centrifugal flotation method), with following difference:

Instead of MgSO₄ s.g. 1.18 , use MgSO₄ s.g. 1.28

Procedure

1. Wash the fixed sample, for example using the Oostenbrink elutriator (§ 2.3.4)
2. Concentrate the suspension and transfer it to two (or another even number of) centrifuge tubes. Equalize the tubes for weight with water.
3. Spin in the centrifuge for 4 minutes at 1800 g.
4. Carefully pour the supernatant over a 10 µm sieve to check whether it contains nematodes. If the sediment is very sandy, the pellet may easily whirl up. To prevent this, add kaolin before the first centrifugation (see § 2.3.5, point 3).
5. Add MgSO₄ 1.28 to the pellet and mix with a vibro-mixer (15 sec). Equalize the centrifuge tubes for weight with MgSO₄ 1.28.
6. Centrifuge for a short period. Switch off the centrifuge as soon as the maximum speed (at 1800 g) is reached (after ± 1 minute).
7. Pour the supernatant off on a 10 µm sieve (the MgSO₄ cannot be reused because the fixative). Carefully rinse the sieve with water to get rid of the MgSO₄ and collect the nematodes in a water-filled beaker (if the suspension has to be stored, put the nematodes again in formalin after counting).
- 7a. If expected that the pellet still contains nematodes (this idea may be well founded, as Claassen 1991 proves), a third centrifugation can be carried out. In that case, repeat steps 5-7.

Literature: Heip *et al.* 1985, Jacobs 1987, Bongers & Van der Haar 1990, Claassen 1991.

2.4. EXTRACTION OF CYSTS

2.4.1. Baunacke method

Area of application

The Baunacke method (or white bowl method) is used for the extraction of cysts from dried soil (max. 50 g) and sometimes for isolating cysts from dried debris.

Principle

The method makes use of floating properties of dried cysts and difference in size, shape, and colour between cysts and other fractions in the sample.

Fine particles are removed by sieving the sample. The debris on the sieve is washed into a water-filled plastic bowl. The cysts keep afloat along the edge of the bowl, whereas the heavy particles sink.

Requirements

175-180 μm sieve (\varnothing 20 cm)

White bowl with a sloping edge, capacity 2-3 litres

Fine painting brush (no. 00, 0 or 1)

Petri dish or watch glass containing moist filter paper

Clean water

Procedure

Dry (§ 1.4.4) a (sub)sample of up to 50 g. Put it on a 175 μm sieve and wash it with water, forcing fine particles through the sieve. Wash the debris off the sieve into a white bowl. The cysts float along the edge of the bowl (a lamp giving top light is useful); they can be picked with a painting brush and transferred to a moist filter paper in a Petri dish or onto a watch glass. The sediment should be stirred, because cysts may be trapped under the settled soil particles. Please note: time to pick cysts is limited, because especially filled ones sink rapidly.

Advantages and disadvantages

The method is very simple, quick, cheap, and water saving. Nevertheless, extraction efficiency and sample size are limited and results depend on the individual carrying out the method. If there are many cysts, a lot of time is required to retrieve them.

Literature: Baunacke 1992, Oostenbrink 1950

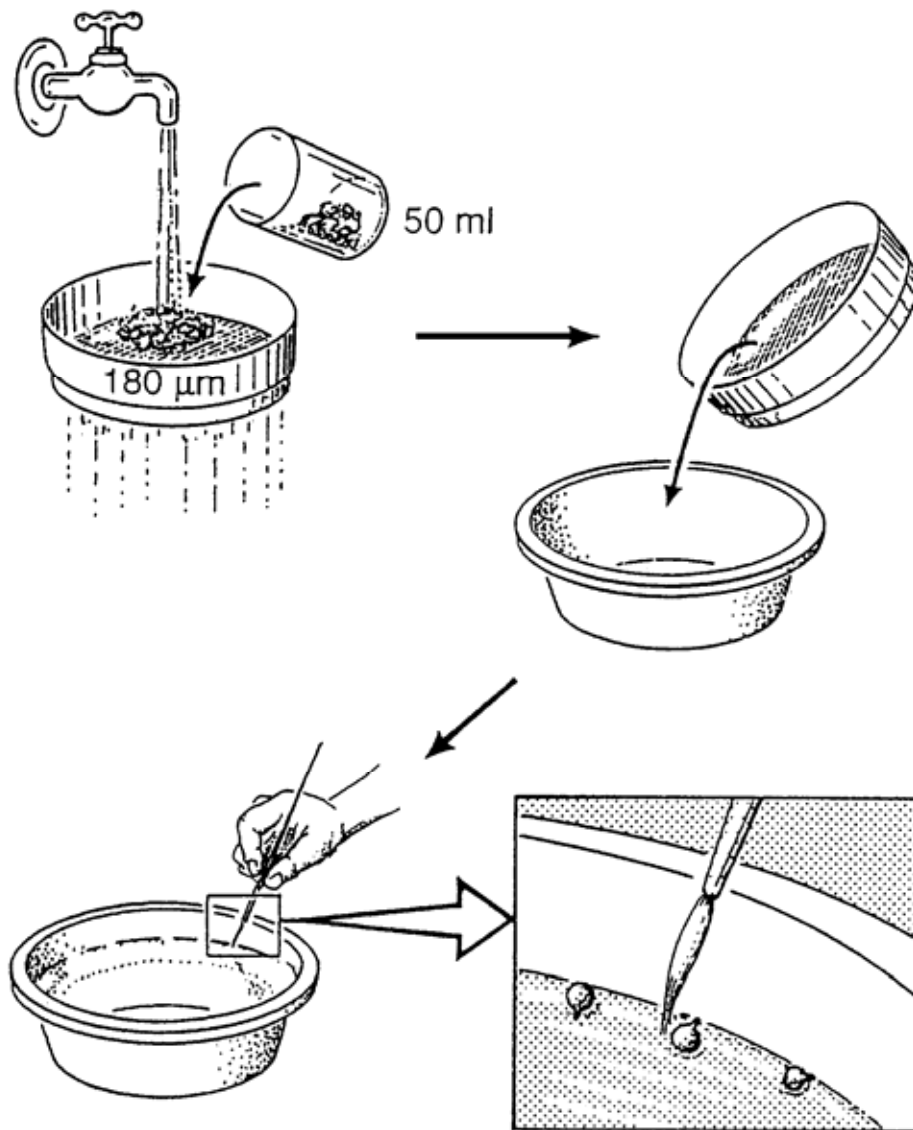


Figure 20
Baunacke or "white bowl" method

2.4.2. Fenwick can

Area of application

The Fenwick can is used for the extraction of cysts from dried soil (max. 300 g). The standard sample size used in potato cyst nematode research is 200 ml.

Principle

The method makes use of floating properties of dried cysts and of difference in size between and other fractions of the sample. In this method, coarse sample material is retained on the sieve, heavy particles passing through sink to the bottom of the can, and fine and light particles, like cysts, keep afloat. When the can overflows, the floating cysts are carried off over the overflow collar, and drop on a sieve with a pore size smaller than the cyst diameter.

Requirements

Modified Fenwick can (Van Bezooijen en Stemerding, 1971,
Catalogue No. 400)

Sieve of 175-180 μm (\varnothing 20 cm)

Procedure

1. Dry a (sub)sample with a maximum weight of 300 g (§ 1.4.4).
2. Clean the can with water. Close the outlet and fill the can to the rim with water. Place a 175 μm sieve under the outlet of the overflow collar, so ar runs on the sieve. The sieve should be slightly inclined (by placing a piece of wood underneath) to facilitate the water running through.

For *Heterodera carotae* and *H. urticae*, which form small cysts, it is better to use a 100 μm sieve. On the other hand, for species forming big cysts (e.g. *H. schachtii*) a sieve with a pore size of 250 μm can be used (the advantage being that more dirt passes through the sieve, but with the risk that immature cysts get lost).

3. Wash the sample through the top sieve using the spray nozzle. Continue spraying for about 5 minutes.
4. Carefully rinse the funnel and the collar with water to ensure that all cysts are washed on the sieve. After this the outlet can be unplugged and the can rinsed with water.
5. The cysts in the debris on the sieve can be isolated in different ways, e.g.:
 - Dry the debris, wash it into a white bowl and retrieve the floating cysts (§ 2.4.1), or:
 - Dry the debris and isolate the cysts using organic solvents (§ 2.4.5.1), or:
 - Wash the debris onto a filter paper and pick the cysts under a dissecting microscope, or:
 - Use the centrifugal flotation method for cysts (§ 2.4.5.2)

Advantages and disadvantages

Large samples can be handled in a standardized way. The method requires large amounts of water. The disadvantages mentioned for dry extraction (see § 2.1.4) also apply here.

Literature: Fenwick 1940, Oostenbrink 1950

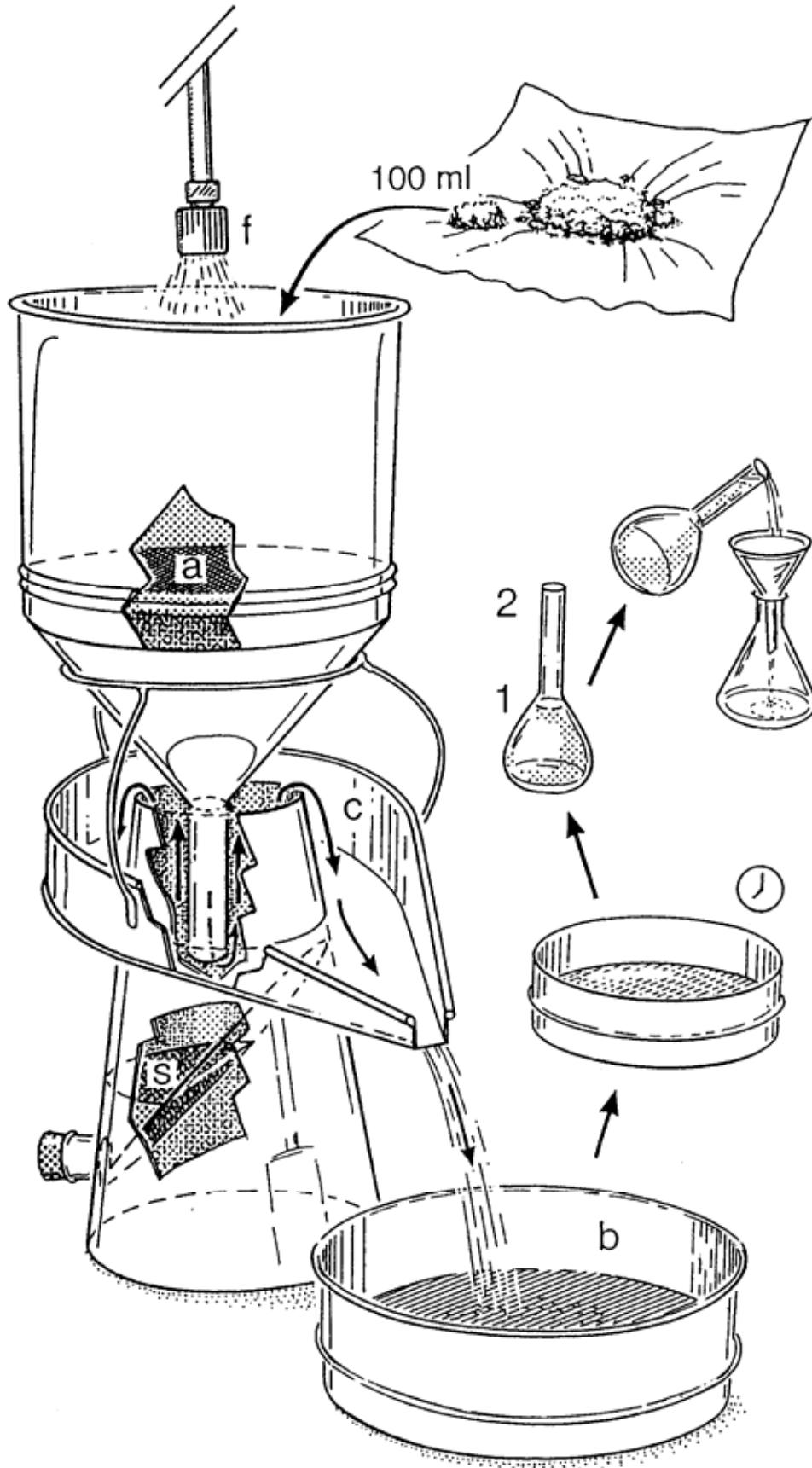


Figure 21
The Fenwick method. Extraction of cysts from dried soil, followed by the acetone method.

2.4.3. Kort's cyst extraction elutriator

Area of application

The Kort's cyst extraction elutriator (Kort elutriator) is used for extraction of cysts from soil (max 300 g). Drying the sample is not necessary. Therefore this method is recommended for the extraction of *Heterodera* and *Punctodera* (if a living cyst content is required).

Principle

The method makes use of differences in sedimentation rate and size between cysts and soil particles. In the Kort elutriator, an undercurrent keeps the cysts afloat, while soil particles settle. The cysts are carried away with the overflowing water, and stay behind on a sieve with a pore size smaller than the cyst diameter.

Requirements

Kort elutriator (Van Bezooijen en Stemerding, 1971,
Catalogue No. 500)

175-180 µm sieve (Ø 20 cm)

Procedure

1. Take a (sub)sample following the guidelines in § 1.4.
2. Clean the Kort elutriator with water. Close the outlet at the bottom of the funnel, and fill the funnel with water. Start the undercurrent and adjust it at 3500 ml/min; position the 175 µm sieve under the overflow collar.

For *Heterodera carotae* and *H. urticae*, which form small cysts, it is safer to use a 100 µm sieve. On the other hand, for species forming big cysts (e.g. *H. schachtii*) a sieve with a pore size of 250 µm may be used (the advantage being that more dirt passes through the sieve, but with the risk that immature cysts might get lost).
3. Wash the sample through the top sieve using the spray nozzle. Stop the sprayer and wait 5 more minutes.
4. Carefully rinse the collar with water, to ensure all cysts are washed onto the sieve. After this the outlet can be unplugged and all parts of the apparatus are rinsed with water.
5. The cysts in the debris on the sieve can be isolated in different ways, e.g.:
 - Dry the debris, wash it into a white bowl and retrieve the floating cysts (§ 2.4.1), or:
 - Dry the debris, and isolate the cysts using organic solvents (§ 2.4.5.1), or:
 - Wash the debris onto a filter paper and pick the cysts under a dissecting microscope, or:
 - Use the centrifugal flotation method for cysts (§ 2.4.5.2)

Advantages and disadvantages

Large samples can be handled in a standardized way. Compared to the 'dry' extraction methods, this method yields more full cysts. Another advantage is that the sample does not need to be dried. But the method requires large amounts of water and the equipment is expensive. To isolate the cysts from the debris, further cleaning is necessary.

Literature: Kort 1960

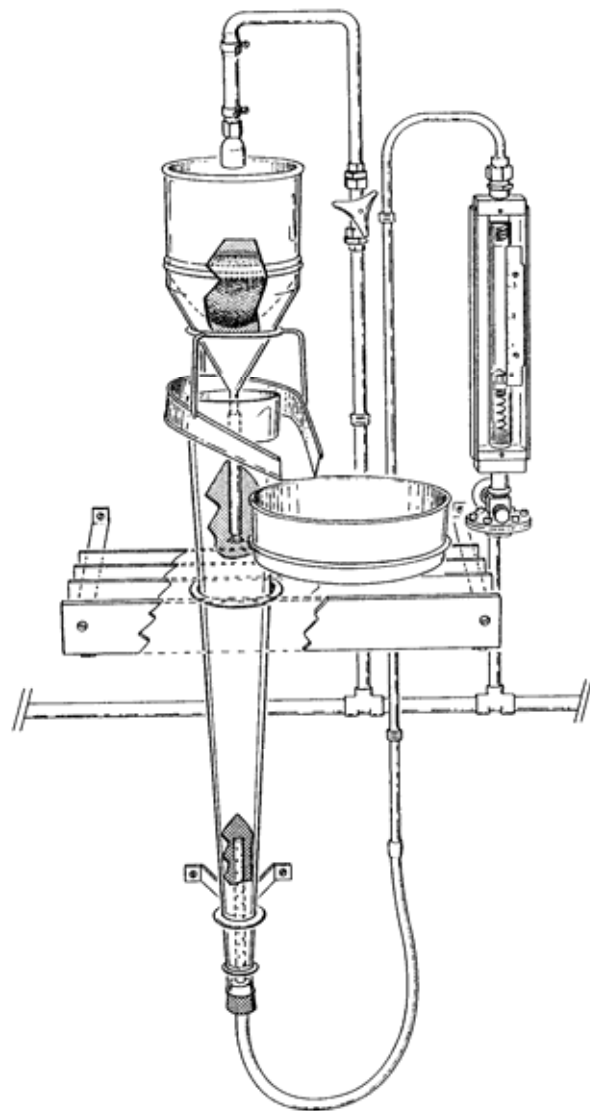


Figure 22
Kort's extraction apparatus, for the extraction of cysts from non-dried soil

2.4.4. Seinhorst cyst extraction elutriator

Area of application

The Seinhorst cyst extraction apparatus (Seinhorst elutriator) is used for the extraction of cysts from soil (max 300 g). The sample does *not* need to be dried; therefore it is recommended for the extraction of *Heterodera* and *Punctodera* (if a living cyst content is required).

Principle

The method makes use of differences in sedimentation rate and size between cysts and soil particles. In the Seinhorst funnel, an undercurrent keeps the cysts afloat while soil particles settle. The cysts are carried away with the overflowing water and stay behind on a sieve with smaller pore size than the cyst diameter. According to Seinhorst, (1964) mainly the light (half) empty cysts are washed onto the sieve, while heavy, full cysts keep afloat in the upper half of the funnel (see figure 23). For that reason the elutriator has a side-outlet to tap the content of the upper half of the funnel, which is also passed through the sieve.

Requirements

Seinhorst elutriator for cysts (Van Bezooijen en Stemerding, 1971,
Catalogue No. 600)

2-mm domestic sieve (Ø 20 cm)

175-180 µm sieve (Ø 20 cm)

Procedure

1. Take a (sub)sample following the guidelines in § 1.4.
2. Clean the Seinhorst elutriator with water. Close both the side-outlet and the outlet at the bottom and fill the funnel with water. Start the undercurrent at 3500 ml/min; Put the 175 µm sieve under the outlet of the overflow collar, so the water from both the overflow collar and the side-outlet (when it is opened) flow on the sieve. The sieve should be slightly inclined (by placing a piece of wood underneath) to help the water passing through.

For *Heterodera carotae* and *H. urticae*, which form small cysts, it is better to use a 100 µm sieve. On the other hand, for species forming big cysts (e.g. *H. schachtii*), a sieve with a pore size of 250 µm can be used (the advantage being that more dirt passes through the sieve, but with the risk that immature cysts get lost).

3. Put the sample in a domestic sieve and place the sieve above the open top side of the elutriator. The sieve, which is submerged in water, should be moved horizontally to make the sample pass through the sieve into the elutriator. When the sample has passed through, wait 5 more minutes.
4. Carefully rinse the collar with water to ensure all cysts are washed onto the sieve. Open the side-outlet, so the suspension passes through the 175 µm sieve. Open the outlet at the bottom and clean the elutriator with water.

5. The cysts in the debris on the sieve can be isolated in different ways, e.g.:
- Dry the debris, wash it into a white bowl and retrieve the floating cysts (§ 2.4.1), or:
 - Dry the debris, and isolate the cysts using organic solvents (§ 2.4.5.1), or:
 - Wash the debris onto a filter paper and pick the cysts under a dissecting microscope, or:
 - Use the centrifugal flotation method for cysts (§ 2.4.5.2)

Advantages and disadvantages

Large samples can be handled in a standardized way. Compared to the ‘dry’ extraction methods, this method yields more full cysts. Another advantage is that the sample does not need to be dried. The method, however, requires large amounts of water and the equipment is expensive. To isolate the cysts from the debris, further cleaning is necessary.

Literature: after Seinhorst 1964

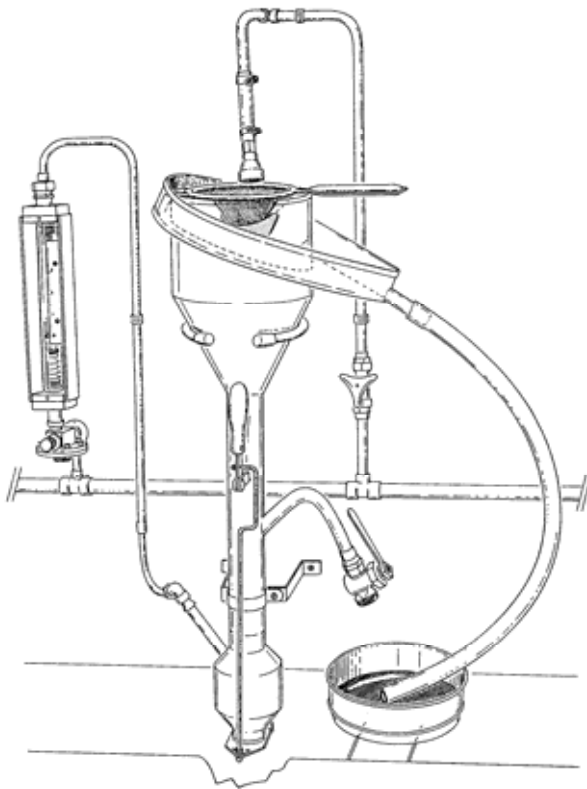


Figure 23
Seinhorst's extraction apparatus for the extraction of cysts from non-dried soil

2.4.5. Isolating cysts from the debris

2.4.5.1. Isolating cysts with organic solvents

Area of application

The method is used for isolating cysts from debris left on the sieve after using one of the extraction methods for cysts described earlier. The debris needs to be well-dried. The method is especially reliable for *Globodera* spp. For many other cysts species, for example the pea cyst (*H. goettingiana*), the cysts are not sufficiently ‘closed’ (they have open ‘windows’, weak spots, and cracks); the extraction fluid rapidly enters the cyst, causing it to sink, so that it is lost for analysis.

Principle

The method makes use of difference in structure between cysts and the debris retained after extraction with one of the methods described earlier. The dried debris is submerged in an organic solvent. Because of the low surface tension the ‘closed’ cysts keep afloat, while the porous organic debris absorbs the fluid and sinks. Commonly used extraction fluids are acetone (CH_3COCH_3), alcohol 96% ($\text{C}_2\text{H}_5\text{OH}$) and mixtures of acetone or ethanol with carbon tetrachloride (CCl_4). In the following example the used extraction fluid is acetone.

Attention: Acetone is very flammable and carbon tetrachloride vapour is poisonous. It is therefore necessary to work in a suitable fume chamber (without ventilation motor producing sparks) and to be careful with fire sources.

Requirements

- 2 glass funnels
- 2 pieces of filter paper (\varnothing 18.5 cm)
- Tea strainer
- 2 glass flasks (250 ml)
- Petri dish (\varnothing 20 cm)
- \pm 500 ml acetone
- Fine painting brush (No. 0 or 1)
- Dissecting microscope

Procedure

1. Wash the soil sample following one of the extraction methods for cysts described earlier.
2. Wash the debris from the extraction sieve into a funnel that contains a filter paper folded in four parts. Let the debris on the filter paper dry at room temperature for at least one night.
3. Bring the dried debris via a tea strainer and a funnel into a 250 ml flask (make sure it does not blow away). The glassware needs to be completely dry.
4. Add acetone up to level 1 (see fig. 21). Stir well and add acetone up to level 2. Wait for 1/2 minute.

5. Pour the floating material with a smooth movement on a filter paper in a funnel, placed on a second flask. To clean the collar from cysts stuck on the side, rotate the first flask slowly while pouring. Pour about half the content of the bottle.
6. Shake the liquid with the remaining debris well and refill with acetone up to level 2. Wait for 1/2 minute and pour the floating material off with a smooth movement. Acetone can be reused.
7. Transfer the filter paper with the cysts to a humid petri dish. Pick the cysts from the debris with a painting brush, under a dissecting microscope.

Advantages and disadvantages

It is a quick, simple and relatively inexpensive method. However, the required extraction fluids have disadvantages (flammability, toxicity); ethanol 96% is the safest, but most expensive alternative.

Literature: den Ouden 1954 (acetone), Oostenbrink 1960 (acetone – carbon tetrachloride mixture), Seinhorst 1975 (ethanol 96%)

2.4.5.2. Isolating cysts with the centrifugal flotation method

Area of application

The method is used for isolating cysts from debris that is retained after cyst extraction. Contrary to the method using organic solvent, it is *not* necessary to dry the debris, and the cysts do *not* need to be ‘closed’. Therefore the method is recommended for the separation of *Heterodera* and *Punctodera* spp. and for separating cysts infected with fungi, if the fungus needs to remain intact for research on biological control.

Principle

The method makes use of difference in specific gravity (s.g.) between cysts and the debris, which remains after nematode extraction. By putting the debris into an extraction fluid with a higher specific gravity than the cysts, they start floating. Particles with a higher specific gravity than the fluid will sink. Centrifugation speeds up this separation process.

The method involves two steps. In the first step, the debris is centrifuged in water. All particles with a specific gravity higher than 1 (including the majority of the cysts) precipitate and the water (supernatant) can be poured off. In the second step, the sediment (pellet) is brought in suspension in the extraction fluid and centrifuged. After centrifugation the cysts float in the supernatant, whereas most other particles are precipitated in the pellet. The cysts are collected by passing the supernatant through a sieve.

For details about the choice and density of the extraction fluid and the centrifugal speed, see § 2.3.5.

Requirements

- Centrifuge
- Vibro-mixer
- Balance
- Densimeter
- Dissecting microscope
- At least two centrifuge tubes
- 2 pipettes for taring (for water and MgSO₄)
- 125 µm sieve (Ø 10 cm)
- Beaker
- Wash-bottle
- Filter paper (Ø 18.5 cm)
- Painting brush (No. 0 or 1)
- MgSO₄ solution, s.g. 1.28

Procedure

1. Wash the sample following one of the extraction methods for cysts described earlier
2. Transfer the debris to two (or another even number) centrifuge tubes and equalize for weight (with water) on a balance. Centrifuge for 4 minutes at 1800 g.

3. Decant the supernatant through a 125 µm sieve, to collect empty (and floating) cysts.
4. Add MgSO₄ (1.28) to the pellet and mix thoroughly with a vibro-mixer (15 sec). Rinse the head of the vibro-mixer with MgSO₄, above a centrifuge tube.
5. Equalize the centrifuge tubes for weight with MgSO₄, on the balance; centrifuge for 3 minutes at 1800 g.
6. Pass the supernatant through the 125 µm sieve. Rinse it with water and collect the cysts from the sieve in a water-filled beaker. The MgSO₄ passing through the sieve can be collected and reused.
7. Place a filter paper folded in four parts in a funnel and pour the beaker's content into the funnel. Use a painting brush to collect the cysts from the debris under a dissecting microscope.

Advantages and disadvantages

Since the debris does not need to be dried, the method is also suitable for separation of cysts that cannot stand dehydration. The equipment used is expensive but is usually part of the standard laboratory inventory. The centrifuge tube size must be sufficiently large, e.g. 100 ml tubes.

Literature: after Dunn 1969

2.5. ANALYSIS OF EXTRACTS

If the extract involves a suspension with living nematodes, it must be analysed as soon as possible after extraction. The watery suspension is perishable (due to fungal and bacterial growth) and the original numbers of nematodes change because of mortality and recoreuction. Store the suspension in the refrigerator (at 4°C) or fix the nematodes (§ 3.4.1). *Cysts*, when dried, can be stored for a long period, although the vitality of the cyst content may decrease.

2.5.1. Counting of nematodes in the suspension

Requirements

Dissecting microscope
Aquarium pump or compressed air
100 ml beaker
10 ml aspirator pipette
Siphon with water
Counting dish
Clean water

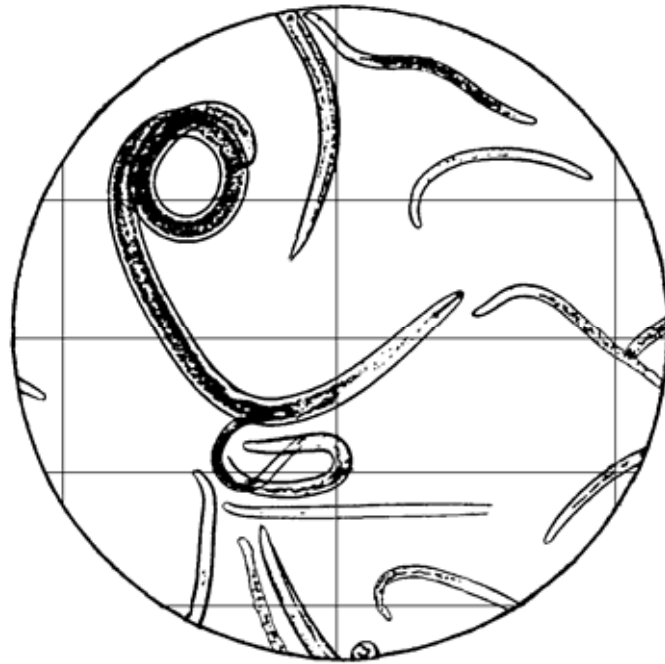


Figure 24

Nematode suspension under a dissecting microscope

Procedure

1. Add water to the nematode suspension with a wash-bottle, up to a particular volume, e.g. 100 ml. Rinse the pipette and the mouthpiece of the air-hose with water.

2. Carefully mix the suspension in the beaker, using air from an aquarium pump or compressed air and by sucking the suspension up and squirting it out with a pipette a couple of times.
3. Keep the tip of the pipette in the middle of the suspension (*not* in the air bubble stream) and quickly suck up a certain amount of liquid, depending on the density of the suspension and the size of the counting dish. Immediately deposit the pipette content in a counting dish. Squeeze the sucker a couple of times, so that no liquid is left in the pipette. Fill a second counting dish following the same procedure.
4. Let the nematodes settle and count them at 25-50x magnification. Carry out at least two counts * (from two different dishes).

* Two counts usually don't have exactly the same number, but what difference is still acceptable? Usually the following rule is used: if the number of a count differs more than 5%, a third count is required. The final result then is the average of three instead of two counts. If one count differs extremely from the others (e.g. because of a pipette error), it is permitted to exclude that count and calculate the average of the two remaining counts (again, the earlier described rule applies for these two counts!). Southey (1986) indicates that, if the suspension is homogenous, the counts follow a Poisson distribution, meaning that the minimum standard deviation for each count (x) is \sqrt{x} . For numbers below 100, the 5 % rule can not be used. The minimum SD (\sqrt{x}) is then a better alternative.

Count x	1	4	16	25	100	400
Minimum SD = \sqrt{x}	1	2	4	5	10	20
5 %	-	-	-	1.25	5	20

2.5.2. Estimating and counting the cyst content

Estimating

Requirements

Dissecting microscope
 2 glass slides
 Fine painting brush (No. 0 or 1)
 Clean water
 If available: cyst squasher

Procedure

Transfer the (determined) cysts to a glass slide, using a fine painting brush. Keep a reasonable distance between cysts and leave each cyst in a water droplet. Place a second glass slide on top of the first and move slightly back and forth, so the cysts burst open and eggs and larvae are released. Instead of the second glass slide, a cyst squasher can be used. Estimate, under a dissecting microscope, the numbers of eggs and larvae per cyst.

Estimating the numbers is less time-consuming than counting and it is relatively reliable for numbers of eggs and larvae below 250 per cyst. At higher numbers per cyst, the deviation becomes more significant.

Counting

Requirements

See § 2.5.1 (Counting of nematodes in the suspension), and also:
Cyst homogeniser (Huijsman 1957)
Fine painting brush (No. 0 or 1)
Wash-bottle with water

Procedure

Transfer a counted number of cysts and a few water droplets onto the piston tip of the cyst homogeniser. Put the piston in the homogeniser and carefully rotate, so the eggs and larvae are released from the bursting cysts. Wash the eggs and larvae into a 100 ml beaker, using a wash-bottle. Rinse the piston and the homogeniser with plenty of water above the beaker and fill the beaker up to the 100 ml level. Take two sub samples from the suspension, following a procedure described under 'Counting of nematodes in the suspension'. Count the numbers of eggs and larvae using a 25-50x magnification. Do not include empty eggshells in the count and repeat the counting procedure at least once (see § 2.5.1).

2.5.3. Presentation of numbers

Per VOLUME:

$$N_{vol} = \left(\frac{v_2}{v_1} \times n_1 \right) \times \frac{100}{v_3} \text{ nematodes per } 100 \text{ ml sample}$$

with n_1 = number of nematodes in v_1
 v_1 = volume (ml) of the counted suspension obtained from v_2
 v_2 = volume (ml) of the extracted sample total suspension
 v_3 = volume (ml) of the sample

Per WET WEIGHT

$$N_{ww} = \left(\frac{v_2}{v_1} \times n_1 \right) \times \frac{100}{W} \text{ nematodes per } 100 \text{ g of wet sample}$$

with n_1 = number of nematodes in v_1
 v_1 = volume (ml) of the counted suspension obtained from v_2
 v_2 = volume (ml) of the extracted sample total suspension
 W = weight (g) of the sample

Per DRY WEIGHT

$$N_{dw} = N_{ww} \times \frac{1}{DM} \text{ nematodes per } 100 \text{ g of dry sample}$$

with N_{dw} = the number of nematodes in 100 grams of wet sample, see above
DM = dry matter fraction of the sample (= 1 – liquid fraction):

$$DS = \frac{w2 - p}{w1 - p}$$

with p = weight of the empty jar
 $w1$ = weight of the jar *with* soil, *before* drying
 $w2$ = weight of the jar *with* soil, *after* drying

Per SURFACE area (Please note: the (sample) *depth* is variable):

$$N = \left(\frac{v2}{v1} \times n1 \right) \times \frac{10000}{surf} \text{ nematodes per } m^2$$

with $n1$ = number of nematodes in $v1$
 $v1$ = volume (ml) of the counted suspension obtained from $v2$
 $v2$ = volume (ml) of the extracted sample total suspension
surf = sample surface (cm²) = surface of the cores used for taking the sample
(if other sampling equipment is used, the formula must be adapted):

$$surf = ns \times (\pi \times (\frac{1}{2}d)^2)$$

with ns = the number of cores
 d = diameter of the core (cm)

Please note: For calculating numbers of larvae and eggs **from cysts**, the same formulae may be used, after adding the term (total number of cysts in the sample) divided by (number of cysts used to make the suspension)

3.1. PICKING NEMATODES

To pick ('fish') nematodes from a suspension, a good handling ('fishing') needle is essential.

'Fishing' needle

A fish needle or handling needle can be made in several ways, for example:

- Cut with a razor blade a fine tip to a bamboo splinter (under a dissecting microscope) and fix it in a needle holder, or:
- Take a hair (eyebrow hair or a pig hair; the last one is sturdier but splits easy), a fine fishbone, a nylon hair from a toothbrush or a fine metal thread. Mount this with a drop of glyceol on a small bamboo stick (± 3 cm), which is fixed in a needle holder, or on the tip of a dissecting needle. If necessary the tip can be cut slantwise under a dissecting microscope. Soft material (hairs) reduce the chance of damaging nematodes, but makes it harder to pick heavy nematodes. When working under sterile conditions, for example if nematodes have to be transferred to agar plates (Chapter 4), a handling needle with a metal thread is required, because it can be sterilized in a flame. A cat's whisker, which is spatulate, is suitable for picking nematode eggs.

Picking nematodes is easiest from the middle of a shallow dish, under a microscope at low magnification. Bring the nematode to the water surface with the needle (keep following the nematode by adjusting the focus of the microscope), and pull the animal with a smooth movement through the meniscus. Picking living nematodes is easier than dead ones, because they curl around the needle and do not slip off that easily. For picking cysts and other swollen stages, a (moistened) fine painting brush (No. 00, 0 or 1) is used. Hesling (1952) used an aspirator for picking cysts from debris. The method was improved by Bijloo 1954.

3.2. CONCENTRATING NEMATODE SUSPENSIONS

For concentrating nematode suspensions, e.g. to a standard volume for counting, or to a few droplets for fixation, the nematode sedimentation rate is used. The amount of fluid can be reduced by sieving the suspension, or by decanting or sucking the liquid after the debris has settled.

Sieving: The suspension is sieved over one or more fine ($\leq 45\mu\text{m}$) sieves. The nematodes are washed off the sieves with a little water (e.g. with a wash-bottle) and collected.

Removing liquid: The time required for proper settling of the suspension depends on the volume, the size and form of the beaker or bowl, and the nematode species (especially Dorylaimids and Monochids tend to float for a long period because of their greasy cuticle). Depending on the required accuracy, a settling time of 1 to 24 hours is used. Alternatively, the suspension can be centrifuged (3 minutes at 1800 g). Removing excess fluid by suction is more accurate than decanting. Decanting has to be done with one steady and smooth movement, to avoid whirling up of nematodes.

Sucking the liquid can be done with a (micro) pipette, a siphon bottle or a water jet pump. The opening size has to be smaller for sucking smaller quantities of liquid (more accurately).

Micropipette

A micropipette can be made from a pasteur pipette. Turn its narrow end around in a flame and, as soon as the glass starts softening, stretch the tip with forceps to form a glass thread. The tip, which is closed by melting, is cracked open with forceps.

When sucking up the liquid, the tip of the pipette has to be kept slightly under the water surface, and the sediment should not whirl up. The sediment can be put in small jars, and sucking can be followed under the dissecting microscope, to make sure that no nematodes are sucked away. Instead of a jar, Bongers *et al.* (1989) use glass funnels with the stem removed and closed by melting. The nematodes sink in the little pit at the bottom, and the liquid can be removed with a micropipette, without disturbing the sediment. The funnel fits under a microscope, so it is possible to follow sedimentation and sucking up.

3.3. ANAESTHETIZING

Nematode body structures, for example the oesophagus or the spear, can be observed more easily in living nematodes than in dead or fixed ones. Living nematodes can be anaesthetized to keep them quiet, making it possible to observe them in a temporary mount (see § 3.6.2). Transfer the nematodes to a glass slide, into a droplet of:

- Dichloro-diethyl-ether solution (2-10 drops in 50 ml water, stir well) (Southey 1986), or
- 0.5-1% propylene-phenoxetol solution (Ellenby & Smith 1964, Townshend 1984), or
- 0.01 M solution of sodium-azide (Nelson *et al.* 1983)

The nematodes recover after adding fresh tap water (their recovery may take some hours).

3.4. KILLING AND FIXING

If a nematode suspension cannot be counted immediately, if it has to be sent to a taxonomist, or if it has to be prepared for further conservation in mounts, nematodes have to be killed and fixed. The result (effect on different body structures, shelf life) depends on the method; none of the existing techniques is equally suitable for all nematodes. Usually the best results are obtained by killing the nematodes rapidly (generally through heating, up to 65-90°C) followed by immediate fixing. With a hot fixative, these two steps are combined. As a result of the heat shock the nematodes assume a characteristic shape, depending on the species; e.g. straight, C-shaped, or spiral-shaped. Cooling must happen fast, because an extended heating period causes deformations.

Formalin (4-5%) is most used as a fixative. It has a hardening effect and causes slight shrinking of nematodes. A combination with substances having contrary effects, such as acetic acid and propionic acid, are used for neutralizing the shrinking effect as much as possible (§ 3.4.2).

3.4.1. Mass fixation of nematode suspensions

In this method, killing and fixing nematodes happens simultaneously with a hot fixative. The technique is derived from the Seinhorst (1966, 1973) method for fixing individual nematodes.

Requirements

Small lockable tube (content 10 ml)
Test tube in a beaker with water
Pipette, water jet pump or siphon bottle
Micropipette
Formalin 4%, in wash-bottle

Procedure

1. Let the nematode suspension settle for at least 1 hour (or a shorter period if lower accuracy is acceptable). Carefully suck the liquid until \pm 10 ml remains.

A pipette, a siphon bottle, or a water jet pump may be used to suck the liquid. The pipette's tip must just touch the water surface and the sediment must not whirl up (see § 3.2).

2. Stir the remaining suspension and pour it into a tube (content 10 ml). Let the suspension settle for at least 1 hour.
3. Carefully remove as much liquid as possible with a micropipette (see § 3.2) under a dissecting microscope to prevent nematodes from being sucked away. To achieve optimal killing and fixing of nematodes, leave as little water as possible; the nematodes must remain in a water film.
4. Heat a few ml of formalin 4% (or a formalin / propionic acid mixture 4:1, F.P. 4:1, see § 3.4.2) in a test tube placed in a water-filled beaker, until little bubbles appear (the liquid is then 90°C). **This must be done in a fume chamber or at a well-aerated location, because formalin is harmful to human health!**
5. Pour the hot fixative over the nematodes and immediately cool down (to prevent deformation of nematodes) by adding cold formalin 4% and/or by submerging the tube in cold water. Carefully close the tube to avoid evaporation of the liquid, and insert a label, written in pencil, to store the sample.

Netscher (1971) concentrated the suspension on a filter placed on a vacuum bottle, avoiding the time-consuming phase of settling and sucking. F.P. 4:1 was added shortly before the filter was dry. After filtering off the F.P. 4:1, he washed the nematodes from the filter into a small bottle with formalin.

3.4.2. Recipes of fixatives

SOME FIXATIVES (notably FORMALIN) ARE HARMFUL TO HUMAN HEALTH! YOU MUST THEREFORE WORK IN A FUME CHAMBER!

Formalin 4%

Formalin (= 37% formaldehyde)	10.8 ml
Distilled water	89.2 ml

The body structure of some species becomes grainy when using formalin as a fixative. This is probably due to the formation of 'free formic acid', and can be prevented by adding a bit of calcium carbonate to the stock solution (Baker 1945).

F.A. 4:1 or F.A. 4:10

Formalin (= 37% formaldehyde)	10.8 ml
Glacial acetic acid	1 or 10 ml
Distilled water	Add to obtain a total volume of 100 ml

Acetic acid neutralizes the shrinking effect caused by formalin. The disadvantage of this mixture (especially 4:10) is that nematodes become brown and the posterior part of the spear fades.

F.P. 4:1

Formalin (= 37% formaldehyde)	10.8 ml
Propionic acid	1 ml
Distilled water	88.2 ml

Propionic acid neutralizes the shrinking effect caused by formalin, and slightly enhances the contrast. F.P. is useful for year-long fixation, although Netscher and Seinhorst (1969) prefer fixing in formalin 4%, after killing with hot F.P. 4:1 (or F.A. 4:1).

F.G. 4:1

Formalin (= 37% formaldehyde)	8.5 ml
Glycerine	2 ml
Distilled water	89.5 ml

If other components of the liquid evaporate (e.g. if the jar was not properly closed), nematodes kept in this fixative (De Grisse 1969) stay in the glycerine, preventing them from drying out.

T.A.F.

Formalin (= 37% formaldehyde)	7.6 ml
Tri-ethylamine	2 ml
Distilled water	90.4 ml

Fixing in T.A.F. (Courtney et al. 1955) gives good results, but Hooper et al. (1983) detected that the cuticle of some nematodes had degenerated after 20 years of storage. Therefore, Southey (1986) does not recommend T.A.F. as a long-term fixative.

F.A.A. (Ditlevsen's)

Formalin (= 37% formaldehyde)	6.5 ml
Ethanol 96%	20 ml
Glacial acetic acid	1 ml
Distilled water	39.5 ml

Because of the alcohol, F.A.A. has a dehydrating, thus shrinking, effect. This may be useful, among others, for making annulation or natural indentations more visible (Southey 1986).

3.5. TRANSFERRING TO GLYCERINE

The intestinal structure of fixed nematodes often becomes grainy. By transferring them to glycerine the structure becomes more clear, so organs like gonads become visible again (although light-refracting structures such as the spear fade). Glycerine is also a long-lasting medium, suitable for making permanent mounts.

Recently fixed nematodes, which have not yet hardened, cannot be directly brought into glycerine, because they would shrink due to the higher osmotic value. There are two methods to encounter this problem:

With the glycerine-ethanol method (Seinhorst 1959) the nematodes are stepwise dehydrated and infiltrated with glycerine by using two solutions (S.1 and S.2, see (§ 3.5.1)). This method is time consuming and is used to transfer individual nematodes for permanent mounts.

With the glycerine-formalin method (Bongers 1993) the nematodes first remain in formalin for 6 weeks to harden. After that, pure glycerine is added to obtain a 1:1 glycerine-formalin mixture. This method is mainly used to simultaneously transfer a number of nematodes for mass mounts (§ 3.6.3.2). Preliminary results indicate that this direct method yields good images but its suitability for long-term storage is not yet known.

3.5.1. Glycerine-ethanol method

Requirements

- Incubator (40°C)
- Dessicator
- Glass fixation dish and petri dish
- Micropipette, handling ('fishing') needle
- S.1** (20 ml ethanol 96%, 1 ml glycerine, 79 ml distilled water)
- S.2** (93 ml ethanol 96%, 7 ml glycerine)
- Ethanol 96%
- Dehydrated glycerine
- Silicagel

Procedure

1. Fix the nematodes according to the Seinhorst method (see § 3.4.1) and let the nematodes harden for at least 2 weeks, but preferably longer.

2. Transfer the nematodes to a fixation dish and add S.1. Place the fixation dish in a dessicator (or any other vessel that can be tightly sealed, e.g. a plastic box) with a small layer of ethanol 96%. Put this in the incubator (at 40°C).

Because of the ethanol at high temperature, a saturated alcohol vapour is formed, increasing the alcohol concentration in the fixation dish, and dehydrating the nematodes. In step 3 (using S.2) the alcohol will evaporate, leaving the nematodes in the glycerine.

3. After 16-24 hours, the S.1 has to be sucked away under the dissecting microscope using a micropipette, and the S.2 is added. Place the fixation dish in a closed petri dish and put it back in the incubator. After 2 hours 2 drops of dehydrated glycerine are added.
4. Once the alcohol has evaporated (after 16-24 hours), place the fixation dish in the dessicator, containing silicagel or another type of drying agent (e.g. calcium chloride) to allow the last water traces to vaporize.

Please note: The method can also be used for transferring nematode masses, by adding S.1 and S.2 to concentrated suspensions. However, the Glycerine-formalin method is much faster:

3.5.2. Glycerine-formalin method

Requirements

Micropipette
Pasteur pipette with tip broken off
Dehydrated glycerine

Procedure

1. Fix the nematodes following the Seinhorst method (see § 3.4.1) and let them harden for 6 weeks.
2. Suck the formalin until approximately 1 ml remains in the tube, and add a few drops of glycerine with a Pasteur pipette without tip. After two days the nematodes can be transferred to a (mass) mount.

3.6. MOUNTING

3.6.1. Materials

Nematode mounts can be made on ordinary glass slides or on Cobb slides (‘aluminium double cover slip slides’, fig. 25). Glass slides are cheaper, and are used for temporary mounts. Permanent mounts are made on Cobb slides. The advantage is that the mounts can be observed from either side and breaking is less probable as the glass slide is fixed between pieces of cardboard, thicker than the glass. The mounts can also be piled up without touching each other. When carrying out routine analysis of nematode communities, it is common to use mass mounts enclosing hundreds of nematodes on one large (76 x 50 mm) glass slide (Bongers *et al.* 1989). To avoid crushing nematodes, the cover slip must be supported and to prevent drying out, the mount must be sealed (Table 3.1). The paraffin ring suits both purposes. It also has the advantage that the nematode is enclosed in a small area (making detection easier) and that the mount becomes stronger. To make the paraffin ring, the heated copper tube is dipped in solid paraffin and stamped on the glass slide. The result is long lasting and the method is relatively fast, but the mount is sensitive to temperature because of the relatively low melting point of paraffin (52-64°C). The mounts of the University’s Nematology Department are all made with a paraffin ring, and sealed with glyceel.

Another method to make permanent mounts is by supporting the cover slip with little pieces of glass fibre (Table 3.1) and by sealing the mount with glyceel. This yields long-lasting slides but the method is labour intensive.



Figure 25.
Prepared Cobb slide, and an aluminium holder with two cardboard pieces.

Table 3.1 Different materials for supporting the cover slip and sealing the mount.

PURPOSE	MATERIAL	Remarks	Durability*
Supporting the cover slip	Paraffin ring	Also seals the mount (after melting and solidification); Adjust the thickness of the ring to the diameter of the nematode(s)	+++
	Pieces of glass fibre	Select thickness according to the nematode diameter; arrange the glass fibre pieces radially	+++
	Cover slip pieces	Used for thick nematodes (e.g. mermitid nematodes)	+++
	Glyceel (nail varnish etc) ring	Prepare the ring on a turntable or make two parallel strips	+++
Sealing the mount	Candle wax	Use a recently extinguished candle and use the candle-wick as a brush	-
	Nail varnish	Dissolves in acetone	+
	Paraffin ring	Make the paraffin melt (hot plate or flame) and solidify	++(+) (1)
	Glyceel	Dissolves in alcohol and butyl-acetate (3); Clean the brush with alcohol	+++ (2)

*: Durability is related to two aspects (amongst others): the cover slip must be sufficiently supported, so nematodes are not crushed and the mount must be properly sealed, so desiccation is prevented, even after storage for decades.

(1): Durability is further enhanced by encircling with glyceel.

(2): Apply at least two layers (unless the glyceel is thick enough).(1)

(3): Take this into account when cleaning the mount, e.g. after use of oil immersion!
Preferably clean the mount by wiping off the oil as much as possible with a dry tissue, and polish it up with another clean tissue. If alcohol (70%) is required for cleaning, avoid contact with the glyceel ring.

3.6.2. Temporary mounts

3.6.2.1. Mounts in water

Living nematodes can be easily identified and studied in a water mount. In this type of mount, a number of structures like the spear, the oesophageal lumen, and the excretory pore can be seen more easily than in dead and fixed nematodes. Water mounts are usually made on common glass slides, with a paraffin ring (see § 3.6.3.1). They are sealed with nail varnish or candle wax.

Requirements

- Clean glass slide and cover slip
- Handling ('fishing') needle, pair of forceps
- Filter paper
- Nail varnish or candle

Procedure

1. Place a droplet of water on a glass slide

Esser & MacGowen (1973) treat the glass slides with 'Siliclad', a silicone paste keeping the water droplet spherically-shaped. This reduces water evaporation and the chance of air bubble formation when placing the cover slip.

2. Put the nematode(s) with a handling needle in the centre of the water droplet. Make sure that they do not float, but remain on the bottom.

If the nematodes move too much, they can be anaesthetized (see § 3.3) or they can be killed by heating the glass slide 1-2 seconds above a flame or on a hot plate (60°C). It is better to use a hot plate (low risk of overheating or not killing the nematode). The plate needs to be switched on fifteen minutes before heating the slide.

3. Apply the cover slip with a pair of forceps. Remove excess water with a filter paper and seal the cover slip with nail varnish or candle wax (fix it first at three points, and then apply the whole ring). Let the ring dry well (10 minutes) before placing the mount under a microscope.

3.6.2.2. Mounts in lacto phenol

These types of mounts can be conserved longer than mounts in water. They are used for enclosing perineal patterns (see § 3.6.3.3), and sometimes for enclosing whole nematodes. For the latter, certain structures may start fading after a few weeks. Therefore, glycerine is used for permanent mounts. Sometimes it is useful to have a semi-permanent mount. Unlike the transfer to glycerine, the transfer of fixed nematodes to lacto phenol is a quick process: transfer the fixed nematodes to a drop of warm lacto phenol on a glass slide and leave it on a hot plate for half an hour (30°C). Produce the mount as described under section 3.6.3.1 (with lacto phenol).

Phenol is toxic; work in a fume chamber!

3.6.3. Permanent mounts

3.6.3.1. Mounts in glycerine

Mounts in glycerine can rightly be called ‘permanent’; if they are sealed well, they can be permanently stored. They are usually made in Cobb slides. Different materials may be used to support the cover slip, e.g. a paraffin ring (*a*) or glass fibre (*b*).

Requirements

- Cobb slide: Aluminium holder
 - Square cover slip (25 x 25 mm) No. 1
 - Circular cover slip (Ø 18 mm) No. 1
 - Two pieces of cardboard (25 x 25 mm)
- Pair of forceps
- Handling (‘fishing’) needle
- Painting brush (No. 1)
- Cotton wool
- Dehydrated glycerine
- Ethanol 96%
- Glyceel
- (*a*) Hot plate
- Alcohol flame
- Stamp (copper tube, interior Ø 10 mm, exterior Ø 12 mm)
- Petri dish with paraffin
- Dissecting needle with flattened and sharpened point
- White spirit
- (*b*) Glass fibre
- Filter paper
- Hot plate (at 80°C)

Procedure (*a*) (paraffin ring)

1. Fix the nematodes and transfer them to glycerine (see sections 3.4.1 and 3.5.1).
2. Place a square cover slip on a Cobb slide. Heat the stamp in a flame, gently press it on the paraffin and stamp a small ring on the cover slip.
3. Deposit a droplet of dehydrated glycerine in the centre of the ring. Transfer the nematode(s) to the middle of the droplet with a handling needle.
4. Apply a (warm) round cover slip with a pair of forceps. Put the mount on the hot plate (65°C) until the paraffin starts melting. Gently press the cover slip with the forceps and let the mount cool down, so the paraffin solidifies.
5. Scrape the excess paraffin off with a flattened dissecting needle and clean the edges first with white spirit and then with ethanol (use a piece of cotton wool, wrapped around a forceps leg). Place two pieces of cardboard on either side of the cover slips.

6. Encircle the round cover slip with glyceel. The glyceel needs to dry for one night; after that, the mount can be studied. Fold the sides of the mount-holder and use the pieces of cardboard to write specifications on with waterproof ink.

Procedure (b) (glass fibre)

1. Fix the nematodes and transfer them to glycerine (see sections 3.4.1 and 3.5.1).
2. Place a square cover slip between two pieces of cardboard on a Cobb slide. Put a drop of dehydrated glycerine on the cover slip and transfer the nematode(s) with a handling needle to the droplet bottom.
3. Arrange 3 pieces of glass fibre around the nematodes on the bottom of the droplet. The glass fibres are approximately 1 mm long, and the thickness should be selected according to the nematode diameter. Apply a cover slip with a pair of forceps. Redundant glycerine can be removed with filter paper first, and then with ethanol (use a piece of cotton wool, wrapped around a forceps leg).
4. Fix the cover slip at three points with glyceel and after that apply the glyceel ring in 1-2 layers. The glyceel must dry for one night before the mount can be studied. Fold the sides of the mount-holder and use the pieces of cardboard to write down the specifications with waterproof ink.

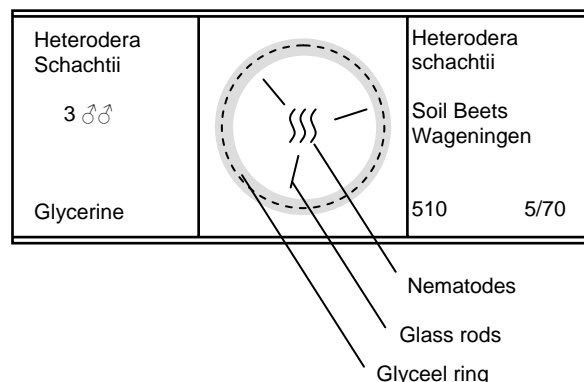


Figure 26
Permanent mount in glycerine, using a Cobb slide

3.6.3.2. Mass mount

Requirements

- Hot plate
- Alcohol flame
- Petri dish with paraffin
- Square paraffin stamp (45 x 45 mm)
- Glass slide 76 x 50 mm
- Cover slip 45 x 45 mm, No. 1
- Pasteur pipette, forceps
- (Painting brush No. 1)
- (Glyceel)
- Hot plate (at 80°C)

Procedure

1. Fix the nematodes and after 6 weeks transfer them to glycerine-formalin (see sections 3.4.1 and 3.5.2). Leave the nematodes in this solution for 2 days.
2. Heat the stamp in a flame, gently press it on the paraffin and stamp a small square on the glass slide.
3. With a Pasteur pipette, carefully pick the nematodes from the bottom of the tube and put 3 droplets of nematode suspension in the middle of the paraffin square.
4. Apply a cover slip with a pair of forceps. Place the mount for a short period on a hot plate (65°C). When the paraffin starts melting, gently press the cover slip down with the forceps, remove from the hot plate, and let the mount cool down for the paraffin to solidify.
5. If necessary, the cover slip can be sealed with glyceel. The glyceel needs to dry for one night; after that the mount can be studied. Label the mount by writing on the glass with a permanent marker, or with a sticker.

3.6.3.3. Mount of perineal patterns

The cuticular patterns around the anus and vulva (perineal patterns) are one of the characteristics studied to identify *Meloidogyne* spp. For this purpose, the mount is enclosed in lacto phenol-cotton blue.

Phenol is harmful; work in a fume chamber!

Requirements

Hot plate
Alcohol flame
Stamp (copper tube, interior Ø 10 mm, exterior Ø 12 mm)
Petri dish with paraffin
Fixation dish with lacto phenol – 0.03% cotton blue
Dissecting needles and pair of forceps
Scalpel, painting brush (No. 1), (soft) handling ('fishing') needle
Dissecting needle with flattened and sharpened point
Cobb slide (for parts required, see § 3.6.3.1)
Piece of Perspex (cutting plate)
Cotton wool
Ethanol 96%
Glyceel, white spirit

Procedure

1. Carefully wash the roots containing galls, and dissect the *Meloidogyne* females from the galls (or: blend the roots in a beaker with water (5 sec) and pick the females from the suspension). Pierce the nematodes at the cervical end and press the body contents out to allow staining from both the inside and the outside. Transfer the females to the fixation dish containing lacto phenol-cotton blue and leave it for 24 hours or longer at room temperature.

2. Put a stained, female nematode in a droplet of lacto phenol-cotton blue 0.03% on a piece of Perspex, and cut off the posterior end under a dissection microscope. Trim this posterior end to obtain a square piece of cuticle with the vulva and anus (dark coloured) in the middle. Carefully remove the remaining pieces of body tissue with a handling needle.
3. Stamp a small ring of paraffin on a square cover slip in a holder (see § 3.6.3.1), put a droplet of lacto phenol-cotton blue in the centre and push the piece of cuticle to the bottom of the droplet, *with the exterior side facing up*.
4. Apply a cover slip with a pair of forceps. Place the mount on a hot plate (65°C) until the paraffin starts melting. Gently press the cover slip down with the forceps and let the mount cool down, so the paraffin can solidify.
5. Excess paraffin is removed with a flattened dissecting needle; clean the edges first with white spirit and then with ethanol (use a piece of cotton wool, wrapped around a forceps leg). Put pieces of cardboard on either side of the cover slip, apply a ring of glyceel, and fold the holder. Before studying the mount, the glyceel must be left to dry for one night.

Literature: s'Jacob & van Bezooijen 1984, Southey 1986

3.6.3.4. Mount of the vulval cone of cyst nematodes

Apart from being based on specific shape and colour, identification of cyst nematodes is also based on structure of the vulva and fenestra, and surrounding internal and external structures. Because the cuticle of dried cysts is hard, it is possible to transfer the vulval cone directly to the glycerine.

Requirements

Hot plate
 Alcohol flame
 Stamp (copper tube, interior Ø 10 mm, exterior Ø 12 mm)
 Petri dish with paraffin
 Dissecting needles and pair of forceps
 Scalpel, painting brush (No. 1), handling ('fish') needle
 Dissecting needle with flattened and sharpened point
 Cobb slide (for parts required, see § 3.6.3.1)
 Piece of Perspex (cutting plate)
 Cotton wool
 Dehydrated glycerine
 Ethanol 96%
 Glyceel, white spirit

Procedure

1. Wash the cysts out of the soil (§ 2.4) and let them dry well.
2. Put a cyst in a droplet of dehydrated glycerine on a piece of Perspex, and cut off the posterior end under a dissection microscope. Trim this posterior end to obtain a piece of cuticle with the vulva and fenestra in the middle. Remove the remaining pieces of body tissue with a (soft) handling needle.
3. Stamp a small ring of paraffin on a square cover slip in a Cobb slide (see § 3.6.3.1; a somewhat thicker paraffin ring must be applied, because the vulval cone is relatively thick). Put a droplet of dehydrated glycerine in the centre, and push the piece of cuticle to the droplet bottom, *with the exterior side of the cuticle facing up*.
4. Apply a cover slip with a pair of forceps. Place the mount on a hot plate (65°C) until the paraffin starts melting. Gently press the cover slip down with the forceps, remove from the hot plate, and let the mount cool down, so the paraffin can solidify.
5. Excess paraffin is removed with a flattened dissecting needle; clean the edges first with white spirit and then with ethanol (use a piece of cotton wool, wrapped around a forceps leg). Put pieces of cardboard on either side of the cover slip, apply a ring of glyceel, and fold the holder. Before studying the mount, the glyceel must dry for one night.

Source: H.H.B. van Megen (pers. comm.)

Further reading: s'Jacob & van Bezooijen 1984 use fresh cysts (extracted from roots), which are first fixed, and then stained (eosine).

3.6.3.5. Mount of the head and cross-sections

With this mount, it is possible to study the head of a nematode viewed from above. The method can also be used for studying other cross-sections.

Requirements

Alcohol flame

Turntable

Glass slide

Cobb slide (for parts required, see § 3.6.3.1)

Dissecting needles and pair of forceps

Scalpel, painting brush (No. 1), handling ('fishing') needle

Glass fibre or hair of a painting brush

Glyceel

Glycerine-gelatine

Soak 20 g of gelatine in 40 ml water for 2 hours. Add 50 ml of glycerine and 1 ml of phenol (**harmful, work in fume chamber!**). Warm this mixture in a water bath for 15 minutes and stir to obtain a homogenous mixture (Southey 1986).

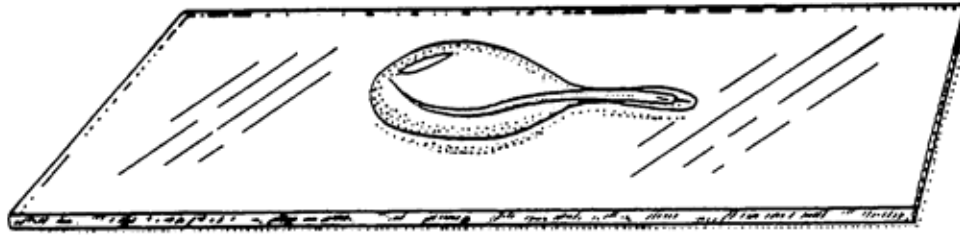


Figure 27

Glycerine-gelatine drop with nematode for making head sections and cross sections

Procedure

1. Melt a drop of glycerine-gelatine on a glass slide above a flame. Spread out the droplet at one point with a dissecting needle. Bring a nematode into this part of the droplet (fixed and transferred to glycerine), with the head pointing towards the spread-out portion of the drop (Fig. 27). Let the jelly harden. For cross-sections, select the part to be mounted.
2. Cut the head off with a scalpel blade (the length should be about 1-3 head widths); try to cut *at a straight angle*. Transfer the head with a handling needle into a drop of melted glycerine-gelatine, lying on a square cover slip in a Cobb slide.
3. Select 3 pieces of glass fibre or hairs of a painting brush (having a diameter equal to the height of the head section) and arrange them in the drop. Carefully place the head section upright, with the lip region facing up. Let the jelly harden.
4. Apply a cover slip with the forceps. Place the mount under a microscope (low magnification) and if necessary, move the cover slip until the head is standing completely upright. Touch the cover slip above the head section with a hot dissecting needle; this melts the jelly and makes the cover slip rest on the glass fibres or hairs.
5. Fix the cover slip at three points with glyceel and check again whether the head is standing upright. After half an hour, the cover slip is sealed with glyceel on a turntable; apply 1-2 layers of glyceel. Before studying the mount, the glyceel must dry for one night.

Literature: s'Jacob & van Bezooijen 1984

Further reading: Southey 1986 (pages 78-79)

3.6.3.6. Perspex slides for mounting cysts

Requirements

Turntable

Piece of Perspex (76 x 25 x 3 mm) with a flat-bottomed round cavity of max. 2 mm depth, made with an 8-mm drill with a blunt cutting tip, or a glass slide with a depression.

Circular cover slip (Ø 19 mm)

Painting brush (No. 1)

Cotton wool dots

Acetone

Glyceel

Transparent varnish

Procedure

1. Transfer 15-20 thoroughly dry cysts, using a painting brush, into the cavity of the Perspex piece or the depression in the glass slide.
2. Apply the cover slip. Fix it first at three points with glyceel (use tiny drops of glyceel to avoid spreading under the cover slip and over the cysts) and apply a glyceel ring after 1 hour. Leave to dry.
3. Roughen the Perspex slide on either side of the cavity with a fine sandpaper, and clean it with acetone moistened cotton wool, so it can be written on with water-resistant ink. If necessary, cover the label with transparent varnish.

Literature: s'Jacob & van Bezooijen 1984

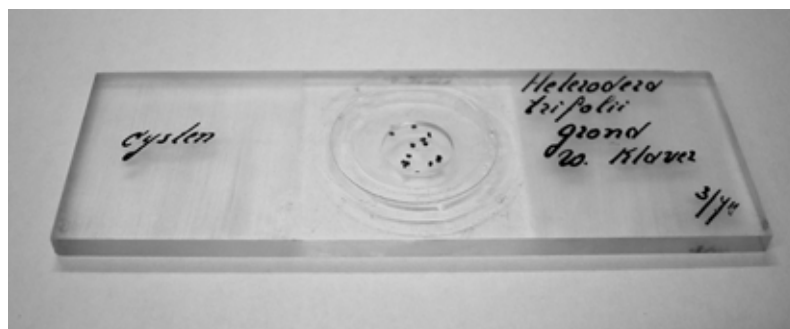


Figure 28

Perspex slide with mounted cysts

3.7. STAINING OF ROOT MATERIAL

Staining simplifies microscopic detection of nematode infections. Staining agents which stain nematodes are used. Plant tissues are not, or only slightly stained. Examples are cotton blue, acid fuchsin and iodine.

3.7.1. Staining in lacto phenol-cotton blue or lacto phenol-acid fuchsine

Phenol is harmful: work in a fume chamber!

This method is most commonly used, although working with lacto phenol is unpleasant. The results are good and the condition of the nematodes extracted from the roots is usually good enough to make identification at high magnification possible (Southey 1986). The nematodes are stained blue (or red when using acid fuchsin) and the plant tissue remains more or less unstained, with the exception of the meristemic regions. The method is used to check roots for infections and to create demonstration material of known infections.

Requirements

Beaker
2 petri dishes
Pair of scissors or knife
Forceps
Lacto phenol – 0.1% cotton blue (or 0.1% acid fuchsine)
Lacto phenol
Glycerine – water 1:1
Water

Recipe lacto phenol

Liquid phenol	94 ml
Lactic acid	83 ml
Glycerine	160 ml
Distilled water	100 ml

Store this solution in a dark bottle and/or in a dark place. The solution turns yellow when exposed to light.

Procedure

1. Carefully wash the root system with water and cut off the small roots
2. Put the small roots in hot (80°C) lacto phenol – cotton blue with forceps and leave them in this solution for 1-3 minutes, allowing the stain to penetrate the material.
OR: ('cold staining')
Put the small roots with forceps in lacto phenol – cotton blue and leave them in this solution for at least 12 hours up to several months (this is possible because the lacto phenol also has fixing properties).
3. Wash off the excess stain with water and transfer the material to pure lacto phenol, removing the stain from the plant tissue, but not from the nematodes.

Leave for at least 15 minutes. The best contrast is obtained when the roots are left in the lacto phenol for 24 hours.

4. Put the roots in a petri dish filled with glycerine/water 1:1 and observe them under the dissecting microscope. The glycerine/water solution has the same refractive index as lacto phenol, but lacks its toxicity and unpleasant odour. Glycerine/water has a short shelf life. The root preparations have to be thrown away after observation. If the material has to be preserved, it can be placed in lacto phenol again.

Literature: Bridge *et al.* 1982, s'Jacob & van Bezooijen 1984, Southey 1986

3.7.2. Staining in Lugol's solution

This method is mainly used for the detection of *Globodera/Heterodera* stages in thin roots. The nematodes are stained dark brown, whereas the plant tissue remains unstained, except for the tissue in the meristemic regions. The method must not be used for demonstration material.

Requirements

Beaker
Petri dish
Pair of scissors or knife
Forceps
Lugol's solution:
 Iodine 1 part
 Potassium iodine 2 parts
 Distilled water 200 parts
Water

Procedure

1. Carefully wash the root system with water and cut off the small roots (showing symptoms such as nodes or lesions).
2. Put the small roots in Lugol's solution with forceps and leave them for 15 minutes.
3. Observe the roots under the dissecting microscope in a water-filled petri dish. Please note: The time available for observation is limited, because the stain soon dilutes (\pm 15 min.).
4. The material is not fixed by this staining procedure, so after observation, the material is discarded.

Literature: s'Jacob & van Bezooijen 1984

3.8 HISTOLOGY

Cutting with the microtome

Good hand-cut sections of diseased plant material provide a lot of information. If the material is too hard to cut by hand or if serial sections are needed, the material can be embedded in paraffin. A microtome is used to make sections with the required thickness. These sections can be observed in the correct sequence.

3.8.1. Preparing the plant material for making microtome sections

Requirements

Diseased plant material, in small pieces (max 0.5 cm); the smaller and thinner, the better the results.

Glass tube, approximately 10 ml content

Warm water bath

F.P. 4:1 or F.A. 4:1 (see § 3.4.2.)

Formalin 4% (see § 3.4.2.)

Bouin's fixative (see recipe on page 92)

Vacuum dessicator

Ethanol 50%, 60%, 70%, 80%, 90% and 96%

Methyl benzoate

Methyl benzoate with 2% celloidin

Toluene

Paraffin (e.g. with a melting point of 56°C)

Porcelain embedding dish

Heating table

Glycerine

Procedure

Be aware of vapours emitted from solutions and work in a fume chamber or in a well-ventilated place.

1. Put the small pieces of diseased plant material in a glass tube. Heat 1.5-2.0 cc of F.P. 4:1, in a warm water bath at 90°C. Pour the hot liquid over the material and cool immediately by adding 4-8 cc cold formalin 4%.
2. Pour the solution off in a chemical waste container. Pour Bouin's fixative onto the material.
3. Put the material in a vacuum dessicator with an underpressure of 25-30 cm and leave it for 30 minutes. The material sinks to the bottom. If not, wait another 15 minutes.

4. Dehydrate the material by passing it through ethanol solutions with increasing concentrations.
 - Ethanol 50%, three changes of 30 minutes each; the yellow colour of the fixative disappears
 - Ethanol 60%, for 30 minutes
 - Ethanol 70%, for at least 3 hours, but longer is possible, e.g. leave the material in the solution until the next day
 - Ethanol 80%, for 30 minutes
 - Ethanol 90%, for 30 minutes
 - Ethanol 96%, two changes of 15 minutes each.
5. Clear the material by drying on a non-fibrous filter paper and immediately transfer it into a clean and dry bottle containing methyl benzoate. In the beginning the material floats, but after 10-60 minutes it gets a glassy appearance and sinks to the bottom.
6. Pour the solution off and immerse the plant material in methyl benzoate 2% celloidin. Leave it in this solution for at least 3 days.
7. Use a pair of forceps to carefully transfer the material to another tube containing toluene, and renew this solution twice, at ten-minute intervals.
8. Transfer the material to a dish filled with warm melted paraffin and leave it for 15 minutes.
 - The type of paraffin used should be identical to the one used for embedding. Avoid overheating of paraffin because it leads to hardening of plant tissues.
9. Transfer the material to another dish filled with paraffin. Again leave it for 15 minutes.
10. Thoroughly clean the porcelain embedding dishes with 96% ethanol, let the dishes dry, take a drop of glycerine and apply a thin film to the dish.
11. Pour the melted and well-filtered paraffin in the dish and as soon as a thin film of solid paraffin appears at the bottom, transfer the plant material. Arrange the plant pieces and note their location in the paraffin (imbedding plan). Make sure that the material is well-enclosed with sufficient paraffin.
12. Arrange the material in the proper position and when the paraffin is completely solid, submerge the whole dish in a water-filled container. After a few minutes, the paraffin automatically detaches from the dish.
13. Place the paraffin blocks in a refrigerator at 4°C, where they can be stored for a long period.

3.8.2. Cutting sections with the microtome

To carry out microtome cuttings, each object is mounted individually on a cutting block. The final result depends on the level of accuracy of mounting. It is important to work in a neat and steady way.

Requirements

- Metal saw
- Wooden blocks boiled in paraffin
- Spatula with a width of 1.5 cm
- Alcohol flame
- Tissues
- 2 scalpels
- Microtome
- 2 painting brushes
- Pair of forceps with a fine tip
- Dissecting needles
- Flat smooth boxes to store cutting ribbons
- Diamond pen (scratching)
- Glass slides

Procedure

1. Carefully cut the paraffin block in pieces with the metal saw. Follow the embedding plan.
2. Cut away excess paraffin with the scalpel. Avoid touching the embedded objects. Try to obtain a pyramid-shape.
3. Melt a piece of paraffin on the wooden block, melt the bottom side of the pyramid with a warm spatula and fix it on the paraffin layer on the block.
4. Put the material in a refrigerator at 4°C and leave it for at least 15 minutes.
5. Trim the pyramid with a scalpel. The object should remain embedded in paraffin, but with the surface to be cut as small as possible.
6. Cut sections with a thickness of 5 micron or more with the microtome. The minimum thickness depends on the quality of the material and on the purpose of the observations.
7. Store the ribbons that have been cut in a clean lockable box.
8. Cut the ribbons in lengths of approximately 3 cm (memorize the order)
9. Apply a thin film of glycerine albumen dissolved in water onto a glass slide, cleaned with ethanol. Mount the ribbons in the correct order.
10. Stretch the ribbons on a heating table and remove excess fluid. The ribbons “stick” because of the glycerine albumen.

11. Put the mounts on clean filter paper and let the water drain off. Dry them in an incubator at approximately 30°C for at least 10 hours.
12. Label the mounts with a diamond writer to record which side is up.

3.8.3. Staining of microtome sections

A large number of staining methods are known (see literature) and many of them are labour intensive. Selection of the most suitable method can be made based on the tissue type to be observed. The Cason staining method described below is a very easy and relatively quick method. However, it is very important to work accurately to achieve good results. In the following scheme, a summary is made.

ATTENTION! Xylene is very poisonous; work in a fume chamber.

Requirements

- Alcohol flame
- Large forceps
- 16 beakers of 100-150 ml
- Cason's staining fluid
- Canada balsam
- Glass rod
- Cover slips 24 x 50 mm
- Ethanol series
- Xylene

Procedure

1. Warm the mounts over an alcohol flame to melt the paraffin. Never overheat.
2. Pass the mount through xylene to remove the melted paraffin.
3. Rinse again in clean xylene.
4. Hydrate the mounts by passing them, for at least 5 seconds, through successively:
 - Ethanol 96% I
 - Ethanol 96% II
 - Ethanol 90%
 - Ethanol 80%
 - Ethanol 70%
 - Distilled water

5. To stain the mounts, leave them in Cason's fluid for 5 minutes.
6. Dehydrate the mounts by passing them QUICKLY (1-2 sec) through successively:
 - Tap water
 - Distilled water
 - Ethanol 70%
 - Ethanol 80%
 - Ethanol 90%
 - Ethanol 96% I
 - Ethanol 96% II
 - Xylene I
 - Xylene II
7. Clean a cover slip with ethanol, apply Canada balsam with a glass rod and carefully put it on the wet mount.
8. Leave to dry for at least 12 hours in an incubator at 30°C. After that the mounts can be cleaned with a piece of cloth with xylene and are ready for use.

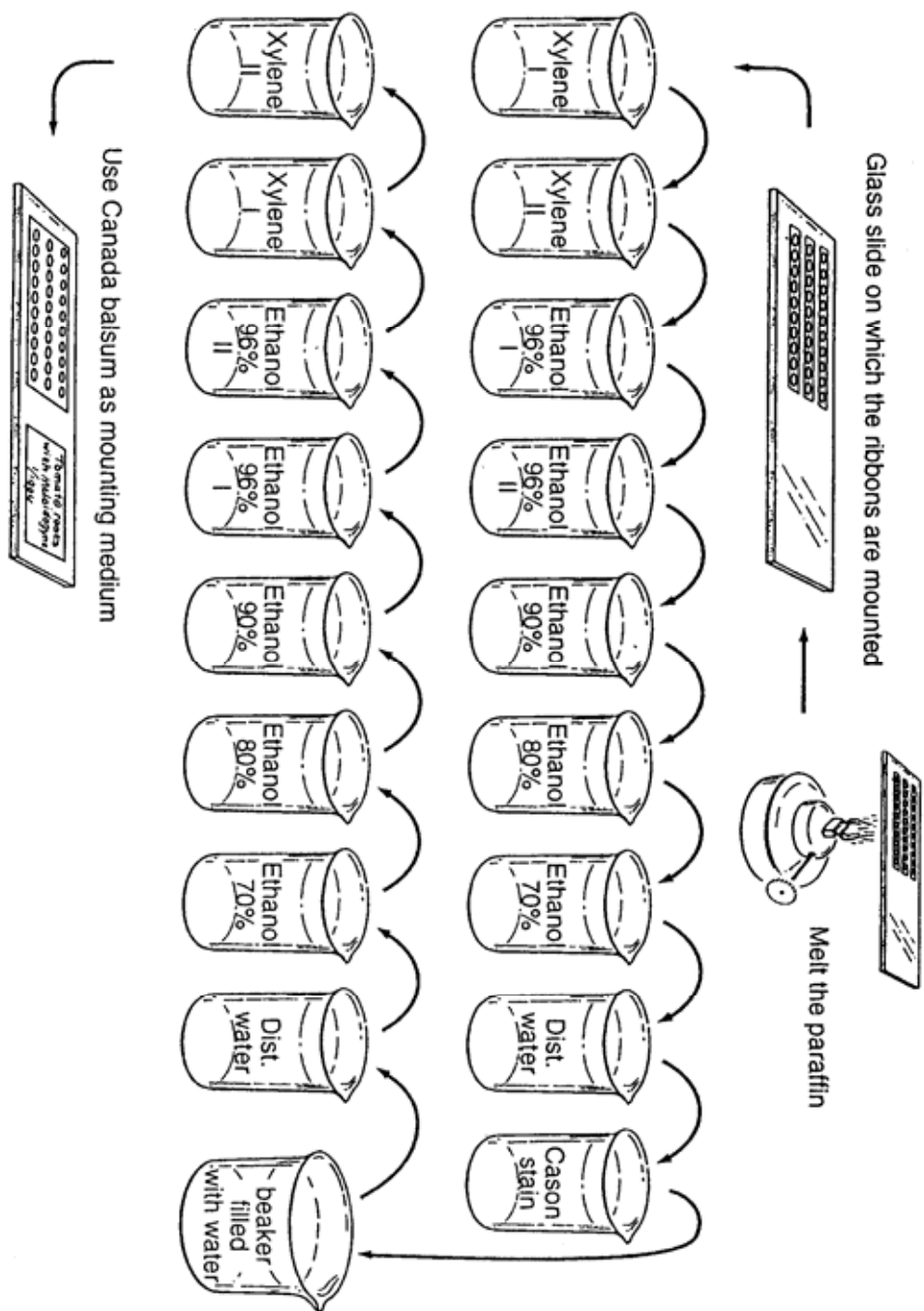


Figure 29
Staining of microtome sections

3.8.4. Recipes for histology

Bouin's fixative:

Picric acid, saturated solution	15 ml
Formalin (formaldehyde 37%)	5 ml
Glacial acetic acid	1 ml

Methyl benzoate 2% celloidin:

Celloidin flakes*	2 g
Methyl benzoate	98 ml

* Celloidin flakes are explosive and therefore its sale has been discontinued. So far not much experience is gained with its substitute, the ready-to-use coreuct Necoloidine, on plant/nematode material.

Cason's staining fluid:

Phosphotungstic acid crystals AR	1 g
Orange G	2 g
Anilin blue, water soluble	1 g
Acid fuchsin	3 g
Distilled water	200 ml

Glycerine albumen:

If the ready-made coreuct cannot be purchased, it can be prepared in the lab. Take the egg white of a fresh chicken egg, add a thymol crystal and mix to obtain a foamy substance. Leave for some hours and drain off the fluid. Mix this filtrate with an equal amount of glycerine. Add some more thymol crystals, and store at 4°C. The coreuct can be kept for a long period.

To glue mounts, add 6 drops of the stock solution to 10 ml of distilled water.

Table for preparing an ethanol series

Grade required	Volume of ethanol 96% (ml)	Volume of distilled water (ml)
90%	93.5	6.5
80%	83.5	16.5
70%	72.9	27.1
60%	62.5	37.5
50%	52.1	47.9

4.1. THE VINEGAR EELWORM *Turbatrix aceti*

The vinegar eelworm (*Turbatrix aceti*) can be reared in a solution consisting of 50% (v/v) acetic acid (CH_3COOH), inoculated with a strain of the *Acetobacter* bacterium. An Erlenmeyer flask with a capacity of e.g. 400 ml, can be used. Remarkably, *Turbatrix aceti* exhibits negative geotaxis. To maintain the culture, the medium has to be refreshed every 5 months. Transfer 30 ml of the old culture to another Erlenmeyer flask filled with a 50 (v/v) acetic acid solution, to ensure that the bacterial culture, the food source of the nematode, remains alive. Instead of 50 (v/v) acetic acid, common domestic vinegar can be used as well (do not dilute).

4.2. THE FREE-LIVING NEMATODE *Panagrellus redivivus*

Panagrellus redivivus also feeds on bacteria, lactic acid bacteria. These bacteria can be reared on oatmeal. Oat is milled and transferred to a glass test tube (diameter 0.9 cm, height 6 cm) and put in an autoclave at 120°C for 30 min. Add 40 ml of sterile tap water for each 20 g of oatmeal, and inoculate this medium with a strain of lactic acid bacteria and *Panagrellus redivivus*. Incubation at 24°C leads to the growth of the nematode population; nematode recolonization starts after few days.

4.3. RHABDITIS

Rhabditis spp. are also bacterial feeders. Many of its families and species can be reared easily on a simple medium.

Composition of the medium:

- Malt (Difco) 10 g
- Agar 14 g
- Tap water 500 ml

Grind 10 g of malt, and add 500 ml of water. Heat this suspension 'Au Bain Marie'. Filter the suspension through cheesecloth and add distilled water up to 500 ml. Add 14 g of agar and heat. Pour 60 ml portions into Erlenmeyer flasks of 100 ml capacity, and put them in an autoclave for 30 min at 120°C. Cool the medium down, and pour it into petri dishes. After the agar has become solid 5 *Rhabditis* specimens are transferred to agar in the dish, under a dissecting microscope.

Alternative procedures:

- Use non-enriched water agar 1%, and inoculate with *Rhabditis*. Multiplication is slower, but the dishes remain clean for a long period.
- Put small, sterile potato pieces on the agar, and introduce a few *Rhabditis* spp.. Incubate the culture at 20°C and observe the potato pieces and the nematodes daily.

4.4. THE CHRYSANTHEMUM FOLIAR NEMATODE *Aphelenchoides ritzemabosi*

Aphelenchoides ritzemabosi is an obligate plant parasite, so it must be reared on living plant material, in this case tobacco seedlings.

The seedlings can be inoculated as follows:

- Prepare a suspension of *A. ritzemabosi* in a solution of 2% methyl cellulose.
- Inoculate 2-weeks-old tobacco seedlings with a drop of the suspension.
- Place the seedling:
 - Days 0-3: in an incubator at 20°C at a relative humidity of 100 %
 - Days 4-14: in a greenhouse at a temperature of 20°C, and a high humidity, until the first symptoms appear.

4.5. POTATO CYST NEMATODES *Globodera rostochiensis* or *G. pallida*

Following non-sterile, *in vitro* method is suitable for:

- Monitoring the infection process
- Making crosses between different genotypes of *Globodera rostochiensis* and *G. pallida*
- Histological studies (the initial plant material is relatively clean)
- Testing host plant resistance

Procedure:

- a. Remove adhering soil and plant residues and wash the potato tubers. Store the tubers for three weeks at 20°C.
- b. Place a number of potatoes on a water-filled beaker, with the roots hanging into the water, to obtain root exudates (required for egg hatching of nematodes).
- c. Let cysts of *Globodera rostochiensis* or *G. pallida* incubate in a solution of root exudates for 3-14 days. Use petri dishes specially designed for this purpose. Dishes with *G. rostochiensis* are incubated at 22°C, and dishes with *G. pallida* at 18°C.
- d. Use a 3% water agar medium for the potato culture. Pour the sterile water agar into sterile petri dishes, in a sterile flow cabinet. Pour about 20 ml in each petri dish; close the dishes after the agar has solidified to avoid condensation on the lids. Store the petri dishes at room temperature for a couple of days.
- e. Disinfect a scalpel by immersion in an ethanol 96% solution and flame it. Cut wedge-shaped pieces from the potatoes, with a sprout in the middle. Place the pieces on the agar, so the sprouts point towards the centre of the dish.
- f. Incubate the dishes at 20°C. As soon as the roots reach a length of 2 cm, place J2's on the roots, preferably in the root elongation zone. This is done under a dissecting microscope. Swelling of the potato root should become visible within about 18 days.

4.6. THE ROOT-KNOT NEMATODE *Meloidogyne* spp.

Root-knot nematodes can be reared on roots, e.g. tomato roots. The procedure is as follows:

- a. Sterilize the surface of tomato seeds. Soak the tomato seeds in tap water for one night. Immerse the seeds for 10 minutes in a 4-5% Chlorix solution (HClO). Stir the solution continuously with a magnetic stirrer. Pour off the Chlorix solution as soon as the seed surface turns yellow. Rinse the sterile seeds in sterile tap water.
- b. Place the sterile seeds on 2% water agar and allow them to germinate. Germination takes place at 20°C.
- c. Use sterile glass or plastic petri dishes; plastic petri dishes are bought sterile, whereas glass petri dishes have to be sterilized in an autoclave (2.5 hours at 120°C).
- d. Place the seedlings on water agar and cut the roots as soon as they reach a length of 1 cm. The cut roots do not receive nutrients from the seed anymore and must therefore be transferred to the nutrient medium.
- e. Use the nutrient medium Gamborg B5 medium with 20 g/l of sucrose. Fill the petri dishes in the laminar flow room and let them cool down (leave uncovered). Transfer the root segments to the petri dishes, and seal with Parafilm. Place the petri dishes in an incubator at 25°C.
- f. Surface sterilize *Meloidogyne* egg masses collected from the roots. Select full-grown masses which contain hatchable eggs. Make sure they are not too old because sterilization may then be incomplete. Incubate the egg masses in a 1% Chlorix solution, in a vacuum desiccator with 30 cm Hg negative pressure. Rinse the sterile eggs, and place them in sterile water, on the tomato roots. Incubate the dishes at 23°C and carry out regular observations.

Composition of the Gamborg B5 medium (Gamborg, O.L., Miller, R.A., Ojima, K. (1968) Exp. Cell Res. 50, 151):

CaCl ₂	113.23 mg/l
CoCl ₂ ·6H ₂ O	0.025
CuSO ₄ ·5H ₂ O	0.025
FeNaEDTA	36.7
H ₃ BO ₃	3.00
KI	0.75
KNO ₃	2500.0
MgSO ₄ ·7H ₂ O	250.0
MnSO ₄ ·H ₂ O	10.0
NaHPO ₄	130.44
NaMoO ₄ ·H ₂ O	0.25
(NH ₄) ₂ SO ₄	134.0
ZnSO ₄ ·7H ₂ O	2.00
Myo-inositol	100.0
Nicotinic acid	1.0
Pyridoxine HCl	1.0
Thiamine HCl	10.0

For surface sterilization of *Globodera* cysts, see the method of Heungens *et al.* (1996)

Disinfection of *Globodera* larvae from cysts.

Heungens *et al.* 1996

1. Remove the tip of a 20 ml plastic hypodermic syringe with a hot needle.
2. Melt the surface of the cut syringe section cut, and quickly press it on a piece of nylon gauze with a mesh size of 25 μm . Check whether the sides are properly sealed and remove redundant gauze rims.
3. Put the syringe, together with the syringe piston, in the autoclave for 20 minutes. Do not overheat to avoid deformation of the plastic.
4. Surface sterilize the cysts in an inoculation chamber, using sterile materials.
5. Place about 30 dry cysts in the syringe and submerge it in ethanol for 15 seconds; move the piston up and down to make the alcohol flow around the cysts.
6. Transfer the cysts to a 1.3% Chlorix solution until they fade and start breaking open.
Depending on the origin and development stage of the cysts, this takes 7-9 minutes.
7. Rinse the cysts 3 times in sterile water.
8. Cut off the sieve part from the syringe using a hot needle at 1.5 cm height. Put the sieve part in a watch glass, placed in a petri dish.
9. Fill the watch glass with 5 ml sterile water, and close the petri dish. Seal the lid with Parafilm.
10. Incubate the cysts in a dark area for 3 days at 20°C.
11. Transfer the sieve with cysts to a new watch glass, filled with 5 ml of sterile Potato Root Diffusate (PRD) and incubate again at 20°C.
12. Larvae can now be collected in the desired quantities. Refill regularly with PRD.

5.1. INTRODUCTION

Principles

Most nematodes are microscopically small, so a microscope is an essential tool for nematologists. Analysing an experiment usually requires hours of sitting behind a microscope. For that reason it is important to work with a microscope of high quality, and to efficiently use its optical possibilities. Incorrect optical adjustment makes the analysis more difficult and physical complaints such as headaches may occur. Be well aware of these symptoms.

For routine work, the Department of Nematology uses two types of microscopes: the “dissecting microscope” and the “compound microscope”.

The “**dissecting microscope**” is a low-powered stereoscopic microscope for small magnification rates (10-50x), offering a wide-angled vision. This microscope is mainly used for analysing nematode suspensions and for dissection work.

The “**compound microscope**” is a stereoscopic microscope offering magnifications ranging from 40 to 1000x. Working at a magnification of 1000x requires an oil immersion lens. This type of microscope is used for identification of nematodes and other types of detailed observations. Usually the material has first been mounted under a dissecting microscope.

(For other specific purposes, the Department of Nematology uses other types of microscopes, for example the inverted microscope, the fluorescence microscope and the interference/phase contrast microscope; they will not be described here)

Correct adjustment leads to optimum use of the equipment. A high-quality microscope which has not been properly adjusted, yields lower-quality images, and causes physical complaints, such as tiredness, painful eyes and headache. If these symptoms appear, the microscope needs to be adjusted. It needs to be adjusted for each individual user to correct for eye aberrations, even for people not wearing glasses.

5.2. THE DISSECTING MICROSCOPE

The dissecting microscope has a built-in lamp or a separate light unit. The light source needs to be arranged or adjusted, so light distribution in the area of observation is uniform, and light yield maximal. Put the mirror, if any, at a correct angle, and position the separate light unit approximately 8 cm away from the microscope stage to avoid heating of the stage.

Adjustment of the eyepiece tubes (oculars)

1. Place a mount on the object stage.
2. Focus on the mounted object.
3. Close one eye and look with the other eye at a specific point of the object, preferably in the centre of the vision field.
4. Focus with the stage adjustment knob, watch carefully and try to remember the sharpness of the image.

The stage adjustment knob should not be touched again.

5. Observe the same point of the object with the other eye and focus with the adjustable ring on the ocular.

Optimum adjustment for each individual eye has now been achieved.

6. Separate the two oculars as much as possible. When observing the mount, there are two separate circular images.
7. Slowly move both oculars towards each other, while watching the object. The two separate images gradually merge and form one single image.

The dissecting microscope has now been adjusted for the particular distance between both eyes.

If there are doubts regarding the correctness of the adjustment, the whole procedure has to be repeated. It is possible to adjust for the distance between eyes first and carry out the focussing of the oculars afterwards.

Do not place any object on top of the lamp unit, and switch off the light when the microscope is not in use.

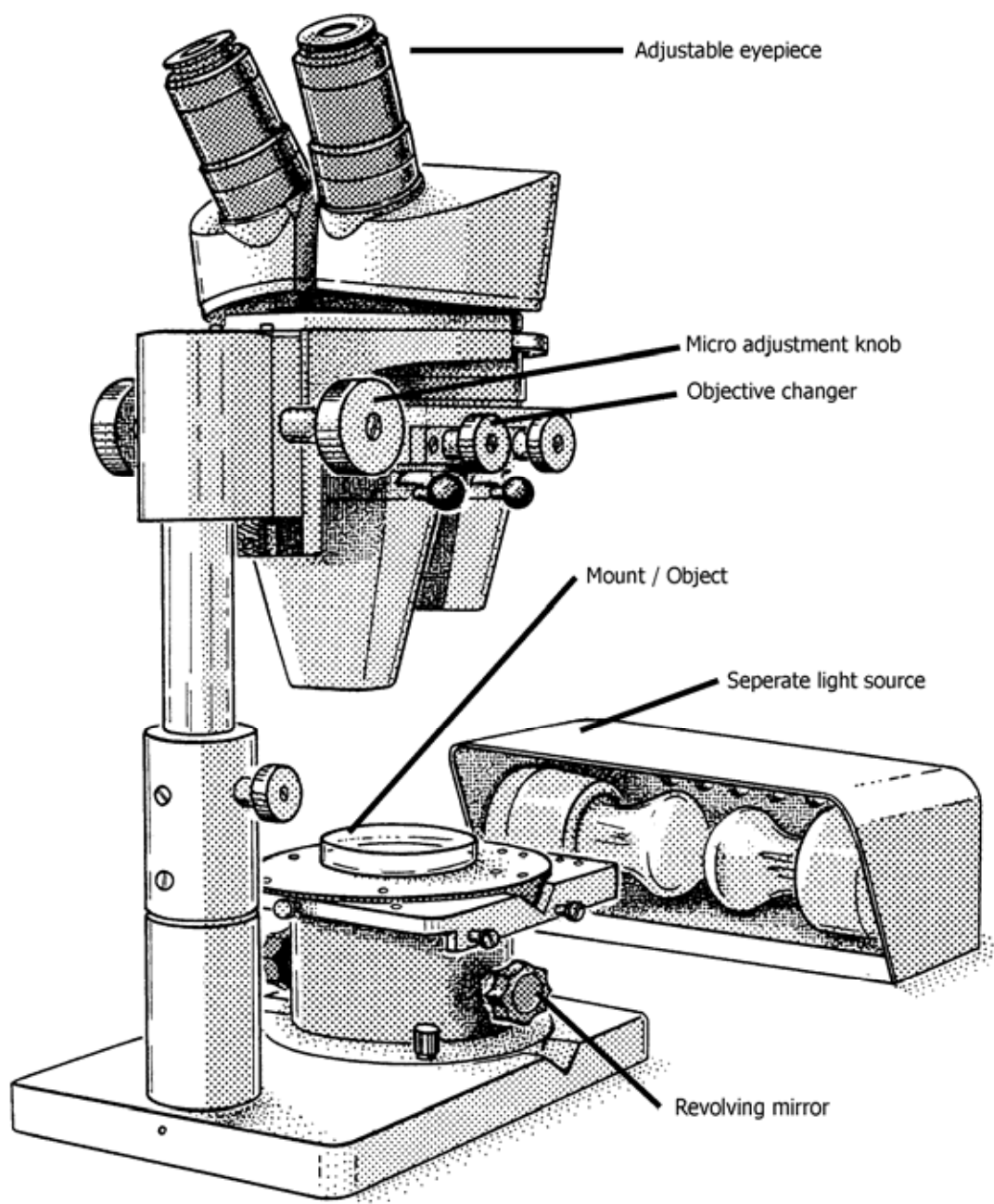


Figure 30
Dissecting microscope with a separate light source, used for observation purpose and for counting nematodes

5.3. THE COMPOUND MICROSCOPE

The microscope has a built-in lamp powered by a built-in or separate transformer.

Centring of the light

1. Adjust the output of the transformer to 2-3 volts.
2. Bring the condenser to its highest level.
3. Swing the condenser lens out of the optical path.
4. Place a mount on the object stage of the microscope.
5. Focus on the mounted object with the 10x objective
6. Swing the condenser lens in the optical path.
7. Focus on the lighted field, and make sure that the borders are well defined. The lighted area must be completely centred in the field of vision. If this is not the case, adjust with the two screws at the front of the condenser.
8. Turn the 25x objective in the optical path. The whole surface must now be uniformly illuminated. If not, the lamp has to be centred. This is not required in modern microscopes.
9. Swing the condenser lens out of the optical path.
10. Put the object exactly in the middle of the field of vision.
11. Observations can now be made with the 40x objective.

Adjusting the oculars

12. Focus on the object.
13. Close one eye and look with the other eye at a specific point of the object, preferably in the centre of the field of vision.
14. Focus with the stage adjustment knobs, watch carefully and try to remember the sharpness of the image.

The stage adjustment knobs should not be touched again.

15. Observe the same point of the object with the other eye and focus with the adjustable ocular.

Optimum adjustment for each individual eye has now been achieved.

16. Separate the two oculars as much as possible. When observing the mount there are two separate circular images.
17. Slowly move both oculars towards each other while watching the object; the two separate images gradually merge and form one single image.

The microscope has now been adjusted for the particular distance between both eyes.

18. If oil immersion is to be used, the object has to be properly centred under the 40x objective.
19. Turn the 10x objective in the optical path and move the object stage downward with the adjustment knob.
20. Swing the condenser lens in the optical path; be careful not to move the object stage.

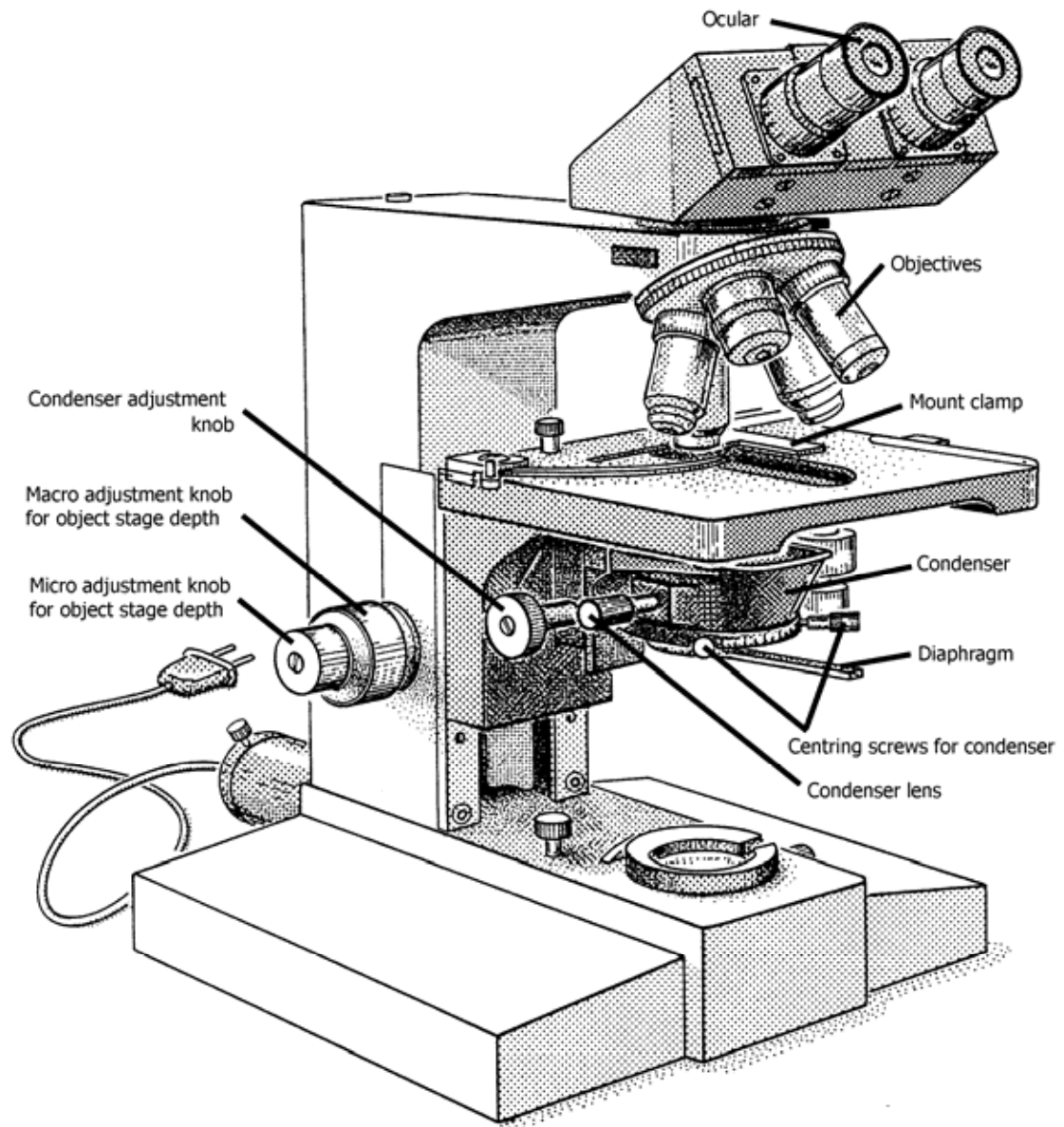


Figure 31
Microscope for observations at high magnification

21. Deposit a SMALL droplet of oil on the lighted surface, and swing the condenser lens out.
22. Turn the 100x objective in the optical path and move the object stage upward. Watch from the side when the oil droplet starts touching the objective.
23. Move the stage further up while looking through the microscope. The object will become visible as a dark structure.
24. Swing the condenser lens in the optical path and adjust the amount of light with the diaphragm and the transformer.

Repeat this procedure if there are doubts whether the adjustment of the microscope is correct.

5.4. MAINTENANCE OF MICROSCOPES

Dirt and dust interfere with microscope observations. It is important to keep the microscope in a clean environment. Any suspected malfunctions of the microscope should be reported immediately.

Lenses are cleaned from oil residues with a tissue and some white spirit. The body of the microscope is regularly cleaned with a clean cloth or tissue.

After use the microscope has to be covered with a plastic hood.

5.5. MEASURING WITH THE MICROSCOPE

For making measurements on nematodes, an eyepiece micrometer is required. Inside the eyepiece, a glass disc with a scale with 100 divisions is mounted. Nematode measures can be expressed in terms of numbers of scale units. These measures may be sufficient for own use and for making comparisons, but for measuring absolute values, e.g. for identification purpose, the eyepiece micrometer has to be calibrated. This is done with a stage micrometer, a slide with a 2-mm scale, subdivided in 200 divisions.

To indicate measurements in microns, the magnification factor for the microscope and for each objective has to be established with the stage micrometer. Each division on the micrometer slide is exactly 10 microns. The value of one division of the eyepiece micrometer can be expressed in microns by comparing the scale divisions of the eyepiece micrometer with its corresponding value in microns. Calibrating all objectives of a microscope is necessary to prevent small mechanical aberrations from influencing the measuring results.

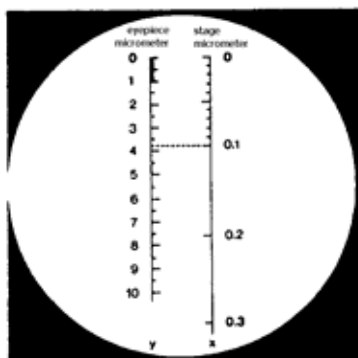


Figure 32
Eyepiece micrometer

In this example 38 ocular divisions coincide with 100 microns. This means that 1 division of the eyepiece micrometer is equal to $100/38 = 2.63$ microns

5.6. NEMATODE MEASUREMENTS (Ratios)

Descriptions of nematodes include a number of measurements. These measurements are expressed in ratios. De Man (1880) is the founder of the use of ratios. Many taxonomists continued using them, and further extended the number of ratios.

The ratios most commonly used are mentioned below.

- L = Total body length in microns or millimetres
- a = Body length \div greatest width
- b = Body length \div oesophagus length
- b' = Body length \div oesophagus length from the lips to the end of the oesophageal glands
- c = Body length \div tail length
- c' = Body length \div body width at the anus
- G¹ = Overall length of the anterior ovary from vulva \times 100 \div body length
- G² = Overall length of the posterior ovary from vulva \times 100 \div body length
- V = Distance of the vulva from the lips \times 100 \div body length

CHAPTER 6. OTHER TECHNIQUES

6.1. CONCENTRATING AND SURFACE STERILIZING NEMATODE SUSPENSIONS

To reduce the risk of culture contamination of sterile cultures, it may be necessary to carry out surface sterilization of nematodes, reducing the risk of culture contamination. This method does not disinfect populations completely, because the disinfection agent only reaches the nematode surface. If carried out properly, however, the risk of infections is small. This method is also suitable for concentrating nematode suspensions to one drop of water.

Requirements

Glass tube with a diameter of approximately 15 mm, and about 50 cm length
Sterilization fluid (e.g. 1% commercial Chlorix)
Stand with clamp
Watch glass
Cotton wool dot

Procedure

1. The glass tube, drawn to a very fine point and closed at the tip, is attached to the stand (Fig. 33).
2. Fill the tube almost until the rim with disinfecting solution. In the lower tip an air bubble remains.
3. Introduce a small piece of cotton wool in the tube until it touches the liquid.
4. Bring a previously concentrated nematode suspension on the cotton wool wad with a pipette or by directly pouring it.
5. Let the nematodes crawl through the cotton wool and settle in the fine point of the glass tube. This may take 30 minutes to several hours, depending on the cotton wool thickness and compactness and on the nematode activity.
6. Keep the upper opening of the glass tube closed with your thumb and break off the lower part of the thin point with a pair of forceps. Let a bit of air in the tube by moving your thumb and collect the nematodes in a watch glass covered with a small layer of cotton wool.

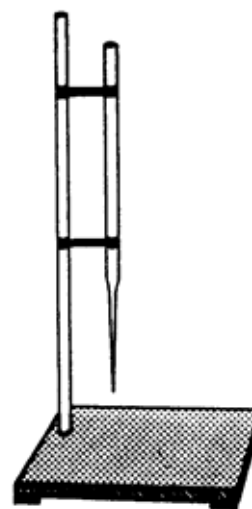


Figure 33

If possible, this procedure should be carried out in a clean air chamber or any other, very clean room.

6.2. QUANTITATIVE EXTRACTION OF RESTING SPORES OF NEMATODE PARASITIC FUNGI WITH A CENTRIFUGAL FLOTATION METHOD

Biological control of nematodes has received increasing attention in recent years. For fungi groups forming resting spores, it is possible to extract spores from soil samples. The method is labour intensive, but makes it possible to collect quantitative information from experiments. This information can be used to explain biological control effects.

Requirements

Soil sieve with 5 mm mesh size
Sheet for mixing soil
Balance
Sieves with mesh sizes of 1000, 150, 35 and 10 μm
Vibro-mixer
Centrifuge with tubes of at least 100 ml capacity
Magnesium sulphate solution, s.g 1.30

Procedure

1. Take a soil sample of 25 grams for extracting the resting spores. Follow the guidelines from § 1.4.
2. Take another soil sample of 100 grams for moisture determination.
3. Wash the 25 gram sample through the sieves with mesh sizes 1000, 150 and 35 μm , respectively.
4. Collect the suspension after each sieve, and after the 35 μm sieve, pass it passed through a 10-micron sieve.

If the suspension does not flow easily through the 10 μm sieve, placing a sponge against the lower side of the sieve may accelerate the process. Holding the sieve against a vibro-mixer has the same effect.

5. Wash the sediment remaining on the 10 μm sieve is washed into a centrifuge tube with a wash-bottle filled with magnesium phosphate s.g. 1.30.
6. Spin for 4 minutes at an R.C.F. value of 39 g (this corresponds with 465 revolutions on the Centaur centrifuge at the Department of Nematology)
7. Pour the supernatant on a 10-micron sieve and collect the sediment in a small, conical shaped, and graduated centrifuge tube (if a graduated tube is not available, indicate 0.5 ml on a common tube). Wash the material into the tube, using a water-filled wash-bottle and as little water as possible.
8. Repeat the spinning procedure (6-7), as spores may have remained in the pellet. Collect what is left on the sieve in the same graduated centrifuge tube.
9. Spin the small tube for 5 minutes at an RCF value of 850 g (this corresponds with 2200 revolutions on the Centaur centrifuge at the Department).

10. Suck the supernatant, except for the last 0.5 ml.
11. Mix the remainder well by blowing air through the suspension with a small pipette.
12. Take a 0.01 ml sample, and transfer it to a spore counting dish. A self-made counting dish, a glass slide with painted lines, can also be used. Bring the sample in a small drop of a 50% glycerine/water mixture. The glycerine protects the sample from desiccation.
13. Apply a cover slip and count the spores under a microscope, with a magnification of about 250x. Count at least three sub-samples.
14. After establishing the dry weight with the 100-gram sample, the number of spores per gram of soil (dry weight) can be established.

6.3. PHYTOTOXICITY TEST

If the soil has been treated with chemicals, such as soil disinfectants, which inhibit germination and crop growth, it is good to perform a phytotoxicity test, to check whether the soil is suitable for sowing or planting crops.

Requirements

Two airtight glass bottles with a minimum capacity of 0.5 litres
Cotton wool
Thin thread (yarn)
Garden cress (*Lepidium sativum* L.) seeds

Procedure

1. Take soil samples at several places in the treated field, at a depth of 3-10 cm. Fill a bottle with a 5 cm layer of treated soil. Immediately seal the bottle airtight.
2. Take a second bottle and fill it in the same way with untreated soil. This is the control.
3. Form small cotton wool and attach these to a thin thread. Moisten the cotton wool and roll them through the Garden cress seeds. Spread some seeds on the soil surface in the jars as well.
4. Hang the cotton wool with the adhering seeds in the bottles and close the bottles again. If necessary, the soil in the bottle can be slightly moistened.
5. After 24-48 hours (depending on the temperature) seeds start to germinate. Inhibition of germination can be established by comparing germination rates in both bottles.
6. If no germination takes place in the treated soil, the experiment can be repeated after soil tillage, or after an extra waiting period.

LITERATURE

- Alkemade, R. (1993). *Bemonsteren van nematodenpopulaties*. RIVM Bilthoven.
- Andrássy, I. (1956). Die Rauminhalts- und Gewichtsbestimmung der Fadenwürmer (Nematoden). *Acta Zoologica, Budapest* 2: 1-15.
- Ayoub, S.M. (1980). Plant Nematology. An Agricultural Training Aid. NemaAid Publications, P.O Box 23085, Sacramento, California 95823.
- Baermann, G. (1917). Eine einfache Methode zur Auffindung von *Ankylostomum* (nematoden) Larven in Erdproben. *Geneesk. Tijdschr. Ned-Indië* 57: 131-137.
- Baker, J.R. (1945). *Cytological technique*. London, Methuen.
- Barker, K.R., Campbell, C.L. (1981). Sampling nematode populations. In: Plant Parasitic Nematodes, Vol. III (B.M. Zuckerman, R.A. Rohde, eds.), pp. 451-474. Academic Press, New York.
- Barker, K.R., Nusbaum, C.J., Nelson, L.A. (1969). Seasonal population dynamics of selected plant-parasitic nematodes as measured by three extraction procedures. *J. Nematology* 1: 232-239.
- Barker, K.R., Townshend, J.L., Michell, R.E., Norton, D.C., Rhuele, J.L., Bird, G.W., Chapman, R.A., Dunn, R.A., Oostenbrink, M., Powell, W.M., Thomason, I.J., Fisher, K.D., Southards, C.J., Mai, W.F., Smart, G.C. Jr., Rhode, R.A., Dickson, D.W., Malek, R.B., Griffin, G.D., Taylor, P.L., Thames, W.H. Jr., Murad, J.L. (1978). Determining nematode population responses to control agents. In: *Methods for Evaluating Plant Fungicides, Nematicides and Bactericides* (E.I. Zehr, G.W. Bird, K.D. Fisher, K.D. Hickey, F.H. Lewis, R.F. Line, S.F. Rickards, eds.), pp. 114-125. American Phytopathological Society, St Paul.
- Baunacke, W. (1922) Untersuchungen zur Biologie und Bekämpfung der Rübennematoden, *Heterodera schachtii* Schmidt. *Arb. Biol. Reichsanst. Berlin* 11: 185-288.
- Van Bezooijen, J., Stermerding, S. (1971). *Catalogue for equipment used in work with plant and soil nematodes*. Wageningen, 53 pp.
- Van Bezooijen, J., Ettema, C. (1996). *Practicumhandleiding Nematologie (Gedeeltelijk herziene versie)*.
- Bijloo, J.D. (1954). A new method for estimating the cyst contents of the potato-root eelworm *Heterodera rostochiensis* Wollenweber. *J. Helminthology* 28: 123-126.
- Bongers, T. (1993). *Praktikumhandleiding identificatiecursus*. Vakgroep Nematologie, Wageningen.
- Bongers, T., de Goede, R.G.M., Kappers, F.I., Manger, R. (1989). Ecologische typologie van de Nederlandse bodem op basis van de vrijlevende nematodenfauna. Rapportnr. 718602002, RIVM, Bilthoven.
- Bongers, T., van de Haar, J. (1990). On the potential of basing an ecological typology of aquatic sediments on the nematode fauna: an example from the river Rhine. *Hydrobiological Bulletins* 24: 37-45.

Bridge, J., Page, S., Jordan, S. (1982). An improved method for staining nematodes in roots. *Rep. Rothamst. exp. Stn for 1981*, Part 1, p 71.

Byrd, D.W. Jr., Nusbaum, C.J., Barker, K.R. (1966). A rapid flotation-sieving technique for extracting nematodes from soil. *Plant Disease Rept.* 50: 954-957.

Byrd, D.W. Jr., Barker, K.R., Ferris, H., Nusbaum, C.J., Griffin, W.E., Small, R.H., Stone, C.A. (1976). Two semi-automatic elutriators for extracting nematodes and certain fungi from soil. *J. Nematology* 8: 206-212.

Carbonell, T.E., Angulo, A.E. (1979). Tamaño optimo relativo de muestra para evaluar nematodos en vamous caneros. *Nematropica* 9: 8-15.

Catalogue, zie VAN Bezooijen, J., Stemerding, S. (1971).

Claassen, Y. (1991). Effecten van verontreinigd sediment op de nematodenfauna van het land van Saefthinge. Vakgroep Bodemkunde en Geologie, Wageningen.

Cobb, N.A. (1918). Estimating the nema population of the soil. *Agric. Tech. Circ. Bur. Pl. Ind. U.S. Dep. Agric. No.1.* 48 pp.

Coolen, W.A. (1979). Methods for the extraction of *Meloidogyne* spp. and other nematodes from roots and soil. In: *Root-knot nematodes (Meloidogyne species) systematics, biology and control* (F. Lamberti, C.E. Taylor, eds.), pp. 317-329. Academic Press, London & New York.

Coolen, W.A., d'Herde, C.J. (1972). A method for the quantitative extraction of nematodes from plant tissue. State Agricultural Research Centre, Ghent, Belgium.

Coolen, W.A., d'Herde, C.J. (1977). Extraction de *Longidorus* et *Xiphinema* spp. du sol par centrifugation en utilisant du silica colloidal. *Nematologia Mediterranea* 5: 195-206.

Courtney, W.D., Polley, D., Miller, V.L. (1955). TAF, an improved fixative in nematode technique. *Plant Disease Rept.* 39: 570-571.

Dickerson, O.J. (1977). An evaluation of the direct centrifugal-flotation methods of recovering nematodes from soil. *Plant Disease Rept.* 61: 1054-1057.

Dunn, R.A. (1969). Extraction of Heterodera species from soils by centrifugation in high density solutions. *J. Nematology* 1: 7.

Ellenby, C., Smith, L. (1964). A narcotic and an immersion medium for living nematodes with some observations on the refractive index of the cuticle. *Nematologica* 10: 342-343.

- Elliott, J.M. (1979). Statistical analysis of samples of benthic invertebrates. Freshwater Biological Association Scientific Publication No. 25, pp. 14-93; 126-137. The Ferry House, Ambleside, Cumbria.
- Elmiligy, I.A., de Grisse, A. (1970). Effect of extraction technique and adding fixative to soil before storing on recovery of plant-parasitic nematodes. *Nematologica* 16: 353-358.
- Esser, R.P., MacGowen, J.B. (1973). Mountant control on glass. *J. Nematology* 5: 76.
- Fenwick, D.W. (1940). Methods for the recovery and counting of cysts of *Heterodera schachtii* from soil. *J. Helminthology* 18: 155-172.
- Ferris, H. (1984). Nematode damage functions: The problem of experimental and sampling error. *J. Nematology* 16: 1-9.
- Freckman, D.W., Kaplan, D.T., Van Gundy, S.D. (1977). A comparison of techniques for extraction and study of anhydrobiotic nematodes from dry soils. *J. Nematology* 9: 176-181.
- Goodell, P.B. (1982). Soil sampling and processing for detection and quantification of nematode populations for ecological studies. In: *Nematodes in Soil Ecosystems* (D.W. Freckman, ed.), pp. 178-198.
- Gooris, J., d'Herde, C.J. (1972). A method for the quantitative extraction of eggs and second stage juveniles of *Meloidogyne* spp. from soil. State Agricultural Research Centre, Ghent, Belgium.
- Greco, N., D'Abbaddo, T. (1990). Efficient procedure for extracting *Tylenchulus semipenetrans* from citrus roots. *J. Nematology* 22: 590-593.
- Griffiths, B.S., Boag, B., Neilson, R., Palmer, L. (1990). The use of colloidal silica to extract nematodes from small samples of soil or sediment. *Nematologica* 36: 465-473.
- de Grisse, A.T. (1969). Redescription ou modification de quelques techniques utilisées dans l'étude des nématodes phytoparasitaires. *Meded. Rijksfaculteit Landbouwwetenschappen Gent* 34: 351-369.
- Heip, C., Vincx, M., Vranken, G. (1985). The ecology of marine nematodes. *Oceanogr. Mar. Biol. Ann. Rev.* 23: 399-489.
- d'Herde, C.J., van den Brande, J. (1964). Distribution of *Xiphinema* and *Longidorus* spp. in strawberry fields in Belgium and a method for their quantitative extraction. *Nematologica* 10, 454-458.
- Hesling, J.J. (1952). An improved method of separating eelworm cysts from debris. *J. Helminthology* 26: 69-70.
- Hooper, D.J., Cowland, J.A., Spratt, J. (1983). Nematode specimen collection. *Report Rothamsted Experimental Station for 1982*, Part I, 167.
- Huijsman, C.A. (1957). Veredeling van de aardappel op resistentie tegen *Heterodera rostochiensis* Wollenweber. *Meded. Sticht. Pl. Vered. Wageningen* No. 14: 85 pp.
- Hussey, R.S., Barker, K.R. (1973). A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Disease Rept.* 57: 1025-1028.

- s'Jacob, J.J., van Bezooijen, J. (1984). *Manual for practical work in nematology, revised (1984) edition*. Department of Nematology, Agricultural University, Wageningen.
- Jacobs, L. (1987). *Inleiding tot de biologische kwaliteitsbeoordeling van onderwaterbodems*. DBW-RIZA, Lelystad.
- Kleijburg, P. (1960). Soil sample examination as a base for advisory work about stem eelworms. *Nematologica* 5: 22-27.
- Kort, J. (1960). A technique for the extraction of *Heterodera* cysts from wet soil and for the estimation of their egg and larval content. *Versl. Meded. PlZiektenk. Dienst Wageningen No. 233*: 6 pp.
- McSorley, R. (1987). Extraction of nematodes and sampling methods. In: Principles and Practice of Nematode Control in Crops (R.H. Brown, B.R. Kerry, eds.), pp. 13-41. Academic Press, Australia.
- McSorley, R., Parrado, J.L. (1981). Effect of sieve size on nematode extraction efficiency. *Nematologica* 11: 165-174.
- McSorley, R., Parrado, J.L. (1982). Estimating relative error in nematode numbers from single soil samples composed of multiple cores. *J. Nematology* 14: 522-529.
- McSorley, R., Parrado, J.L., Dankers, W.H. (1984). A quantitative comparison of some methods for the extraction of nematodes from roots. *Nematologica* 14: 72-84.
- Nelson, F.K., Albert, P.S., Riddle, D.L. (1983). Fine structure of the *Ceanorhabditis elegans* excretory system. *J. Ultrastruct. Res.* 82: 156-171.
- Netscher, C. (1971). A rapid technique for mass-killing of nematodes. *Nematologica* 16 (yr 1970): 603.
- Netscher, C., Seinhorst, J.W. (1969). Propionic acid better than acetic acid for killing nematodes. *Nematologica* 15: 286.
- Oostenbrink, M. (1950). Het aardappelaaltje (*H. rostochiensis* Wollenweber), een gevaarlijke parasiet voor de eenzijdige aardappelcultuur. *Versl. Meded. PlZiektenk. Dienst Wageningen No. 115*. 230 pp.
- Oostenbrink, M. (1954). Een doelmatige methode voor het toetsen van aaltjesbestrijdingsmiddelen in grond met *Hoplolaimus uniformis* als proefdier. *Meded. LandbHoges. Gent* 19: 377-408.
- Oostenbrink, M. (1960). Estimating nematode populations by some selected methods. In: Nematology (J.N. Sasser, W.R. Jenkins, eds.), pp. 85-102. The University of North Carolina Press, Chapel Hill.
- Oostenbrink, M. (1970). Comparison of techniques for population estimation of soil and plant nematodes. In: Methods of study in soil ecology (J. Phillipson, ed.), pp. 249-255. UNESCO (Proc. Paris Symp., 1967).
- Den Ouden, H. (1954). Het bieten cysteaaltje en zijn bestrijding. I. Methoden te gebruiken bij het onderzoek naar kunstmatige en natuurlijke lokstoffen. *Meded. Inst. Suikercore. Bergen-o.-Zoom* 24: 101-120.

- Proctor, J.F., Marks, C.F. (1975). The determination of normalizing transformations for nematode count data from soil samples and of efficient sampling schemes. *Nematologica* 20: 395-406.
- Prot, J.C., Ferris, H. (1992). Sampling approaches for extensive surveys in nematology. *J. Nematology* 24(4S): 757-764.
- Schouten, A.J., Arp, K.K.M. (1991). A comparative study on the efficiency of extraction methods for nematodes from different forest litters. *Pedobiologia* 35: 393-400.
- Seinhorst, J.W. (1955). Een eenvoudige methode voor het afscheiden van aaltjes uit grond. *Tijdschrift voor Plantenziekten* 61:188-190.
- Seinhorst, J.W. (1956). The quantitative extraction of nematodes from soil. *Nematologica* 1: 249-267.
- Seinhorst, J.W. (1959). A rapid method for the transfer of nematodes from fixative to anhydrous glycerine. *Nematologica* 4: 67-69.
- Seinhorst, J.W. (1962). Extraction methods for nematodes inhabiting soil. In: *Progress in Soil Zoology* (P.W. Murphy, ed.), pp. 243-256. Butterworths, London.
- Seinhorst, J.W. (1964). Methods for the extraction of *Heterodera* cysts from not previously dried soil. *Nematologica* 10: 87-94.
- Seinhorst, J.W. (1966). Killing nematodes for taxonomic study with hot f.a. 4:1. *Nematologica* 12: 178.
- Seinhorst, J.W. (1973). How small is a small drop of water? *Nematologica* 19: 121.
- Seinhorst, J.W. (1975). Separation of *Heterodera* cysts from dry organic debris using ethanol. *Nematologica* 20: 367-369.
- Southey, J.F. (ed.) (1986). *Laboratory methods for work with plant and soil nematodes*. Ministry of Agriculture, Fisheries and Food, Reference Book 402, London.
- Stemerding, S. (1964). Een mixer-wattenfilter methode om vrijbeweeglijke endoparasitaire nematoden uit wortels te verzamelen. *Versl. Meded. Plziektenk. Dienst Wageningen No. 141 (Jaarboek 1963)*, pp. 170-175.
- Stolk, M. (1989). *Methodiek-ontwikkeling voor bemonstering en verwerking van nematodenmonsters*. Stageverslag RIVM Bilthoven.
- Townshend, J.L. (1984). Anaesthesia of three nematode species with propylene phenoxetol. *Nematologica* 29 (Yr 1983): 357-360.
- Viglierchio, D.R., Schmitt, R.V. (1983). On the methodology of nematode extraction from field samples: Baermann funnel modifications. *J. Nematology* 15: 438-444.
- Viglierchio, D.R., Yamashita, T.T. (1983). On the methodology of nematode extraction from field samples: density flotation techniques. *J. Nematology* 15: 444-449.