Bulletin OEPP/EPPO Bulletin (2013) **43** (3), 471–495 ISSN 0250-8052. DOI: 10.1111/epp.12077

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/119 (1)

Diagnostics
Diagnostic

PM 7/119 (1) Nematode extraction

Specific scope

This standard describes procedures for nematode extraction[1].

Specific approval and amendment

Approved in 2013-09.

1. Introduction

Nematode diagnosis requires efficient recovery of the plant-parasitic nematodes from the plant or soil sample. The easiest and most simple method is to submerge a plant sample in water in a Petri dish and directly select the nematodes for further identification using the microscope. Although this procedure can provide results in a very short time, it is only suitable for small samples, cannot be standardized and its overall efficacy is low. For soil samples it is not possible to observe nematodes directly due to the dense cloud of soil particles. For routine diagnostics, such as in the case of regulated plant pathogens, standardized methods are required that provides high nematode recovery under varying sample conditions. At the same time, those methods should allow only minimal individual failure and results should be reproducible. Over past years, many methods have undergone test performance studies nationally and internationally, but only few results have been published (e.g. Müller, 1983; Ladeveze & Anthoine, 2010; den Nijs & van den Berg, 2012). Nevertheless, it became evident from these tests that results between laboratories can vary greatly. Reasons for that might be differences in the custom-made equipment used, laboratory-specific adaptations of the method or different experience of the operators.

Unfortunately, there is no one method that is ideal for all nematodes species under all conditions. Nematodes vary in size, surface structure and motility; plant and soil samples in composition, compactness and organic matter content. Furthermore, the choice of the method also depends on the aim of the extraction, on time and equipment available, the required efficiency and preferences of the person performing the extraction. There are several excellent reviews on common extraction techniques available that also discuss the strength and limitations of each technique (e.g. Oostenbrink, 1960; Coolen & D'Herde, 1972; Ayoub, 1980; Southey, 1986; Seinhorst, 1988; Turner, 1998; Hooper *et al.*, 2005; Van Bezooijen, 2006).

With the exception of direct examination, extraction methods are indirectly using one of the following principles or a combination of them:

- Specific density of the nematodes (flotation/centrifugation, elutriation, sedimentation);
- Size and shape of the nematodes (sieving);
- Motility of the nematodes (Baermann funnel, mistifier, incubation).

These principles lead to a variety of different methods such as those recommended by EPPO in its Diagnostic Protocols for plant-parasitic nematodes (Table 1).

The overall goal of this EPPO standard is to provide guidance for the proper application of methods mentioned in EPPO Diagnostic Protocols regarding the extraction of plant-parasitic nematodes from plant and soil samples. Routine operations require standardized procedures, especially when it comes to quality assurance such as accreditation. Within this, the given instructions describe the methods in their ideal form as agreed upon by the member experts of the EPPO Panel on Diagnostics in Nematology. However, depending on available equipment and laboratory experience modifications are still possible as long as they are in line with the purpose of the EPPO diagnostic protocols and any regulations associated with quarantine organisms. Modifications could comprise parameters such as centrifugation

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

Table 1 Methods for the extraction of plant-parasitic nematodes from plant and soil samples currently included in EPPO Diagnostic Protocols

Plant samples	
Motile nematodes	Baermann funnel/Oostenbrink dish, mistifier, incubation of plant parts
Motile and immotile nematodes	Direct examination, maceration and filtration, maceration and centrifugal flotation, enzymatic digestion
Soil samples	
Motile nematodes	Baermann funnel/Oostenbrink dish, flotation and sieving, Flegg modified Cobb technique, Oostenbrink elutriator
Motile and immotile nematodes	Centrifugal flotation
Cysts from soil	
Dried soil	Baunacke method, paper strip method, Fenwick can, Schuiling centrifuge
Wet or dried soil	Seinhorst elutriator, centrifugal flotation, Wye washer

speed and duration, blending speed and duration or sieve aperture size.

1.1 Selection of an extraction method

An ideal extraction method would allow extraction of 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, hence nematology laboratories have to select the most appropriate method for each and every situation (Van Bezooijen, 2006). Although numerous papers have been published on the comparison of different methods, the conditions of all those studies varied considerably while general conclusions cannot be drawn (e.g. Oostenbrink, 1960; Ayala *et al.*, 1963; Kimpinski & Welch, 1971; Harrison & Green, 1976; Viglierchio & Schmitt, 1983b; McSorley *et al.*, 1984; Riggs *et al.*, 1997; Tenente *et al.*, 2007; Viaene *et al.*, 2007; Bellvert *et al.*, 2008a). Nonetheless, some trends can be indicated.

For methods based on nematode 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 easier to control than methods without an undercurrent (decanting, Baunacke, Fenwick), although the former 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 some nematodes may be damaged by the extraction fluid (dorylaimids are relatively sensitive) (Van Bezooijen, 2006). Average sedimentation rates in water for selected plant-parasitic nematodes are given by Viglierchio & Schmitt (1983a) as follows:

Meloidogyne incognita juveniles	0.3 cm min^{-1}
Heterodera schachtii juveniles	0.4 cm min^{-1}
Ditylenchus dipsaci J4	0.9 cm min^{-1}
Mesocriconema xenoplax mixed stages	2.6 cm min^{-1}
Xiphinema index J4/adult	5.2 cm min^{-1}

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 juveniles of *Meloidogyne*, *Tylenchus*, or *Rotylenchulus*. They recommend 38 μm sieves (Van Bezooijen, 2006). In many laboratories, sets of four sieves of 50 μm are used. That way, nematodes washed through the first sieve, most probably stay behind on the second or subsequent sieve (also see Oostenbrink, 1954; Seinhorst, 1956).

Methods based on nematode motility do not capture slow and inactive nematodes or eggs. The number of nematodes moving out of a sample depends on extraction duration 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 motility in the sample and therefore numbers in the final suspension. Optimal temperatures for motility may differ between nematode species (McSorley, 1987). Finally, a number of nematode species prefer moving up instead of crawling down (negative geotaxis), so they will never be found in the extraction disk (*Bursaphelenchus cocophilus*, the redring nematode, and a number of insect parasites) (Van Bezooijen, 2006).

Apart from these considerations, extraction yield also depends on the type of soil and properties of nematode species. Generally, for many methods 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 (Van Bezooijen, 2006). All those methods vary considerably in terms of costs such as for equipment, labour and water but also in terms of extraction efficacy and quality (Table 2). In general, methods using the motility of nematodes are cheapest, whereas methods involving an undercurrent (elutriation) or centrifugation are more expensive. On the other hand, the latter methods often have higher extraction

Table 2 Costs and benefits of the extraction methods mentioned in the EPPO Diagnostic Protocols for plant-parasitic nematodes (modified after Van Bezooijen, 2006)

Extraction method	Principle	Maximum sample size	Extraction efficacy	Cost of equipment	Labor costs	Water use	Time until evaluation*	Quality of extraction (clean)
Plant material								
Direct examination	Motility	10 g	+	+	+	+	10 min	+
Baermann funnel/Oostenbrink dish	Motility	50 g	++	+	++	+	24 h	+++
Root incubation	Motility	20 g	++	+	++	+	72 h	++
Mistifier	Motility	50 g	+++	++	+	+++	24 h	++
Maceration and filtration	Size and shape	50 g	+++	++	++	++	15 min	+
Maceration and centrifugal flotation	Density	50 g	+++	+++	++	++	30 min	++
Enzymatic digestion	Size and shape	10 g	++	++	++	+	72 h	+
Soil								
Baermann funnel/Oostenbrink dish	Motility	250 mL	+	+	++	+	24 h	+++
Flotation and sieving	Density and size and shape	200 mL	++	++	+++	++	15 min	+
Flegg modified Cobb	Density and size and shape	1000 mL	++	++	+++	+++	24 h	++
Oostenbrink elutriator and Baermann funnel	Density	250 mL†	+++	+++	++	++	24 h	+++
Oostenbrink elutriator and centrifugation	Density	250 mL†	+++	+++	++	++	60 min	+++
Centrifugal flotation	Density	250 mL	++	+++	++	++	15 min	+++
Cysts								
Baunacke method Paper strip method	Density and size and shape	100 mL	+	+	++	+	10 min	+
Fenwick can	Density	250 mL	++	++	++	++	15 min	++
Schuiling centrifuge	Density	500 mL	++	+++	++	+++	15 min	++
Seinhorst elutriator	Density	500 mL‡	+++	+++	++	+++	15 min	++
Centrifugal flotation	Density	250 mL	++	+++	++	++	15 min	++
Wye washer	Density	1000 mL§	++	++	++	+++	15 min	++

+ small (low); equipment costs <100 EUR.
++ medium; equipment costs 100–5000 EUR.
+++ large (high); equipment costs >5000 EUR.
*The time given is that needed to extract a sample and receive a suspension ready for evaluation. In many cases additional cleaning steps (e.g. Baermann funnel, Oostenbrink dish, centrifugal flotation) are required that will prolong the process.
†Upscaled versions of the Oostenbrink elutriator can process 1000 mL soil.
‡Upscaled versions of the Seinhorst elutriator can process 2000 mL soil.
§Upscaled versions of the Wye washer can process 2000 mL soil.

efficiency and are more suitable for larger samples (Van Bezooijen, 2006).

2. Extraction of plant-parasitic nematodes from plant material and insect vectors

In general, only migratory and sedentary endoparasites are recovered from plant material. Nevertheless, few ectoparasites might also be extracted, such as specimens still feeding on epidermal cells or nematodes trapped under collapsed root tissue. The extraction of motile nematodes requires different methods than extraction of sedentary immotile nematode stages. In addition, the plant tissue also affects the extraction procedure as roots, bulbs, wood or seeds require different methods to release the nematodes. Unfortunately, there is no single method suitable for all purposes. For quantitative analysis, it also needs to be considered that a significant part of the nematode population within plant tissue is in the form of eggs that will hatch over time. If those numbers are of interest, samples need to be incubated for 3–4 weeks allowing nematodes to hatch.

For comparison of methods and extraction efficacy see Böhmer & Weil (1978), McSorley *et al.* (1984), Oostenbrink (1960), Penas *et al.* (2002), Tarjan (1960, 1972) and Viaene *et al.* (2007).

The methods recommended by EPPO for the extraction of regulated plant-parasitic nematodes from plant material such as seeds, foliage, wood, roots, bulbs and tubers as well as insect vectors in the case of *Bursaphelenchus xylophilus* are listed in Table 3.

Table 3 Methods recommended for the extraction of plant-parasitic nematodes from plant tissue and/or insect vectors as listed in the corresponding EPPO Diagnostic Protocols

	Direct examination	Baermann funnel/ Oostenbrink dish	Root incubation	Mistifier technique	Maceration and filtration	Maceration and centrifugal flotation	Enzymatic digestion
Seeds							
Aphelenchoides besseyi (PM 7/39)		X					
Ditylenchus destructor/D. dispaci (PM 7/87)		X					
Foliage							
Aphelenchoides besseyi (PM 7/39)	X	X		X			
Root							
Nacobbus aberrans (PM 7/5)	X		X			X	
Meloidogyne chitwoodi/M. fallax (PM 7/41)	X	X				X	
Radopholus similis (PM 7/88)	X	X					
Hirschmanniella spp. (PM 7/94)	X	X		X	X	X	
Meloidogyne enterolobii (PM 7/103)		X				X	
Tuber/bulb							
Meloidogyne chitwoodi/M. fallax (PM 7/41)						X	X
Nacobbus aberrans (PM 7/5)	X	X			X		
Plant tissue							
Ditylenchus destructor/D. dispaci (PM 7/87)		X					
Radopholus similis (PM 7/88)					X	X	
Wood and wood products							
Bursaphelenchus xylophilus (PM 7/4)*		X		X			
Vector beetle							
Bursaphelenchus xylophilus (PM 7/4)	X	X					

*Incubation of wood samples at 25°C for at least 14 days is recommended before extraction (see PM 7/4).

2.1 Direct examination

Plant material and insect vectors can be examined directly for motile and immotile nematodes under a dissecting or inverse microscope at magnifications of 10–50× or 50–400×, respectively, using transmitted and/or incident light.

Materials
- Petri dish;
- Pair of forceps, dissecting needle, scalpel;
- Handling needle for picking nematodes;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure
- Wash roots, bulbs or tubers to remove soil debris; seeds, foliage and wood chips can be processed directly;
- Place plant tissue in water in a Petri dish;
- Tear apart plant tissue with forceps, dissecting needle or scalpel to release nematodes;
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope;
- For insects carrying nematodes, decapitate and transfer the insect into a glass dish with water or 1% saline (NaCl) solution and then cut the insect in pieces with a scalpel to release the nematodes from the insect body.

Advantage
- Simple, fast and cheap;
- Small amount of water.

Disadvantage
- Laborious;
- Suspension is usually dirty due to plant or insect debris;
- Only suitable for small samples.

Remarks
Motile nematodes will be released from the tissue within few minutes. However, resting stages of *Ditylenchus dipsaci* and *D. destructor*, such as in flower bulbs and infected seeds (*D. dipsaci* only), first have to rehydrate to become active which can take 2–4 h. Too long an exposure of nematodes in suspension should be avoided as plant secondary metabolites and decomposition products can kill nematodes.

2.2 Baermann funnel/Oostenbrink dish

This method for the extraction of motile nematodes was introduced by Baermann (1917) using a funnel. In its

original version, the sample was wrapped in a tissue cloth and almost fully incubated in water resulting in very low nematode recovery. Modified versions use a wire basket plus filter to spread the sample over a larger area. In addition, the sample is only immersed half-way into the water. Oostenbrink (1954) replaced the funnel by a dish. Since then, several modifications have been published such as by Whitehead & Hemming (1965), Rodríguez-Kábana (1981) and others.

Materials

- Knife, pair of scissors or blender;
- Cotton-wool milk filter or equivalent (e.g. cheesecloth, filter paper, paper towel);
- Funnel made of glass with a piece of soft polyethylene tube attached to the stem and closed with a spring or screw clip (Fig. 1). Recommended slope of funnel is approx. 30°. For the Oostenbrink dish method plastic or stainless steel dishes (pie pan) are used (Fig. 2);
- Stand to hold the funnel;
- Support, such as plastic sieve or wire basket of large enough aperture to allow nematode passage (i.e. 250 µm);
- 20 or 25 µm aperture sieve;
- 100 mL glass beaker;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure

- Peel and chop plant tissue such as wood chips (for preparation of wood samples for *Bursaphelenchus xylophilus* see Appendix 3 of PM 9/1), leaves, roots, bulbs or tubers in ±1 cm pieces or macerate in a blender for up to 1 min depending on source of plant material; seeds remain intact or can be split longitudinally to facilitate nematode removal (e.g. *Aphelenchoides besseyi*/rice, Hoshino & Togashi, 1999);
- Place plant material on the cotton-wool milk filter placed within a support (sieve);
- Submerge support with sample gently in the water of the funnel/dish;
- Nematodes leave the plant tissue, pass through the cotton-wool milk filter and sink to the bottom of the funnel stem or dish, respectively;

Fig. 1 Modified Baermann funnel for extracting nematodes from plant material or soil (Photo: JKI, Germany).

- Collect nematodes after 24–72 h by opening the spring or screw clip on the funnel stem or by collecting the nematodes of the dish in a glass beaker;
- Let the nematodes settle in the glass beaker and remove the supernatant, or pass suspension in the beaker over a 20 or 25 µm sieve to reduce the volume of water;
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope.

Advantages

- Simple and inexpensive;
- Small amount of water;
- Final suspension is clean;
- Good recovery of motile nematodes from small samples.

Fig. 2 Oostenbrink dish. Left: Set-up showing plastic dish, supporting sieve made of 250 µm polyamide gauze and cotton-wool milk filter (Photo: JKI, Germany). Right: Set-up consisting of plastic dish, plastic basket and cotton-wool milk filter for extracting *Bursaphelenchus xylophilus* from wood chips (Photo: Vladimir Gaar, Diag. Lab. Prague, Czech Rep.).

Disadvantages
- Only suited for samples up to 50 g;
- Lack of aeration in the water reduces nematode movement;
- Rapid bacterial growth for some plant materials (e.g. bulbs), especially after maceration;
- Poor recovery of relatively immotile nematodes (e.g. *Xiphinema*, *Hemicycliophora*, *Criconemoides*);
- Poor recovery from large samples.

Remarks
Alternatively, funnels made of plastic or stainless steel and/or using silicone tubes can be used. However, regarding the latter, diffusion of oxygen into water is lower than for polyethylene (Stoller, 1957) which could slowly lead to asphyxiation. Depending on the plant tissue, most (50–80%) of the motile nematodes present will be recovered within 24 h; however, samples can be left on the funnel for up to 72 h or for wood chips even up to 14 days to increase recovery rate. For longer extraction periods regular tapping and adding of fresh water increases nematode motility and therefore recovery rate. Similar results can be achieved by using a solution of 0.15% H_2O_2 instead of water, or by placing the Baermann funnel/Oostenbrink dish in a mistifier (see 2.4). In general, extraction can be performed at room temperature (20°C). The diameter of the funnel or dish should be chosen so that sample layer will not be more than 1–2 mm. For larger sample sizes use aliquots or divide the sample over several funnels/dishes. The filter paper should retain remaining soil particles and plant debris but allow easy passage of nematodes at the same time. Milk filters made of cotton wool or fleece (e.g. Höschele GmbH, Remshalden, Germany; http://www.hoesch-hele-nonwoven.de/) are commonly used. Usually, one or two layers work well. However, users should be aware that nematode passage can vary highly depending on filter material and thickness of filter. If in doubt, efficacy tests should be performed.

2.3 Root incubation

This method for the extraction of motile nematode stages is especially recommended for *Nacobbus aberrans* and *Meloidogyne* spp., but can also be used for other endoparasitic nematodes, such as migrating endoparasites (*Radopholus* spp., *Bursaphelenchus* spp.). The method was introduced by Young (1954) and later modified by Mountain & Patrick (1959), who used glass jars instead of plastic bags.

Materials
- Pair of scissors or knife;
- Balance (scale);
- Polythene bag;
- 100 mL glass beaker;
- 20 or 25 μm aperture sieve;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure
- Wash roots free from adhering soil and cut into short pieces (1–5 cm);
- Approximately 5 g of roots are moistened and placed in a polythene bag, that is closed and incubated for 3–4 days at room temperature (20°C); during this period, most of the nematodes will leave the root tissue;
- Wash the roots and the inside of the bag with a small amount of water and collect the water containing the nematodes in a glass beaker
- Pass the nematode suspension in the beaker over a 20 or 25 μm sieve to reduce the volume of water;
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope.

Advantages
- Simple and cheap;
- Minimum labor and equipment.

Disadvantages
- Little efficacy;
- Long time period until results are available.

Remarks
Efficacy of extraction can be improved by adding 1–3% H_2O_2 for oxygen supply (Tarjan, 1967, 1972). Oxygen can also be supplied by adding air through a small pump as used in fish aquaria. In modified form, wood chips can be directly emerged in water for extraction of nematodes such as *Bursaphelenchus* spp. (Fig. 3). A comprehensive discussion on this technique can be found in Ayoub (1980).

2.4 Mistifier technique

This technique was originally described by Seinhorst (1950) and is used to extract motile nematodes. It consists in principle of a Baermann funnel or Oostenbrink dish placed in a mist or fog of water to avoid oxygen depletion. The mist is produced by spray nozzles over the plant material or by nozzles spraying upwards so that the droplets fall in a soft curve onto the plant material. Sap and toxic decomposition products of the plant material are washed off with the funnel overflows allowing extraction times of possibly up to 6 weeks (see remarks below).

Materials
- Knife, pair of scissors or blender;
- Mistifier spray apparatus (Figs 4 and 5);

Fig. 3 Extraction of a chopped wood sample in a glass beaker (Photo: Tomasz Konefał, Toruń, Poland).

- Baermann funnel or Oostenbrink dish (Figs 1 and 2);
- Supports (sieves, wire baskets) with legs;
- 20 or 25 µm aperture sieve;
- Glass beaker;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure

- Chop plant tissue such as wood pieces, leaves, roots, bulbs or tuber peels in ±1 cm pieces; seeds remain whole;
- Place plant material on the support;
- Gently place support with sample in the funnel/dish in the mistifier spray apparatus; the legs should prevent the support from touching the water surface;
- Nematodes leaving the plant tissue are washed by the water into the funnel/dish where they settle;
- Collect nematodes every 24–48 h in a glass beaker by opening the spring or screw clip on the funnel stem or by collecting the nematodes on a 20 or 25 µm sieve;

Fig. 5 Mistifier spray apparatus with Oostenbrink dishes used at PPO Lelystad, the Netherlands, with continuous fog (Photo: JKI, Germany).

- Extraction can be continued for up to 4 weeks;
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope.

Advantages

- Nematode extraction over prolonged periods allows juveniles to hatch from eggs (e.g. *Meloidogyne* spp.), i.e. higher extraction efficiency compared with Baermann funnel/Oostenbrink dish;
- Nematodes are usually in a better condition compared to the Baermann funnel/Oostenbrink dish alone;
- Toxic decomposition products are washed off.

Disadvantages

- High use of water;
- Time consuming.

Remarks

Water temperature should be around 20°C to allow optimum motility of nematode specimens. The spray can be left on constantly or regulated at certain time intervals (e.g. 30 s spraying every 5 min). Hollow cone or solid cone

Fig. 4 Left: Mistifier spray apparatus with Baermann funnels used at the Julius Kühn-Institute in Münster, Germany, with water spraying from the top directly onto the samples. Right: Mistifier spray apparatus with Oostenbrink trays used at Wageningen University, the Netherlands, with water spraying from the bottom to the top and then falling onto the sample (Photos: JKI, Germany).

nozzles are generally used at 4–6 L h^{-1} under pressure of about 2.8 kg cm^{-2}. In case of fog, spray nozzles are best placed at the sides spraying into the interior of the apparatus. Fog spray runs constantly. When left for too long (>4 weeks), bacterial cultures can flourish and make the nematode suspension collected at the bottom of the funnel unclear and can even clog the tubing and filter. Complete replacement of the water in the funnel every 3–4 days can improve extraction efficacy.

2.5 Maceration and filtration

This method is suitable for the extraction of motile and immotile stages of *Hirschmanniella* spp., *Radopholus similis*, *Nacobbus aberrans* and other endoparasitic nematodes from roots, tubers and other plant tissues. Further details on this method are given by Coolen *et al.* (1971) and Seinhorst (1988).

Materials
- Pair of scissors or potato knife;
- Domestic blender (e.g. Waring blender);
- 250, 150 and 45 μm aperture sieves;
- 100 mL glass beaker;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure
- Cut and macerate roots and/or tuber peels (0.5 mm thick) in a blender at about 12 000 rev min^{-1} for 30 s;
- Pass the resulting suspension through a set of sieves with the 250 nested on top of the 150 and 45 μm aperture sieves;
- Debris collected on the 250 and 150 μm sieve is generally discarded unless there is specific interest for swollen female stages such as those of *Meloidogyne* or *Nacobbus*;
- Transfer nematodes on the 45 μm sieve into a glass beaker;
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope.

Advantages
- Examination within few minutes possible;
- Small amount of water.

Disadvantages
- Nematodes might get damaged in blender;
- Maceration of plant tissue may release toxic or sticky (e.g. banana roots) compounds;
- Low efficacy.

Remarks
In case of potato peels, starch grains can complicate the examination of the suspension from the 45 μm sieve. If that happens, addition of water can ease evaluation. For better visualization, nematodes within root tissue can be stained prior to extraction with Phloxine B (0.15 g L^{-1} water), acid fuchsin solution (875 mL lactic acid, 63 mL glycerol, 62 mL water, 0.1 g acid fuchsin) or 12.5% McCormick Schilling red food color (Manzanilla-Lopez *et al.*, 2002; Thies *et al.*, 2002; Hooper *et al.*, 2005); however, applicability of this method is questioned by some nematologists. Instead of mechanical maceration, enzymatic maceration can also be used (see 2.7). For *Meloidogyne chitwoodi* extraction from potato tubers, mechanical and enzymatic maceration yielded similar nematode numbers.

2.6 Maceration and centrifugal flotation

This method is well suited to recover both motile and immotile nematode stages from plant tissues. In the first step, nematodes are liberated from plant tissue by maceration in a blender. They are then separated from the macerated plant tissue by centrifugal flotation using a solution of specific weight higher than the nematodes. Further details on this method are given by Coolen & D'Herde (1972), Coolen (1979) and Greco & D'Abbaddo (1990).

Materials
- Pair of scissors or knife;
- Blender (e.g. Waring blender, household blender);
- 1200 μm aperture sieve;
- Centrifuge plus centrifuge tubes (size ranging from 100 to 1000 mL);
- Kaolin;
- $MgSO_4$ solution with a density of 1.15–1.18 (or similar extraction fluid, see 3.5);
- 20 or 25 μm aperture sieve;
- 100 mL glass beaker;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure
- Wash plant tissue and cut into pieces about 1 cm long. Mix the sample carefully if only part of it is used for nematode extraction;
- Macerate plant tissue in a blender at about 12 000 rev min^{-1} for 30 s;
- Pour the resulting suspension through a 1200 μm sieve and collect in a beaker;
- Wash the plant tissue on the sieve carefully with water to collect all nematodes;
- Centrifuge the collected washing water containing the nematodes with 1% kaolin powder (approx. 1 tablespoon, depending on size of tubes) at approx. 1800 g for 4 min;

time and g-force is not that critical, as long as a stable pellet is achieved; time lengths of 2–5 min and g-forces of 700–2900 g can be used;
- Discard the supernatant and re-suspend the sediment in a $MgSO_4$ solution (or similar extraction fluid) with a density of 1.15–1.18;
- Centrifuge again at 1800 g for 4 min;
- Pour the supernatant through a sieve of maximum 20 or 25 μm aperture size and rinse very well with water to remove the excess salt or sugar solution;
- Transfer the nematodes from the sieve into a glass beaker;
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope.

Advantages
- Rapid;
- Small amount of water.

Disadvantages
- Nematodes might get damaged in blender (e.g., *Ditylenchus dipsaci*).

Remarks
Instead of $MgSO_4$, other salts (e.g. $ZnSO_4$), sucrose or colloidal silica can be used (for more information see remarks in chapter 3.5). To reduce the risk of nematode damage, the method can be adapted by first macerating the plant tissue at low speed for 15 sec and collect nematodes on nested sieves of 1 mm, 250 and 20 μm. Then macerate the plant tissue from the 1 mm sieve at high speed for 1 min and collect nematodes again by sieving. Nematodes collected on the 250 and 20 μm sieve are collected for centrifugal flotation as described above.

2.7 Enzymatic digestion of roots and potato peels

This method recovers both motile and immotile stages of migratory and sedentary endoparasitic nematode species from plant tissues. The method was described for potato tuber tissue by Viaene *et al.* (2007) based on similar work published previously (e.g. Araya & Caswell-Chen, 1993; Hussey, 1971; Julio *et al.*, 2003; Kaplan & Davis, 1990).

Materials
- Knife or peeler;
- Balance (scale);
- 200 mL plastic cups;
- Enzymatic solution (can be stored refrigerated for several months): For 50 mL: 10 mL Pectinex (26 000 polygalacturonase units mL^{-1}), 10 mL Celluclast (700 endoglucanase units mL^{-1}), 30 mL phosphate buffer;
- Antibiotic solution: 50 mg tetracycline and 50 mg streptomycin per litre (optional);
- pH meter;
- Incubator;
- Orbital shaker;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure
- Peel the potato;
- Cut the peels into 1 cm pieces and combine them to a bulk sample;
- Transfer about 3 g potato peels into a 200 mL plastic cup;
- Add 50 mL enzymatic solution plus antibiotics (optional);
- Final pH should be 4.5–5; if not, adjust pH using 0.1 N NaOH or 1 N HCl;
- Incubate cups at 35°C on an orbital shaker at 150 rpm for 48 h;
- Extract nematode stages from the potato suspension using sieving, centrifugal flotation or zonal centrifugation (for details see 2.5 Maceration and filtration and 2.6. Maceration and centrifugal flotation);
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope.

Advantages
- Easy and effective;
- Nematodes stay intact;
- Small amount of water.

Disadvantages
- Long extraction time due to incubation;
- Enzymes are expensive.

Remarks
The method described above was originally developed for plant roots by Araya & Caswell-Chen (1993). Optimum incubation time and enzyme concentration might vary with host species, host tissue and nematode species. Modifications made by Anses (Agence nationale de sécurité sanitaire de l'alimentation, de l'environement et du travail, France) include 200 potato peelings in a 500 mL beaker, no antibiotics, enzymes dissolved in water instead of phosphate buffer (no pH adjustment required) and incubation at room temperature for at least 12 h at 100 rpm on an orbital shaker.

In the above described method for potato peels, 48 h incubation yielded more nematode stages than 24 h incubation and zonal centrifugation was more efficient extracting nematode stages then sieving (Viaene *et al.*, 2007).

3. Extraction of vermiform plant-parasitic nematodes from soil

The number of plant-parasitic nematodes that can be recovered from soil depends on soil type, sampling depth, host plant and seasonal factors (Hooper, 1986). High amounts of fresh organic matter in the soil sample (e.g. plant residues after harvest) can influence nematode numbers due to the decomposition processes that might cause nematode toxicity or increase numbers of saprophytic nematodes, or because organic matter hampers extraction by clogging sieves or contaminate the supernatant obtained in density-based methods.

Samples are generally best stored at 5–8°C where nematode metabolism is at a minimum (Elmiligy, 1971; Hooper, 1986). Freezing temperatures need to be avoided. Prior to extraction, bulk soil samples can be carefully passed through a 1 mm sieve and mixed well for homogeneity. Mixing should be done gently to avoid damage to the nematodes. If the sample cannot be processed as a whole, aliquots should be taken.

For optimum results, root fragments should be first separated from soil by hand or using a sieve. Both fractions ('organic' and 'mineral') can then be analyzed separately. For quantitative analysis, it also needs to be considered that a significant part of the nematode population within plant tissue or soil is in form of eggs that will hatch over time. If those numbers are of interest, samples need to be incubated for 3–4 weeks allowing nematodes to hatch.

For comparison of methods and extraction efficacy see Malo (1960), Oostenbrink (1960), Ayala *et al.* (1963), Tobar Jimenez (1963), Elmiligy (1971), Kimpinski & Welch (1971), Chawla & Prasad (1975), Harrison & Green (1976), Viglierchio & Schmitt (1983a,b), Viglierchio & Yamashita (1983), Persmark *et al.* (1992), Yen *et al.* (1998), and Verschoor & De Goede (2000).

EPPO recommends the following methods for nematode extraction in their respective Diagnostic Protocols (Table 4).

3.1 Baermann funnel/Oostenbrink dish

This method for the extraction of motile nematodes was introduced by Baermann (1917) using a funnel. In its original version, the sample was wrapped in a tissue cloth and almost fully incubated in water resulting in very low nematode recovery. Modified versions use a wire basket plus filter to spread the sample over a larger area. Oostenbrink (1954) replaced the funnel by a dish. Since then, several modifications have been published such as by Whitehead & Hemming (1965), Rodríguez-Kábana (1981) and others.

Materials

- Support such as plastic sieve or wire basket of minimum 250 μm aperture to allow nematode passage;
- Cotton-wool milk filter or equivalent (e.g. cheesecloth, filter paper, bathroom tissue);
- Funnel made of glass with a piece of soft polyethylene tube attached to the stem and closed with a spring or screw clip (Fig. 1). Recommended slope of funnel is approx. 30°. For the Oostenbrink dish method, plastic or stainless steel dishes (pie pan) are used, alternatively a plastic bowl (Fig. 2);
- Stand to hold the funnel;
- 20 or 25 μm aperture sieve;
- 100 mL glass beaker;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure

- Place soil on the cotton-wool milk filter which is held by a support; maximum thickness of soil layer is 2–3 mm (Fig. 6);
- Place support with sample into the funnel;
- Add water from the side until bottom of the sieve just touches the water;
- Nematodes leave the soil, pass through the cotton-wool milk filter and sink to the bottom of the funnel stem or dish, respectively;
- Collect nematodes after 24–72 h in a glass beaker by opening the spring or screw clip on the funnel stem or by collecting the nematodes of the dish on a 20 or 25 μm aperture sieve;

Table 4 Methods recommended for the extraction of plant-parasitic nematodes from soil samples as listed in the corresponding EPPO Diagnostic Protocols

	Baermann funnel/ Oostenbrink dish	Flotation and sieving	Flegg modified Cobb	Oostenbrink elutriator	Centrifugal flotation
Nacobbus aberrans (PM 7/5)	X	X			
Meloidogyne chitwoodi/M. fallax (PM 7/41)	X	X		X	X
Ditylenchus destructor/D. dipsaci (PM 7/87)	X	X		X	X
Radopholus similis (PM 7/88)	X			X	X
Hirschmanniella spp. (PM 7/94)	X	X		X	X
Xiphinema americanum sensu lato (PM 7/95)			X	X	
Meloidogyne enterolobii (PM 7/103)	X	X		X	X

Fig. 6 Oostenbrink dishes for extracting nematodes from soil. Left: Oostenbrink dish with 15 cm inner diameter for samples up to 100 mL soil, Right: Oostenbrink dish with 24 cm inner diameter for samples up to 250 mL soil (Photo: JKI, Germany).

- Let the nematodes settle in the glass beaker and remove the supernatant, or pass suspension over a 20 or 25 µm sieve to reduce the volume of water;
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope.

Advantages
- Simple and inexpensive;
- Small amount of water;
- Final suspension is clean;
- Good recovery of motile nematodes from small samples.

Disadvantages
- Only suited for small samples up to 250 mL;
- Lack of aeration in the soil reduces nematode movement;
- Poor recovery of relatively immotile nematodes (e.g. *Xiphinema*, *Hemicycliophora*, *Criconemoides*);
- Poor recovery from large samples.

Remarks
Alternatively, funnels made of plastic or stainless steel and/or using silicone tubes can be used. However, regarding the latter, diffusion of oxygen into water is less than for polyethylene (Stoller, 1957) which could slowly lead to asphyxiation. Depending on soil type and nematode species, 50–80% of the motile nematodes present will be recovered within 24 h (e.g. Verschoor & De Goede, 2000); however, samples can be left on the funnel for up to 72 h. If so, regular tapping and adding of the water increases nematode motility. Choose diameter of funnel or tray so that sample layer will not be more than 2–3 mm to achieve best recovery. For larger sample sizes use aliquots or divide the sample on several funnels/dishes. Covering the soil sample to prevent evaporation can accelerate and increase efficacy of nematode extraction (Robinson & Heald, 1989). As nematodes appear to move towards cold (Robinson & Heald, 1989) a heating source (e.g. light bulbs or similar) installed atop of the Baermann funnel/Oostenbrink dish (see Nielsen, 1947/48; Sohlenius, 1976) can speed up extraction time and improve overall efficacy; however, this method is not commonly used in nematology. Additional information on the different parameters affecting nematode extraction by the Baermann funnel technique is given by Viglierchio & Schmitt (1983a). See also remarks made in 2.2.

3.2 Flotation and sieving

This technique for the extraction of motile nematodes from soil was introduced by Cobb (1918) and is mainly used for Tylenchid species. In principle, soil is washed in water, decanted and nematodes are collected on sieves of different aperture followed by cleaning the suspension with Baermann funnel/Oostenbrink dish. The method makes use of differences in size, shape and sedimentation rate between nematodes and soil particles, and of nematode motility.

Materials
- Bucket of about 10 L;
- Stirring rod;
- 3 × 50 µm aperture sieves;
- 500 mL glass beaker;
- Watch glass (d = 6 cm);
- Baermann funnel/Ostenbrink dish (see 3.1.);
- 100 mL glass beaker;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure
- Add up to 200 mL of soil to a 10 L bucket and add approx. 5 L of water;
- Stir the soil suspension vigorously for 10 s;
- Allow the soil to settle for 45 s;
- Pour the supernatant through a bank of 3 sieves of 50 µm aperture;
- Wash the debris collected on the sieves in a clean glass beaker;
- Carefully pour the suspension from the beaker with the help of a watch glass onto the cotton-wool milk filter supported by a plastic sieve in the Baermann funnel/Oostenbrink dish; if necessary add more water until the bottom of the filter is just covered;
- After 24 h, collect the nematodes from the Baermann funnel or Oostenbrink dish in a glass beaker;
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope.

Advantages
- Simple and rapid;
- High extraction efficiency;
- All nematode genera are recovered;
- No elaborate apparatus needed.

Disadvantages
- Maximum of 200 mL soil;
- Not suitable for clay soil because light particles remain in suspension leading to a dirty final suspension.

Remarks
Aperture size of sieves can be adapted to size of target nematodes, e.g. 150 µm for *Xiphinema* and *Longidorus*. For further comments see remarks on Baermann funnel/Oostenbrink dish (2.2) and Flegg Modified Cobb technique (3.3).

3.3 Flegg modified Cobb technique

This method for motile nematodes is especially recommended for the extraction of *Xiphinema*, but can also be used for other nematode species. It was developed by Flegg (1967) for extracting large Dorylaimid nematodes from soil. The method follows Cobb's washing, decanting and sieving technique (Cobb, 1918) but uses three nested sieves of 150 µm instead of consecutively using five sieves with decreasing aperture size.

Materials
- Plastic beaker of about 1 L;
- Stirring rod;
- 2 mm aperture sieve;

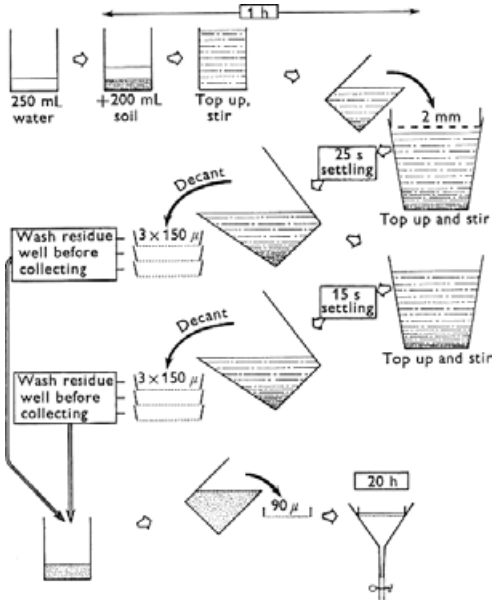

Fig. 7 Scheme of the modified Cobb's washing, decanting and sieving technique (Flegg, 1967).

- 3 × 150 µm aperture sieves;
- 90 µm aperture sieve;
- Wash bottle;
- 5 L plastic bucket with a diameter such that the 2 mm aperture sieve will just fit into the rim;
- Baermann funnel/Ostenbrink dish (see 3.1);
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure
- Fill a 1 L beaker with about 250 mL of water (Fig. 7);
- Add the prepared soil sample (approx. 200 mL soil) and soak for about 30 min (loamy soil) to 60 min (clay soil), stir 2–3 times during soaking period;
- Place the 2 mm aperture sieve on the 5 L plastic bucket;
- Wash the soil suspension through the 2 mm sieve into the 5 L bucket;
- Remove the sieve and top up the bucket with water, then agitate solution by stirring;
- After 25 s sedimentation time, the supernatant suspension is decanted through a bank of three 150 µm aperture sieves; ensure that the sediment stays in the bucket;
- Gently wash the residue on the sieves with a wash bottle to a clean 1 L beaker;
- Top up the residue in the bucket again with water and swirl thoroughly;
- After 15 s sedimentation, decant the supernatant through the same bank of three 150 µm aperture sieves.
- After washing, the residue on the sieves is added to that collected previously;
- Pour the complete content of the litre beaker onto a 90 µm aperture sieve;
- For cleaning the nematode suspension from soil particles, use the Baermann funnel or Oostenbrink dish method (see 3.1);
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope.

Advantage
- High extraction efficiency;
- Little use of water.

Disadvantage
- Labour intensive;
- Experience required to carry out the method in a reliable way.

Remarks
Soil type will affect some aspects of this method (e.g. sieving or suspension); adaptation may then be necessary. Preparing the soil sample and performing the modified

Cobb extraction takes about 1 h. Additional time is required for cleaning the suspension by Baermann funnel/Oostenbrink dish method. Alternatively, the faster centrifugal flotation method can be applied to clean the suspension. For nematodes smaller than *Xiphinema*, sieves with 50 µm aperture should be used. See also remarks made in chapter 2.2.

The reproducibility of the method was tested with four nematode species by three operators in 13 individual tests. In no case was the difference between operators significant (Flegg, 1967).

3.4 Oostenbrink elutriator

This method allows the extraction of motile nematodes from soil samples of 100–1000 mL. Cobb (1924) introduced the principle of flotation with the help of a constant stream of water. Since then, several modifications have been developed such as the one described here by Oostenbrink (1960) as Flotation apparatus III. Nematodes are first separated from heavier soil particles by their specific gravity in an upward current. They are then collected on a nest of sieves and further cleaned by passing on a Baermann funnel/Oostenbrink dish or by centrifugation. This method allows the extraction of large soil samples. Automated versions are available.

Materials

- Oostenbrink elutriator made of stainless steel or acryl glass (e.g. www.mirma.nl, www.meku-pollaehne.de);
- 1 mm aperture sieve (2–4 mm aperture for large nematodes);
- 4 × 45 or 50 µm aperture sieves;
- Plastic bowl;
- Watch glass (d = 6 cm);
- Baermann funnel/Oostenbrink dish (see 3.1);
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure

- Close the side-outlet and the opening at the bottom with a plug (Figs 8 and 9);
- Fill funnel with water up to level 1 where the water level just touches the small funnel;
- Set the undercurrent water stream at 1000 mL min^{-1};
- Wash sample through the top sieve into the funnel using a nozzle;
- Continue until water level has reached level 2, i.e. 2/3 of the funnel;
- Close nozzle;
- After a few seconds, reduce undercurrent to 600 mL min^{-1}; undercurrent fills the funnel;
- Moisten the four 45 or 50 µm aperture sieves to avoid clogging of the mesh and place them under the side-outlet;
- When water has reached level 3, open the side-outlet and let the suspension run on the four nested 45 or 50 µm sieves;
- Incline the sieves by slightly lifting them and tap on the side of the sieves to help the suspension to pass through;
- Immediately wash debris off the sieves into a plastic bowl;
- For cleaning the nematode suspension from soil particles, use the Baermann funnel or Oostenbrink dish method (see 3.1) or centrifugation (see 3.5);
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope.

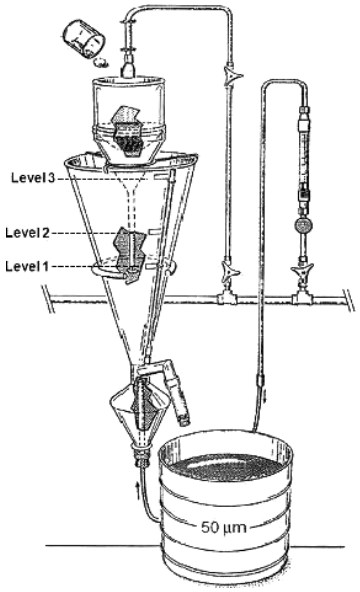

Fig. 8 Schematic overview of the Oostenbrink elutriator (courtesy Van Bezooijen, 2006).

Fig. 9 Oostenbrink elutriator made of stainless steel at Wageningen University, the Netherlands.

Advantages
- Efficient;
- Easy to standardize;
- Good for large samples up to 1000 mL.

Disadvantages
- Expensive equipment;
- High use of water.

Remarks
The apparatus is generally used for samples of about 250 mL soil; however, samples up to 1000 mL are possible. Maximum sample size is determined by the fact that the soil must be washed into the funnel before water level 2 is reached. For small nematodes (e.g. *Paratylenchus*), the water current speed can be reduced, for larger nematodes (e.g. *Xiphinema*, *Longidorus*) it can be increased to 1500–2000 mL min^{-1}. Also for larger nematodes three nested sieves of 160–200 μm are recommended. If nematode suspension needs to be examined the same day, cleaning can be done by centrifugal flotation instead of Baermann funnel/Oostenbrink dish. For detailed information on overall extraction efficacy and parameters influencing extraction efficacy see Verschoor & De Goede (2000).

A similar apparatus was built by Seinhorst: the 'Seinhorst elutriator for free-living nematodes'. This device is used in some laboratories and also gives excellent results.

Modified Oostenbrink elutriator. The modified Oostenbrink elutriator made by MEKU (www.meku-pollaehne.de) processes soil samples of 100–500 mL (Fig. 10). The soil sample is washed with a jet nozzle at 600 mL min^{-1} through a 1 mm aperture sieve (4 mm for *Xiphinema*, *Longidorus*) into the apparatus. At the same time, the undercurrent water stream is set at 1000 mL min^{-1} and shortly later reduced to 600 mL min^{-1} (1500–2000 mL min^{-1} for large nematodes). After 10–15 min, when the water has almost reached the top of the extraction chamber, the side-outlet is opened manually to pass the nematode suspension on four nested 50 μm sieves.

3.5 Centrifugal flotation

This method is used in other areas of biology and was adapted for the extraction of nematodes by Caveness & Jensen (1955). It is the only method that allows extraction of motile and immotile nematodes from soil. Nematode specimens are brought into a suspension with a specific gravity greater than their own, which is about 1.08, so they will float and heavier soil particles will sink. Centrifugation is used to speed up the separation of the sinking fraction and floating fraction. It is also used to clean extracts obtained by sieving or elutriation. The size of the sample that can be processed is limited by the size of the centrifuge tubes. Automated versions are available.

Material (Fig. 11 illustrates equipment used in centrifugal flotation)
- Centrifuge plus centrifuge tubes (size ranging from 100 to 1000 mL);
- Kaolin;
- Stirrer or Vibro mixer;
- $MgSO_4$ solution with a density of 1.15–1.18 (or similar extraction fluid, see Table 5);
- 20 or 25 μm aperture sieve;
- 100 mL glass beaker;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedure
- Fill 1000 mL centrifuge tube with up to 250 mL soil;
- Add about 400 mL water plus a table spoon of kaolin; kaolin forms a visible white layer that separates the sediment and nematodes from the supernatant (water and light organic material);
- Stir suspension thoroughly with stirrer or Vibro mixer to form a homogenous suspension;
- Centrifuge tubes for approx. 4 min at 1800 g; time and g-force is not that critical, as long as a stable pellet is achieved; time lengths of 2–5 min and g-forces of 700–2900 g can be used;
- Gently pour off the supernatant and discard;
- Re-suspend the pellet in about 400 mL of a $MgSO_4$ solution (or similar extraction fluid) with a density of 1.15–1.18;
- Centrifuge tubes again at 1800 g for 4 min;
- Gently pour the supernatant containing the nematodes over a 20 or 25 μm sieve;
- Rinse the sieve immediately with water to remove the $MgSO_4$ solution;
- Nematodes are transferred from the sieve in a glass beaker;
- Examine nematodes within a counting slide at 25–40× magnification using a dissecting or inverse microscope or transfer nematodes with a handling needle to a microscope slide for inspection at higher magnification in a compound microscope.

Advantages
- Sluggish and immotile nematodes are extracted;
- Relatively clean nematode suspension;
- Small amount of water;
- Nematodes are available for examination within 15 min.

Disadvantages
- Extraction fluid can damage nematodes or alter their shape, which hampers identification; it can also influence vitality (important when nematodes are needed for infection studies or cultures);
- Recovery of dorylaimid and triplonchid nematodes may be poor (e.g. *Xiphinema*, *Longidorus*, *Trichodorus*, *Paratrichodorus*);
- Expensive equipment.

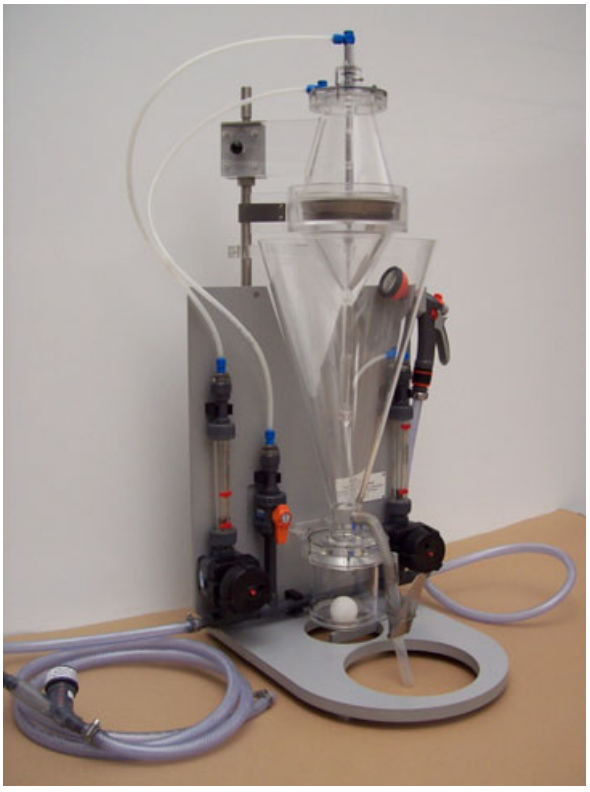

Fig. 10 Modified Oostenbrink elutriator made of acryl glass (Photo: MEKU, Germany).

Remarks

If there are only smaller centrifuge tubes available, reduce sample size accordingly. To mix the soil suspension many laboratories routinely use a household mixer with dough hooks for 1 min or longer, until a homogenous suspension is formed. For the effect of different solutions at certain specific gravities on population recovery see Viglierchio & Yamashita (1983). In general, the higher the density, the higher the osmotic pressure (depending on the chemical used) and the higher the amount of soil particles and debris recovered. Always check the specific density with a densimeter before use at the appropriate temperature (heating will change density).

Regarding choice of the extraction fluid, sugar is cheap, but very sticky and cannot be reused. Its osmotic value is so high that some nematodes (e.g. dorylaimids) might get destroyed. $ZnSO_4$ has lower osmotic value than sugar or $MgSO_4$, but it is acid and toxic. $MgSO_4 \times 7\ H_2O$ (magnesium sulfate heptahydrate) is a common fertilizer and therefore cheap. It is most commonly used for this type of extraction. If solutions are reused, it must be guaranteed that the solution is free of nematodes to avoid cross contamination of other samples. Colloidal silica such as Ludox, Percoll and Ficoll have almost no osmotic effect but are expensive, nevertheless, they are especially suitable for dorylaimids.

Fig. 11 Equipment used for centrifugal flotation; (1) centrifuge, (2) balance (scale), (3) centrifuge tubes, (4) container with $MgSO_4$ solution, (5) Vibro mixer (JKI, Münster, Germany).

Table 5 Required quantity of a particular substance in gram per litre of water, to obtain solutions with the indicated specific density (Southey, 1986)

Specific density (20°C)	1.15	1.18	1.22
Sugar	401	484	588
$MgSO_4$ (pure)	166	200	245
$MgSO_4 \times 7\ H_2O$	339	409	503
ZnSO4 (pure)	156	187	229
$ZnSO_4 \times 7\ H_2O$	279	335	410

Automated zonal centrifugation. A variation of centrifugal flotation is zonal centrifugation (Fig. 12) (Hendrickx, 1995). Nematodes are extracted from a matrix (soil or mixed plant tissues) in one step, based on centrifuging in a large bowl. In this process, the sample (soil or mixed plant tissue suspended in water) is gradually added to two layers with different densities formed inside a spinning centrifuge (17 000 g). The nematodes are separated from the matrix and moved to a layer with density between 1 (water) and that of the separating fluid e.g. 1.15–1.18 ($MgSO_4$). When the spinning is finished, the sediment is sealed off with a layer of kaolin and the layer with the nematodes is removed through the central hole in the zonal centrifuge and collected in a beaker in a watery suspension. The process can be automated by adding carrousels to the zonal centrifuge for delivery of the beakers with samples and nematode suspensions (Fig. 12). The machine handles samples up to 200 mL of soil, suspended in 1 L water.

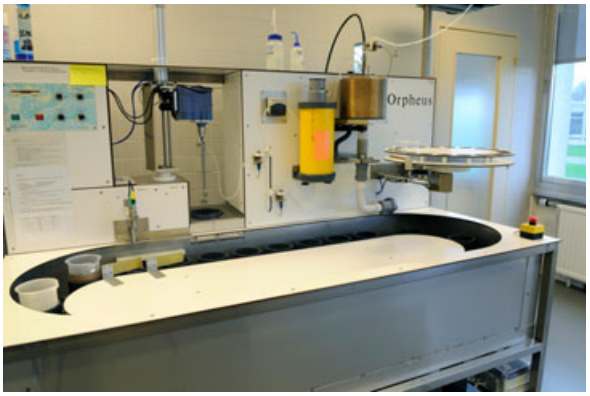

Fig 12 Automated zonal centrifugation. The lower carousel feeds the samples to the centrifuge (1 L beaker) and the upper carousel receives the nematode suspensions (150-mL beaker). The bowl of the zonal centrifuge is kept inside a cupper cage for safety reasons. The sample, kaolin and $MgSO_4$ are fed to the centrifuge bowl through tubes (ILVO, Merelbeke, Belgium).

4. Extraction of cysts from soil

Cysts differ from other nematode stages in size, shape and weight. Therefore, special methods have been developed for extracting cysts from dry (Baunacke method, paper strip method, Fenwick can, Schuiling centrifuge) and wet or dry soil (Seinhorst elutriator, centrifugal flotation, Wye washer). Efficacy of cyst extraction generally decreases with increasing organic content. None of those methods provide satisfactory separation of cysts from organic debris and soils with high content of peat. In those cases, cysts need to be handpicked.

Cyst extraction from dried soil is based on the fact that those cysts contain air bubbles and therefore float on water. The soil must be completely dry. Therefore, keep the sample in a porous (paper) bag at room temperature or in a drying chamber at about 35°C. In case live specimens are required, note that while *Globodera* can resist drying, vitality of the cyst content of *Heterodera* spp. rapidly decreases during drying, especially when dried at temperature higher than 25°C and air humidity lower than 40%. Gentle air circulation accelerates the drying process. Depending on the conditions, the soil will dry within 2–4 weeks. Unfortunately, young, full cysts do not float very well and can be lost; hence the total population can be underestimated.

Methods for wet soil rely on upward water current that keeps the cysts afloat in the suspension or flotation of cysts in a solution with a higher density then their own. These methods extract young full cysts as well as half depleted or empty cysts.

Following extraction, the remainder of the sample left on the sieve (the 'float') often contains high amounts of organic matter. Cysts need to be hand-picked using a pair of forceps, paint brush or a more robust hair (e.g. pig hair in a holder). A great part of the organic matter can also be removed with the help of 96% ethanol (Seinhorst, 1975) or acetone (den Ouden, 1954; Oostenbrink, 1960). Prior to this separation, the float needs to be dried. The float is then placed in a conical flask and the solvent is added and thoroughly mixed with the float (Turner, 1998). Cysts will float to the top while organic matter will sink to the bottom. The cysts are then carefully decanted into a filter paper-lined funnel. Alternatively, centrifugal flotation using a solute with a specific density of 1.22–1.25 can also be used to clean cysts from organic debris.

For comparison of methods and extraction efficacy see D'Errico & Brzeski (1975), Müller (1980), Clayden *et al.* (1985), Winfield *et al.* (1987), Riggs *et al.* (1997), Tenente *et al.* (2007) and Bellvert *et al.* (2008a,b).

EPPO recommends the following methods for cyst extraction in their respective Diagnostic Protocols (Table 6).

4.1 Baunacke method and/or paper strip method

Baunacke (1922) introduced the principle of drying soil for the collection of *Globodera* and *Heterodera* cysts from field soil. The method makes use of the fact that dried cysts float in water. They can then be decanted and collected on sieves. The method was improved by Buhr (1954) using a paper strip to collect the cysts.

Materials

- 200 or 250 μm aperture sieve for collecting the cysts;
- Plastic beaker or bowl;
- Stirring rod;
- Paper strip (e.g. made from filter paper) in case of paper strip method;
- Detergent;
- Pair of forceps or fine painting brush;
- Dissecting microscope.

Table 6 Methods recommended for the extraction of nematode cysts from dry and wet soil as listed in the corresponding EPPO Diagnostic Protocols

	Dried soil only			Wet or dried soil		
	Baunacke method, paper strip method	Fenwick can	Schuiling centrifuge	Seinhorst elutriator	Centrifugal flotation	Wye washer
Globodera rostochiensis/*G. pallida* (PM 7/40)	X	X	X	X	X	X
Heterodera glycines (PM 7/89)	X	X	X	X	X	

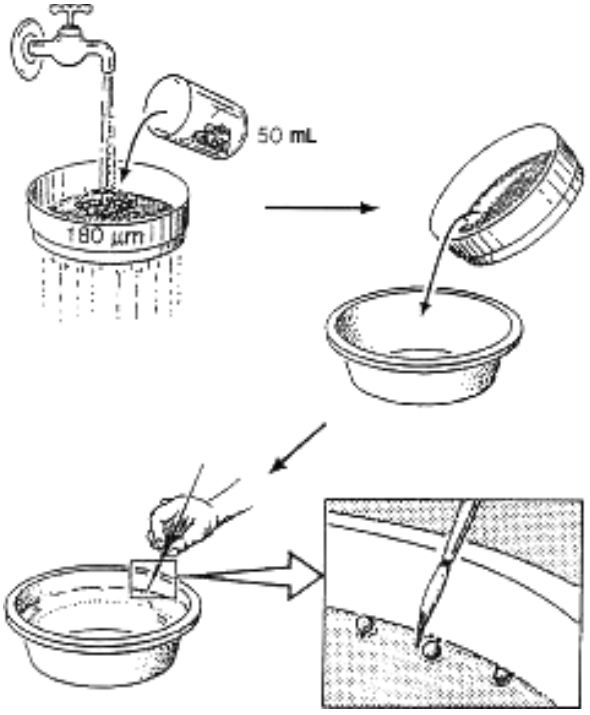

Fig. 13 Baunacke method (courtesy Van Bezooijen, 2006) performed with a 180 µm sieve (for *Globodera* spp. and *Heterodera glycines* a 200 or 250 µm sieve is recommended).

Procedure
- Pass the dried soil with a jet of water through a 200 or 250 µm sieve to eliminate soil and small organic particles (Fig. 13);
- Transfer the debris remaining on the 200 or 250 µm sieve into a plastic beaker or bowl;
- Stir the suspension thoroughly;
- Let the suspension settle for 30 s to some min; depending on the soil type, the water is cleared and the liquid will only contain the floating organic debris and cysts;
- Add a drop of detergent that causes the cysts to move to the edge;
- Pick the cysts by hand under a dissecting microscope using forceps or a fine painting brush.

Advantages
- Simple, quick and cheap;
- Little use of water.

Disadvantages
- Sample size is limited to about 100 mL soil;
- Results depend highly on individual person.

Remarks
Alternatively, use a paper strip around the beaker and raise the water level so cysts can adhere to it (Buhr, 1954). Carefully remove the paper strip with the attached cysts from the beaker. Sieves with smaller aperture up to 100 µm should be used for catching smaller cysts (e.g. *Heterodera carotae*).

4.2 Fenwick can

A widely used apparatus for extracting cysts that was originally described by Fenwick (1940). The method is suitable for dried soil samples up to 250 mL. Extraction is based on the floating properties of dried cysts (containing air) and the difference in size between other fractions of the sample. Most of the cysts in the soil sample will be collected this way. The soil at the base of the Fenwick can is elutriated by means of water flowing rapidly through a long glass or metal tube which is inserted deep into the can. The water flow stirs the sediment and releases any trapped cysts. The cysts move upward, into the collar and end up on the 250 µm aperture sieve. An automated version (carousel) is available.

Materials
- Fenwick can (Fig. 14);
- 1 mm aperture top sieve;
- 200 or 250 µm aperture sieve (d = 20 cm) for collecting the cysts;
- 840 µm aperture sieve (facultative);
- Filter paper;
- Dissecting microscope.

Procedure
- Clean the can with water, close the outlet at the bottom and fill the can to the rim with water;
- Place a 200 or 250 µm aperture sieve under the outlet of the overflow collar;
- Wash the air-dried soil sample through the top sieve (1 mm) into the can with a strong jet of water;
- Leave the water running for approx. 5 min. until water overflow is clean;
- Heavy soil particles will sink to the bottom of the can, cysts and light root debris will overflow;
- Collect overflow including cysts on the 200 or 250 µm sieve beneath the outlet of the collar;
- Rinse the funnel and collar very well to gather all cysts on the 200 or 250 µm sieve;
- Remove stopper at bottom of can to remove the remainder of the sample, rinse the can and the 1 mm sieve before the next sample is processed;
- Transfer the material collected on the 200 or 250 µm sieve on a filter paper;
- Count cysts and process them for identification using a dissecting microscope.

Advantages
- One person can process a large quantity of samples;
- Easy to construct.

Disadvantages
- Soil samples must be dried beforehand;
- Large amount of water.

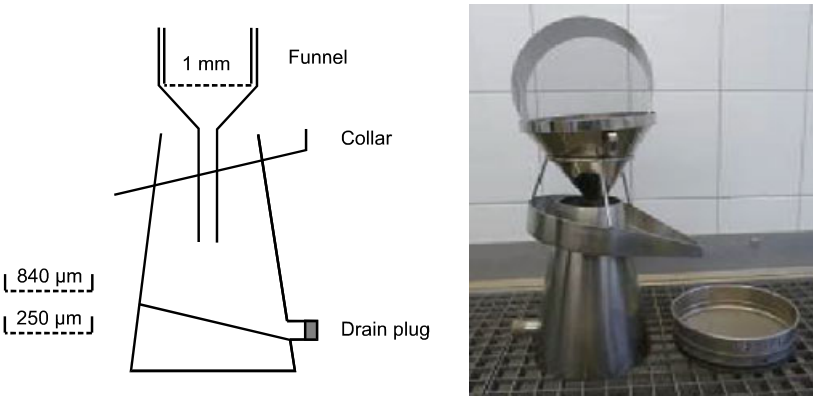

Fig. 14 Left: Vertical-section diagram of Fenwick can. Right: Fenwick can made of stainless steel with 1 mm aperture top sieve displayed in upright position for better visualization (Photo: JKI, Germany).

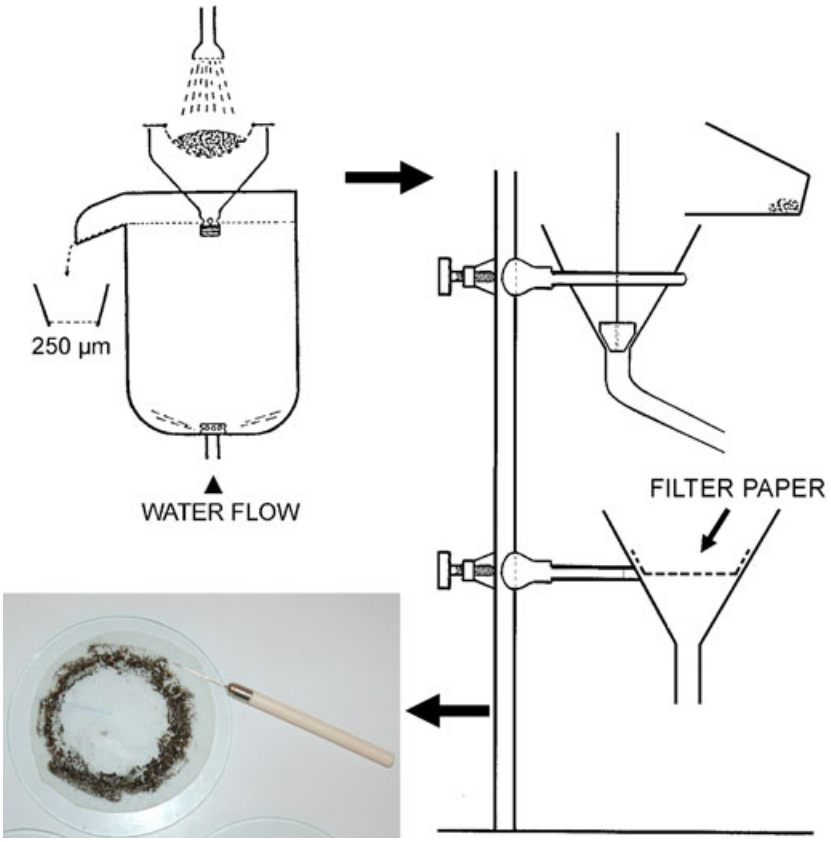

Fig. 15 Semi-automated cyst extraction and a method for separating the cysts from debris at the Agricultural Institute of Slovenia (Photo: AIS, Slovenia).

Remarks

An extra sieve of 840 µm can be added on top of the 200 or 250 µm sieve to remove large debris that moved with the float (e.g. straw, plant roots). This sieve will not retain cysts. Sieves with smaller aperture up to 100 µm should be used for catching smaller cysts (e.g. *Heterodera carotae*). If the cyst suspension is still dirty, dry the suspension at room temperature in case viable eggs and juveniles are needed or identification is based on proteins or otherwise on a heating plate and then separate cysts from organic debris by means of ethanol, acetone or centrifugal flotation. Some automated versions of this apparatus exist (e.g. carousel, see below).

Semi-automated modification by the Agricultural Institute of Slovenia (AIS). This modification of the Fenwick can technique manages the duration of the extraction time and water flow electronically (Fig. 15) (Urek & Širca, 2003). A dry soil sample of 200 mL is washed through a 1 mm sieve into the flotation tank via a plastic funnel using a nozzle. The pressure of the water stream used for extraction is regulated by the quantity of water and the duration of extraction. Cysts plus organic debris and larger soil particles are collected on a 250 µm sieve. The material from the 250 µm sieve is rinsed into a funnel of 18 cm diameter with a flow end stopper. While cysts float, the large soil particles settle at the bottom of the funnel and are removed

Fig. 16 Automated carousel at the Plant Protection Service in Hannover, Germany (Photo: Plant Protection Service, Chamber of Agriculture, Lower Saxony, Germany).

by opening the rubber stopper. The floating material containing the cysts is collected in a beaker and poured into the funnel of 13 cm diameter. Beneath the bent funnel a smaller funnel (10 cm diameter) is placed with a circular filter paper. The floating portion of the debris containing the cysts is rinsed to the filter paper surface and a drop of detergent is added to the centre that causes the float to move to the edge. The water is leaked out of the funnel slowly. The cysts remain in the floating debris at the edge of the filter paper. In proficiency tests, over three consecutive years, the average detection rate of *Globodera* cysts in soil was >95% (S. Širca pers. comm.).

Automated carousel. The fully automated carousel consists of 16 Fenwick can-based extraction units allowing processing of 100–120 samples h^{-1} (Fig. 16). It is manufactured by MEKU (www.meku-pollaehne.de). Dried soil samples are filled into the extraction unit by hand and the automated process starts. Samples are homogenized two times by high pressure water jets and washed through a 1 mm sieve to separate coarse particles. After the sieve is removed automatically, the sample is thoroughly mixed and then allowed to settle. Finally, floating cysts are moved with the help of a fine air stream over the collar onto a 200 µm sieve. Cysts are then transferred manually from the sieve onto filter paper. The extraction unit and the sieves will be automatically cleaned after each cycle. The method was validated by the Plant Protection Service Hannover, Germany (H. Warnecke pers. comm.).

4.3 Schuiling centrifuge

This semi-automatic flotation method for extracting cysts from dried soil samples was developed by J. Schuiling of the Netherlands Inspection Service for Field Seeds and Seed Potatoes (NAK) and is marketed as 'Schuiling Centrifuge' (Hietbrink & Ritter, 1982). It combines flotation with sieving to extract cysts from soil.

Materials

- Schuiling centrifuge (Fig. 17);
- 200 or 250 µm aperture sieve;
- Dissecting microscope.

Procedure

- Fill up to 500 mL air-dried soil into the transparent cylindrical container of the apparatus half-filled with water;
- Within the apparatus, the content is swirled with a rotating two-pronged fork at 450–500 rpm, creating a vortex and causing cysts and similar sized floating particles to be forced to the centre through a wire-mesh cylinder of 1.5 mm aperture. The mesh cylinder is fixed above a tube of the same diameter leading through an outlet to a collecting sieve of 200 or 250 µm;
- While swirling, more water is added around the inside of the main container washing off any adhering debris and

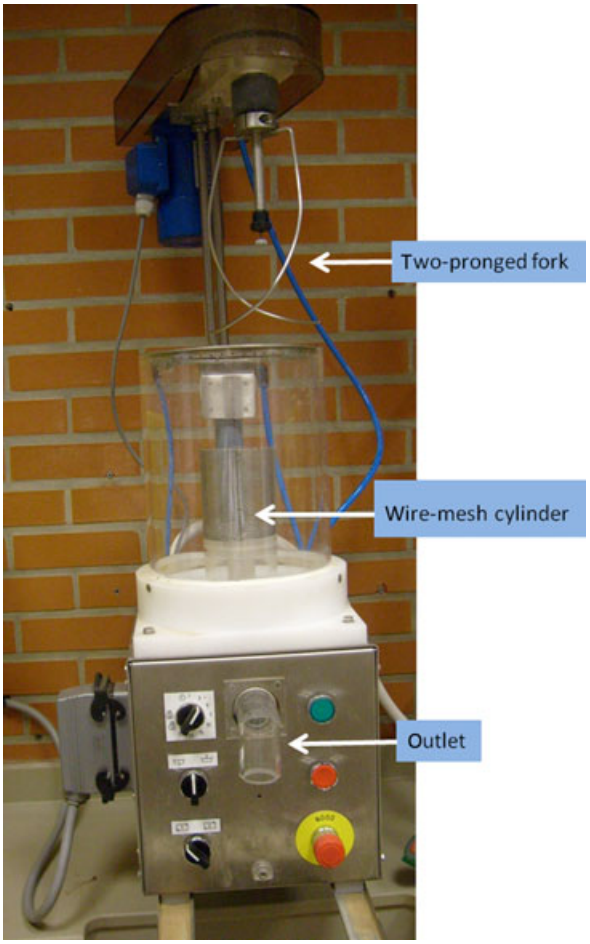

Fig. 17 Schuiling centrifuge for the extraction of cysts from dried soil samples (Photo: Centre pour l'agronomie et l'agro – Industrie de la Province de Hainaut, CARAH, Belgium).

cysts which pass with the others through the outlet to the collecting sieve;
- The apparatus cleans itself after each sample processing;
- Count cysts and process them for identification using a dissecting microscope.

Advantages
- High efficacy;
- Automatic process, individual failure low;
- High throughput of samples; 30 samples h^{-1} per person and unit; one person can operate three units at a time;
- Reduced water requirement compared with Fenwick can or Seinhorst elutriator (approx. 6 L $sample^{-1}$).

Disadvantages
- Expensive;
- Might be less suitable for peat or other organic soils (Clayden *et al.*, 1985);
- Samples containing stones and dried clumps of clay may disturb the operation.

Remarks
Further separation of cysts is possible by a special cleaning process involving the so-called Schuiling can and special sieves. In some laboratories, the Schuiling units have been modified to suit different soils and conditions: the modifications include additional spinning and cleaning time, larger collecting sieves, or an improved plastic cleaning 'can' for reducing the amount of debris. Cysts are collected on a 200 or 250 μm aperture sieve for further processing.

4.4 Seinhorst elutriator

This method is based on the difference in density of cysts in comparison to soil particles. The method is suitable for wet and dry soils. Following the principle of Cobb's flotation technique (Cobb, 1924) the technique was further developed by Kort (1960), Oostenbrink (1960) and Seinhorst (1964). The latter is presented here. At the base of a conical column, water enters through a perforated tube at a constant rate. Soil is added into the column using a funnel. Cysts float and are washed through the overflow to be collected on a 250 μm sieve. A second 'harvest' of cysts that did not reach the collar is collected through a side outlet halfway the column. An automated version of this technique is available.

Materials
- Seinhorst elutriator (Fig. 18);
- 2 mm aperture sieve;
- 200 or 250 μm aperture sieve (d = 20 cm) or bucket with bottom consisting of a 200 or 250 μm aperture stainless steel sieve or gauze;
- Dissecting microscope.

Procedure
- Place the 200 or 250 μm sieve under both the overflow collar and side-outlet;
- Fill the elutriator with water using an undercurrent water stream of 3500 mL min^{-1};
- Pass the sample through a 2 mm sieve into the funnel;
- Wash sample into funnel by moving the sieve up and down;
- Wait for 2–5 min, depending on soil type, until water overflow is clean;
- Close upward water current;
- Rinse outlet collar with water to ensure all cysts are washed onto the 200 or 250 μm sieve;
- Open side-outlet to pass suspension containing heavier cysts through the 200 or 250 μm sieve;
- Collect cysts from the sieve for direct investigation or further cleaning;
- Open bottom outlet and clean elutriator with water;
- Count cysts and process them for identification using a dissecting microscope.

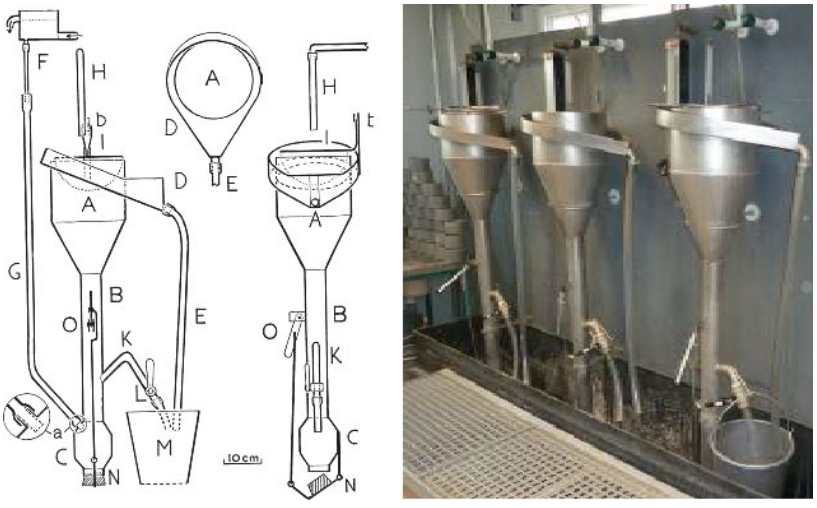

Fig. 18 Left: Diagram of Seinhorst elutriator for the separation of *Heterodera* and *Globodera* cysts from moist soil. Left and right: as seen from the side. Center: as seen from above. In circle: inlet enlarged to show placement of sieve for breaking the water current (Seinhorst, 1964, courtesy Nematologica). Right: Scaled-up Seinhorst elutriator for up to 2.5 kg at Wageningen University.

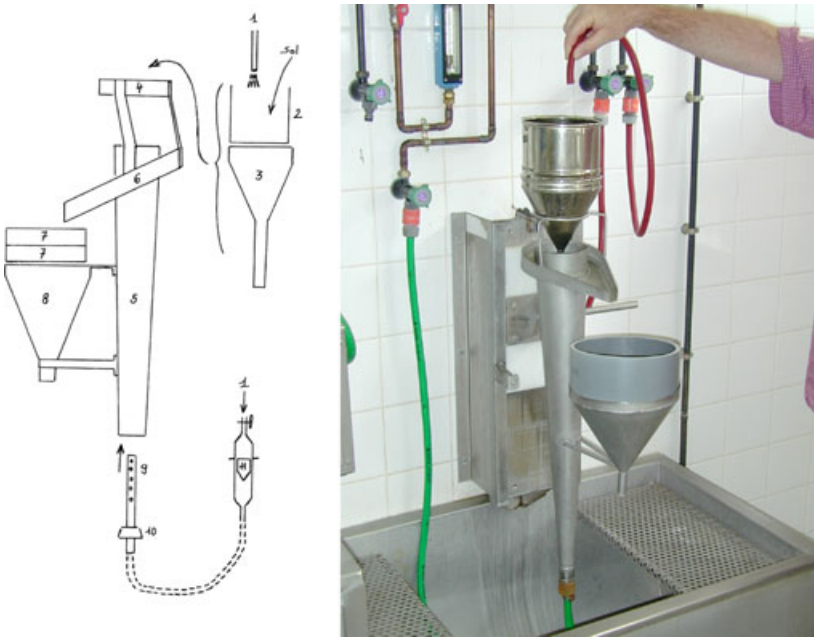

Fig. 19 Left: Drawing of Kort's cyst extraction elutriator. Right: Kort's cyst extraction elutriator in operation at Anses-LSV (Photo: Anses-LSV, France).

Fig. 20 Automated soil sample extractor for cysts (Photo: JKI, Germany).

Advantages

- Large samples up to 1000 mL soil can be handled in a standardized way;
- No need to dry sample.

Disadvantages

- Expensive equipment;
- Large amount of water;
- Large amounts of debris can accumulate along with the cysts; additional cleaning required.

Remarks

Minimum efficacy of this technique is 98% (Seinhorst, 1964). Been *et al.* (2007) developed a scaled-up version of the Seinhorst elutriator allowing up to 2000 mL (2500 g) of soil to be processed at 99% efficacy. Use a 100 µm sieve for small cysts (e.g. *Heterodera carotae, H. urtica*). If cysts on sieve contain lots of debris, it is useful to dry the debris and then recover the floating cysts after transfer into water or isolate the cysts from the debris using ethanol (Seinhorst, 1975) or acetone (den Ouden, 1954). Also centrifugal flotation can be used to separate cysts from the debris (see 4.5).

Similar elutriators. The Kort's cyst extraction elutriator (Kort, 1960; Fig. 19) as well as the Oostenbrink flotation apparatus II (Oostenbrink, 1954, 1960) use the same principle as described above for the Seinhorst elutriator. Those elutriators differ in shape of the funnel, water speed and handling. Unfortunately, no data seems to be available comparing those three techniques.

Automated soil sample extractor. The automated soil sample extractor made by MEKU (www.meku-pollaehne.de) is based on the Seinhorst elutriation principle (Fig. 20). A high pressure water jet homogenizes the soil sample.

Extraction time, resting period and pressure can be adjusted depending on soil type. Due to its automation, operator failures are negligible. The clear acryl glass allows continuous monitoring of the extraction process.

4.5 Centrifugal flotation

Similar to vermiform nematodes (see 3.5), cysts can be separated from wet and dry soil by bringing them in a suspension with a specific density greater than their own (cyst density is about 1–1.28), so that they will float and heavier material will sink. Centrifugation is used to speed up the separation of the sinking and floating fractions. The size of the sample that can be processed is limited by the size of the centrifuge tubes.

Material
- Centrifuge plus centrifuge tubes (size ranging from 100 to 1000 mL);
- Kaolin;
- Stirrer or Vibro mixer;
- $MgSO_4$ solution with a density of 1.22–1.28 (or similar extraction fluid, see Table 5);
- 200 or 250 µm aperture sieve;
- Glass beaker to collect cysts;
- Dissecting microscope.

Procedure
- Fill 1000 mL centrifuge tube with up to 250 mL soil;
- Add about 400 mL water plus a spoon of kaolin; kaolin forms a visible white layer that separates the sediment and cysts from the supernatant (water and light organic material);
- Mix suspension thoroughly with stirrer or Vibro mixer to form a homogenous suspension;
- Centrifuge tubes for approx. 4 min at 1800 g; time and g-force is not that critical, as long as a stable pellet is achieved; time lengths of 2–5 min and g-forces of 700–2900 g can be used;
- Gently pour off the supernatant and discard;
- Re-suspend the pellet in about 400 mL of a $MgSO_4$ solution (or similar extraction fluid) with a density of 1.22–1.28;
- Centrifuge tubes again at 1800 g for 4 min;
- Gently pour the supernatant containing the cysts over a 200 or 250 µm sieve;
- Rinse the sieve immediately with water to remove the $MgSO_4$ solution;
- Collect cysts from the sieve in a glass beaker;
- Count cysts and process them for identification using a dissecting microscope.

Advantages
- Relatively clean cyst suspension;
- Small amount of water;
- No need to dry sample;
- Cysts are available within 15 min.

Disadvantages
- Re-suspending soil pellet can be laborious for loamy soils;
- Expensive equipment.

Remarks
Extraction efficacy, especially for heavy soils, can be improved by adding elutriation prior to centrifugation which will free cysts from soil particles. Alternatively, the suspension can be thoroughly homogenized using with a household mixer with dough hooks for approx. 1 min or longer, until a homogenous suspension is formed. If only smaller centrifuge tubes are available, reduce sample size accordingly. For adjusting the salt solutions to a specific density and the strength and weaknesses of different salt solutions, see remarks made in chapter 3.5. It is recommended to routinely check the supernatant after the first centrifugation step for cysts before being discarded. Sometimes few small or empty cysts can be found in this fraction.

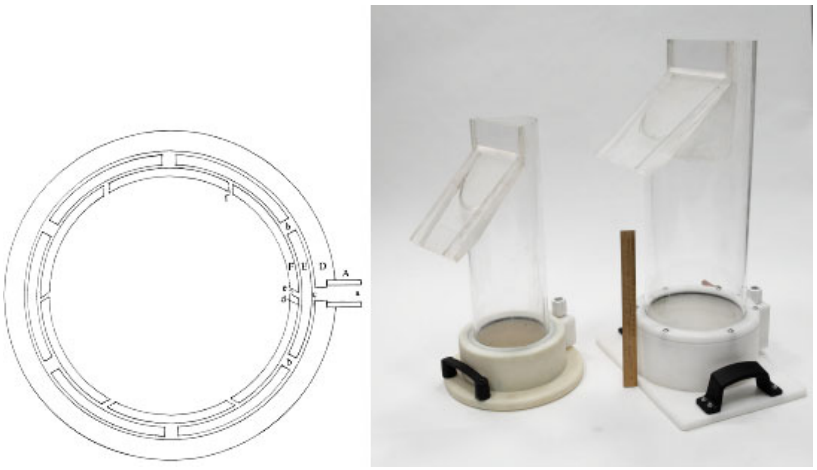

Fig. 21 Left: Cross section of the base of the Wye washer; A = 2.25 cm hose connector, D = PVC base, E = PVC sleeve, F = acrylic tube, a = water supply inlet, b = 9.5 mm diameter hole, c = outer water gallery, d = inner water gallery, e = 3.2 mm diameter hole, f = 2.4 mm diameter hole (Winfield *et al.* 1987, courtesy Annals of Applied Biology). Right: Standard Wye washer for samples up to 1000 mL and 'Giant' Wye washer for samples up to 2000 mL (Photo: fera, Sand Hutton, Great Britain).

4.6 Wye washer

The Wye washer allows extraction of cysts from wet and dry soil. The apparatus is constructed of a 50 cm long, 15 cm diameter wide clear acrylic tube which, at its lower end, is held inside two tight-fitting concentric PVC sleeves (Winfield *et al.*, 1987) (Fig. 19). Water enters through an inlet pipe on the outer sleeve and is caused to swirl by means of an arrangement of grooves and angled holes on the inner sleeve and the acrylic tube. At the top of the tube is a spout which directs overflow onto sieves of similar size to those used with the Fenwick can (i.e. aperture of 840 and 250 μm).

Materials
- Wye washer apparatus (Fig. 21);
- 840 μm aperture sieve;
- 200 or 250 μm aperture sieve;
- 100 mL glass beaker.

Procedure
- Add a soil sample up to 1000 mL to a small quantity of water in the Wye washer;
- Add more water as rapidly as possible, to break up the soil until the rim is reached;
- Briefly stop the flow and then increase water flow gradually to about 10 L min^{-1} for 10 min; the overflow carries: (1) small soil particles, which will pass through both sieves; (2) large organic debris which will be retained by the upper 840 μm sieve; (3) cysts and similar sized organic particles, collected on the 200 or 250 μm sieve;
- Collect cysts from the sieve in a glass beaker;
- Count cysts and process them for identification using a dissecting microscope.

Advantages
- Samples up to 1000 mL can be processed;
- Use for wet and dry soil possible;
- Higher consistency compared with Fenwick can;
- Less operation time than for Fenwick can.

Disadvantages
- High water use, e.g. 10 min extraction at 10 L min^{-1} = 100 L $sample^{-1}$
- High costs, tailor built.

Remarks
A 'giant' version of the Wye washer can process samples up to 2 kg soil (Fig. 21).Validation studies at The Food and Environment Research Agency (Fera), Sand Hutton, Great Britain, have shown that the extraction rate of nematode cysts of the 'standard' Wye Washer was equally reliable to the Fenwick can for sand and sandy-loam soil types and more efficient for extracting cysts from clay-loam soils for sample sizes below 1 kg (S. Hockland, pers. comm.). Cyst recovery was at least 95% for the standard Wye washer and slightly lower for the 'giant' Wye washer. Operator failure was negligible. The Wye washer does not process peat samples.

Further information

Further information on this Standard can be obtained from:

Mr Hallmann Julius Kühn-Institut, Germany johannes.hallmann@jki.bund.de.

Ms Viaene ILVO, Belgium nicole.viaene@ilvo.vlaanderen.be.

Feedback on this Diagnostic Protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

Protocol revision

An annual review process is in place to identify the need for revision of diagnostic standards. Standards identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

Acknowledgements

This Standard was originally drafted by: Mr Hallmann Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institut for Epidemiology and Pathogen Diagnostics, Toppheideweg 88, 48161 Münster Germany johannes.hallmann@jki.bund.de. Ms Viaene ILVO, Plant-Crop Protection, Nematology Burg. Van Gansberghelaan 96 9820 Merelbeke Belgium nicole.viaene@ilvo.vlaanderen.be.

References

Araya M & Caswell-Chen EP (1993) Enzymatic digestion of roots for the recovery of root-knot nematode developmental stages. *Journal of Nematology* **25**, 590–595.

Ayala A, Roman J & Tarjan AC (1963) Comparison of four methods for isolating nematodes from soil samples. *Journal of Agriculture of the University of Puerto Rico* **47**, 219–225.

Ayoub SM (1980) *Plant Nematology. An Agricultural Training Aid*. NemaAid Publications, Sacramento, CA (US).

Baermann G (1917) Ein einfache Methode zur Auffindung von Anklyostomum (Nematoden) Larven in Erdproben. *Geneesk. Tijdschr. Nederlandsch-Indië* **57**, 131–137.

Baunacke W (1922) Untersuchungen zur Biologie und Bekämpfung der Rübennematoden, *Heterodera schachtii* Schmidt. *Arbeiten aus der Biologischen Reichsanstalt Berlin* **11**, 185–288.

Been TH, van Bekkum PJ, van Beers TG & Beniers A (2007) A scaled-up Seinhorst elutriator for extraction of cyst nematodes from soil. *Nematology* **9**, 431–435.

Bellvert J, Crombie K & Horgan FG (2008a) Comparative efficiency of the Fenwick can and schuiling centrifuge in extracting nematode cysts from different soil types. *Journal of Nematology* **40**, 30–34.

Bellvert J, Crombie K & Horgan FG (2008b) Effect of sample size on cyst recovery by flotation methods: recommendations for sample processing during EU monitoring of potato cyst nematodes (*Globodera* spp.). *EPPO Bulletin* **38**, 205–210.

Böhmer B & Weil B (1978) Comparison of methods for the extraction of leaf and bud nematodes (*Aphelenchoides* spp.) from strawberry plant crowns. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* **30**, 85–88.

Buhr H (1954) Untersuchungen über den Kartoffelnematoden – I. Die "Papierstreifen-Methode", ein vereinfachtes Verfahren zur Untersuchung von Bodenproben auf ihren Besatz mit Nematodenzysten. *Nachrichtenblatt für den Deutschen Pflanzenschutzdienst* **8**, 45–48.

Byrd DW Jr, Barker KR, Ferris H, Nusbaum CJ, Griffin WE, Small RH *et al.* (1976) Two semi-automatic elutriators for extracting nematodes and certain fungi from soil. *Journal of Nematology* **8**, 206–212.

Caveness FE & Jensen HJ (1955) Modification of the centrifugal-flotation technique for the isolation and concentration of nematodes and their eggs from soil and plant tissue. *Proceedings of the Helminthological Society of Washington* **22**, 87–89.

Chawla ML & Prasad SK (1975) Techniques in nematology. II. Comparative efficiency of sampling tools and nematode extraction methods. *Indian Journal of Nematology* **4**, 115–123.

Clayden IJ, Turner SJ & Marks RJ (1985) Comparison of the Fenwick can and Schuiling centrifuge methods for the extraction of potato cyst nematodes from soil. *EPPO Bulletin* **15**, 285–287.

Cobb NA (1918) Estimating the nema populations of soil. USDA Technical Circular 1, p. 48.

Cobb NA (1924) Removing nemas from soil by flotation. *Journal of Parasitology* **11**, 103.

Coolen WA, Hendrickx G & d'Herde CJ (1971) Method for the quantitative extraction of nematodes from plant tissue and its application in the testing of rose rootstocks for resistance to endoparasitic root nematodes. Rijksstation voor Nematologie en Entomologie, Publicatie No. W6, Wetteren, Belgium, p. 34.

Coolen WA & D'Herde CJ (1972) *A Method for the Quantitative Extraction of Nematodes from Plant Tissue*. State Agriculture Research Centre, Ghent, Belgium, p. 77.

Coolen WA (1979) Methods for the extraction of *Meloidogyne* spp. and other nematodes form roots and soil. In *Root-knot Nematodes (Meloidogyne species) Systematics, Biology and Control* (eds Lamberti F & Taylor CE), pp. 317–329. Academic Press, London & New York.

D'Errico FP & Brzeski MW (1975) Comparison of four methods for extraction of *Heterodera* cysts from mineral soils. *Roczniki Gleboznawcze* **26**, 165–171.

den Ouden H (1954) Het bietencystenaaltje en zijn bestrijding. I. Methoden te gebruiken bij het onderzoek naar kunstmatige en natuurlijke lokstoffen. *Mededelingen van het Instituut voor Rationele Suikerproduktie* **24**, 101–120.

den Nijs L & van den Berg W (2012) The added value of proficiency tests: choosing the proper method for extracting *Meloidogyne* second-stage juveniles from soil. *Nematology* **15**, 143–151.

Elmiligy IA (1971) Recovery and survival of some plant-parasitic nematodes as influenced by temperature, storage time and extraction technique. *Mededelingen van de Fakulteit Landbouwwetenschappen Gent* **36**, 1333–1339.

Fenwick DW (1940) Methods for the recovery and counting of cysts of *Heterodera schachtii* from soil. *Journal of Helminthology* **18**, 155–172.

Flegg JJM (1967) Extraction of *Xiphinema* and *Longidorus* species from soil by a modification of Cobb's decanting and sieving technique. *Annals of Applied Biology* **60**, 429–437.

Greco N & D'Abbaddo T (1990) Efficient procedure for extracting *Tylenchulus semipenetrans* from citrus roots. *Journal of Nematology* **22**, 590–593.

Harrison JM & Green CD (1976) Comparison of centrifugal and other methods for standardization of extraction of nematodes from soil. *Annals of Applied Biology* **82**, 299–308.

Hendrickx G (1995) An automatic apparatus for extracting free-living nematodes stages from soil. *Nematologica* **41**, 308 (Abstract).

Hietbrink H & Ritter CE (1982) Separating cysts from dried soil samples by a new centrifugation and a flotation method. In: *Proceedings of XVIth International Symposium, European Society of Nematologists*, pp. 28–29. St Andrews.

Hooper DJ (1986) Extraction of free-living stages from soil. In *Laboratory Methods for Work with Plant and Soil Nematodes* (ed. Southey JF), pp. 5–30. Her Majesty's Stationary Office, London (GB).

Hooper DJ, Hallmann J & Subbotin SA (2005) Methods for extraction, processing and detection of plant and soil nematodes. In: *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture*, 2nd edn (ed. Luc M, Sikora RA & Bridge J), pp. 53–86. CAB International, Wallingford (GB).

Hoshino S & Togashi K (1999) A simple method for determining *Aphelenchoides besseyi* infestation level of *Oryza sativa* seeds. *Journal of Nematology* **31**, 641–643.

Hussey RS (1971) A technique for obtaining quantities of living *Meloidogyne* females. *Journal of Nematology* **3**, 99–100.

Julio VA, Freitas LG, Coelho JLC, Muscardi DC & Ferraz S (2003) Extraction of *Meloidogyne javanica* females from tomato roots through maceration with fungal enzymes. *Nematologia Brasileira* **27**, 75–80.

Kaplan DT & Davis EL (1990) Improved nematode extraction from carrot disk culture. *Journal of Nematology* **22**, 399–406.

Kimpinski J & Welch HE (1971) Comparison of Baermann funnel and sugar flotation extraction from compacted and noncompacted soils. *Nematologica* **17**, 319–320.

Kort J (1960) A technique for the extraction of *Heterodera* cysts from wet soil and for the estimation of their egg and larval content. *Verslagen en Mededelingen, Plantenziektenkundige Dienst, Wageningen* Netherlands **233**, 6.

Ladeveze L & Anthoine G (2010) Outcome of a seven-year proficiency test for the detection and identification of potato cyst nematodes. *Aspects of Applied Biology* **103**, *3rd Symposium on Potato Cyst Nematodes*, pp. 97–105.

Malo S (1960) Comparative efficiencies of three methods for extracting nematodes from root and soil samples. *Plant Disease Reporter* **44**, 217–219.

Manzanilla-Lopez RH, Costilla MA, Doucet M, Franco J, Inserra RN, Lehman PS *et al.* (2002) The genus Nacobbus Thorne & Allen, 1944 (Nematoda: Pratylenchidae): systematics, distribution, biology and management. *Nematropica* **32**, 149–227.

McSorley R, Parrado JL & Dankers WH (1984) A quantitative comparison of some methods for the extraction of nematodes from roots. *Nematropica* **14**, 72–84.

McSorley R (1987) Extraction of nematodes and sampling methods. In *Principles and Practice of Nematode Control in Crops* (ed. Brown RH & Kerry BR), pp. 13–41. Academic Press, Sydney, NSW (AUS).

McSorley R & Parrado JL (1981) Effect of sieve size on nematode extraction efficiency. *Nematropica* **11**, 165–174.

Mountain WB & Patrick ZA (1959) The peach replant problem in Ontario. VII. The pathogenicity of *Pratylenchus penetrans* (Cobb, 1917) Filip. & Stek. 1941. *Canadian Journal of Botany* **37**, 459–470.

Müller J (1980) An improved method for the extraction of *Heterodera schachtii*. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* **32**, 21–24.

Müller J (1983) On the problem of estimating population densities of *Heterodera schachtii* by soil sampling and extraction. III Influence of

operator and extraction method. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* **35**, 168–172.

Nielsen CO (1947/48) An apparatus for quantitative extraction of nematodes and rotifers from soil and moss. *Natura Jutlandica* **1**, 271–278.

Oostenbrink M (1954) Een doelmatige methode voor het toetsen van aaltjesbestrijdingsmiddelen in grond met *Hoplolaimus uniformis* als proefdier. *Mededelingen Landbouwhogeschool Gent* **19**, 377–408.

Oostenbrink M (1960) Estimating nematode populations by some selected methods. In *Nematology* (ed. Sasser JN & Jenkins WR), pp. 85–102. The University of North Carolina Press, Chapel Hill, NC (US).

Penas AC, Dias LS & Mota MM (2002) Precision and selection of extraction methods of aphelenchid nematodes from maritime pine wood, *Pinus pinaster* L. *Journal of Nematology* **34**, 62–65.

Persmark L, Banck A, Andersson S & Jansson HB (1992) Evaluation of methods for extraction of nematodes and endoparasitic fungi from soil. *Nematologica* **38**, 520–530.

Riggs RD, Schmitt DP & Mauromoustakos A (1997) Comparison of extraction and shipping methods for cysts and juveniles of *Heterodera glycines*. *Journal of Nematology* **29**, 127–132.

Robinson AF & Heald CM (1989) Accelerated movement of nematodes from soil in Baermann funnels with temperature gradients. *Journal of Nematology* **21**, 370–378.

Rodríguez-Kábana R (1981) A simple incubation method for the extraction of nematodes from soil. *Nematropica* **11**, 175–185.

Seinhorst JW (1950) De betekenis van de toestand van de grond voor het optreden van aantasting door het stengelaaltje (*Ditylenchus dipsaci* (Kühn) Filipjev). *Tijdschrift over Plantenziekten* **56**, 289–348.

Seinhorst JW (1956) The quantitative extraction of nematodes from soil. *Nematologica* **1**, 249–267.

Seinhorst JW (1964) Methods for the extraction of *Heterodera* cysts from not previously dried soil samples. *Nematologica* **10**, 87–94.

Seinhorst JW (1975) Separation of *Heterodera* cysts from dry organic debris using ethanol. *Nematologica* **20**, 367–369.

Seinhorst JW (1988) *The Estimation of Densities of Nematode Populations in Soil and Plants*. Växtskyddsrapporter No 51, Uppsala (SE).

Sohlenius B (1976) A carbon budget for nematodes, rotifers and tardigrades in a Swedish coniferous forest soil. *Holartic Ecology* **2**, 30–40.

Southey JF (1986) *Laboratory Methods for Work with Plant and Soil Nematodes*. Her Majesty's Stationary Office, London (GB).

Stoller BB (1957) An improved test for nematodes in the soil. *Plant Disease Reporter* **41**, 531–532.

Tarjan AC (1960) A comparison of polyethylene plastic bags and glass jars as incubation chambers for obtaining nematodes from roots. *Plant Disease Reporter* **44**, 574–577.

Tarjan AC (1967) Influence of temperature and hydrogen peroxide on the extraction of burrowing nematodes from citrus roots. *Plant Disease Reporter* **51**, 1024–1028.

Tarjan AC (1972) Observations on extracting citrus nematode, *Tylenchulus semipenetrans*, from citrus roots. *Plant Disease Reporter* **56**, 186–188.

Tenente RCV, Gonzaga V & Vilas-Boas L (2007) Evaluation of different methods of cyst extraction of *Heterodera glycines* from soybean seed samples. *Nematologia Brasileira* **31**, 72–77.

Thies JA, Merrill SB & Corley EL (2002) Red food coloring stain: new, safer procedures for stinind nematodes in roots and egg masses on root surfaces. *Journal of Nematology* **34**, 179–181.

Tobar Jimenez A (1963) The behaviour of a soil population and some plant parasitic nematodes in the processes of extraction of five different methods. *Revista Iberica de Parasitologia* **23**, 285–314.

Turner SJ (1998) Sample preparation, soil extraction and laboratory facilities for the detection of potato cyst nematodes. In *Potato Cyst Nematodes: Biology, Distribution and Control* (ed. Marks RJ & Brodie BB), pp. 75–90. CAB International, Wallingford (GB).

Urek G & Širca S (2003) Efficiency of the system of nematode cyst extraction from soil samples used in Slovenia. *Acta Phytopathologica et Entomologica Hungarica* **38**, 209–215.

Van Bezooijen J (2006) Methods and techniques for Nematology. Script, 112 p. http://www.nem.wur.nl/NR/rdonlyres/CC0A519F-3ADD-4FFA-B473-959062BC9C7F/47004/MethodsandTechniquesforNematology.pdf [accessed on 1 Sept 2013].

Verschoor BC & De Goede RGM (2000) The nematode extraction efficiency of the Oostenbrink elutriator-cottonwool filter method with special reference to nematode body size and life strategy. *Nematology* **2**, 325–342.

Viaene N, Mahieu T & de la Pena E (2007) Distribution of *Meloidogyne chitwoodi* in potato tubers and comparison of extraction methods. *Nematology* **9**, 143–150.

Viglierchio DR & Schmitt RV (1983a) On the methodology of nematode extraction from field samples: Baermann funnel modifications. *Journal of Nematology* **15**, 438–444.

Viglierchio DR & Schmitt RV (1983b) On the methodology of nematode extraction from field samples: comparison of methods for soil extraction. *Journal of Nematology* **15**, 450–454.

Viglierchio DR & Yamashita TT (1983) On the methodology of nematode extraction from field samples: density flotation techniques. *Journal of Nematology* **15**, 444–449.

Whitehead AG & Hemming JR (1965) A comparison of some quantitative methods of extracting small vermiform nematodes from soil. *Annals of Applied Biology* **55**, 25–38.

Winfield AL, Enfield MA & Foremann JH (1987) A column elutriator for extracting cyst nematodes and other small invertebrates from soil samples. *Annals of Applied Biology* **111**, 223–231.

Yen JH, Lee MD, Chen DY, Lin CY & Tsay TT (1998) The comparison of three nematode-extraction methods on four selected nematodes. *Plant Protection Bulletin (Taipei)* **40**, 153–162.

Young TW (1954) An incubation method for collecting migratory endo-parasitic nematodes. *Plant Disease Reporter* **38**, 794–795.