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Report 138

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1. Introduction

The endoparasitic root-knot nematodes, genus *Meloidogyne*, constitute worldwide the economically most important group of plant parasitic nematodes. *Meloidogyne chitwoodi* and *M. fallax* are considered especially harmful, because they severely reduce the yield and quality of crops from which the underground plant parts are harvested; for example ware and seed potatoes, carrots and black salsify may become unmarketable because of deformation.
M. chitwoodi and *M. fallax* are difficult to control because they both have a large range of host crops, which makes management by crop rotation difficult. Resistant crops are not yet available. The current solution to deal with this problem, both curatively and precautionary, is the use of pesticides, for instance Aldicarb, Mocap, Vydate, Nemathorin, cis-dichloropropeen and metam-sodium. This strategy conflicts with the European policy to reduce drastically these substances, which endanger both the qualities of the environment and food safety.
Both *M. chitwoodi* and *M. fallax* were described in 1980 (Golden *et al.*) and 1996 (Karssen), respectively. They have already been reported from Portugal (Beek, 1977), France, Germany (Mueller *et al.*, 1996), Belgium (Waeyenberge & Moens, 1997) and the Netherlands (Karssen, 1995, 1996). Because of their harmfulness, their persistence and the risk of their spread by means of infected plant material, they pose a threat to the free movement of plant products, especially seed potatoes, in the European Community. They are subjects of a Commission Directive 1998/1/EC of 8th January 1998 aimed at preventing the spread and further introduction of *M. chitwoodi* and *M. fallax.* As *M. chitwoodi* and *M. fallax* are only recently described (12), the information about their life cycle and epidemiology is limited. As a consequence the recommendations in the Council Directive 2000/29/EC of 8 May 2000 for detection of *M. chitwoodi* and *M. fallax* are provisionally and do not have a sound scientific basis. Inspection services have produced evidence that the EU-recommended inspection methods, which are based on visible external symptoms of plant material (e.g. potato tubers), are unreliable (Van Riel, 1993).

In the Netherlands, the root-knot nematodes *M. chitwoodi* and *M. fallax* cause serious problems in arable farming and the production of field vegetables. Potatoes are known to be good hosts for both species. They can reduce the quantity, but their major effect is reduction of the quality of potato tubers, since they enter tubers and cause galling on the surface. As both eggs and females survive and propagate within the tubers, dispersion of both species with seed potatoes is the primary challenge which has to be met.

Therefore, it is imperative that user-friendly, rapid, reliable and cost-effective tools and methodologies for sampling and detection of these organisms are provided. Firstly, to combat the spread of these quarantine nematodes in the European Community, and thus to enable the execution of the Commission Directive, and secondly, as a basis for future management systems in regions where *M. chitwoodi* and *M. fallax* are established.

This document describes the research that was carried out to provide some of these tools with focus on:
1. Distribution patterns of *Meloidogyne* spp. to develop sampling strategies for detection.
2. Population dynamics in time, especially the natural decline of *Meloidogyne* spp. after harvest.
3. Methods to identify both species in harvested potatoes based on molecular techniques.
4. Providing sound scientific information for a EU-recommended inspection method.

2. Spatial distribution patterns of *Meloidogyne chitwoodi* and *M. fallax*

2.1 Introduction

Recently descriptive models for distribution patterns of *Globodera rostochiensis* and *G. pallida* have been developed. These models discriminate between different scales (small, medium and large-scale) in fields, attribute these patterns to vectors of spread and incorporate parameters with respect to population intensity. The medium-scale distribution patterns, caused by displacement of soil by agricultural machinery, were modelled by regression analysis and parameterised, whilst the small-scale distribution, connected with root growth in soil, was modelled by a discrete random distribution function (Schomaker & Been, 1999). The models were evaluated by uncertainty analysis and incorporated in custom developed simulation software to enable evaluation of existing and development of new sampling methods on demand (Been & Schomaker, 2000). As a result sampling methods with predefined characteristics e.g. the probability of detection or the variability of density estimation could be developed.
In order to develop sampling methods for the detection or population density estimation of *Meloidogyne chitwoodi* and *M. fallax*, knowledge is required concerning the spatial distribution patterns and their variability within an agricultural unit, *e.g.* field. In general three different spatial distribution patterns can be distinguished inside a farmer's field:

2.1.1 Small scale distribution

The small scale distribution describes the distribution pattern over small areas in the field. It is the result of growing the host plant in a grid pattern, defined by the distance in the row between individual plants and the distance between rows and the size of the root system.

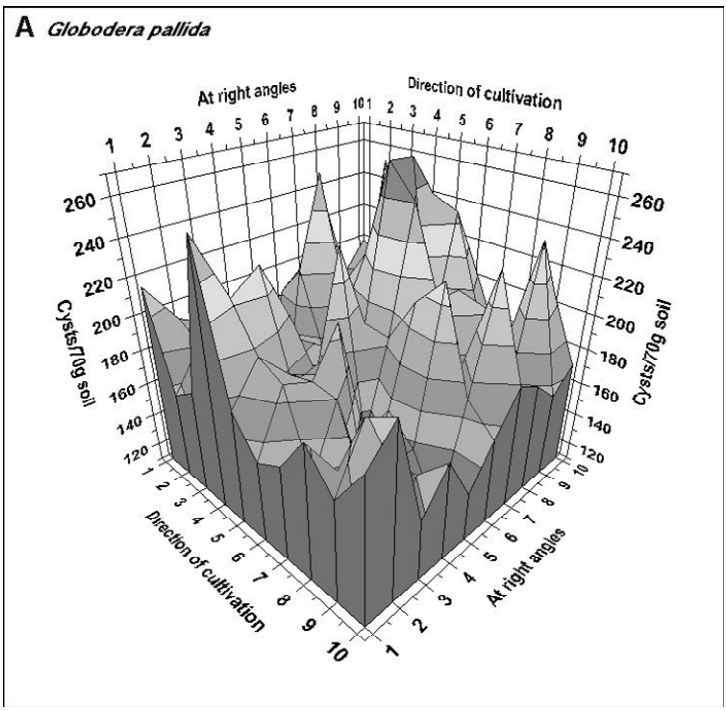

Figure 2.1. Visualization of the mapped small scalle distribution in 1 m² of: Globodera pallida representing the number of cysts per 70 g of dried soil.

In general the area defined by the small scale distribution is the largest area without a defined shape or gradient of population densities or the largest area with an acceptable small variance of the population density estimator. In Figs 2.1 the small scale distribution of *G. pallida* is shown in a 1 m^2 plot with population densities presented per dm^2. The aggregated distribution of cysts in even such a small area is apparent and applies to all nematode species. As a result, the estimation of population densities with a limited margin of error is difficult even in such a small area. In order to estimate these errors, one first needs to describe the small scale distribution of nematodes mathematically. This will enable the calculation of several interesting aspects for that area, e.g. the probability of finding 0, 1, 2 or more nematodes or cysts when taking one sample with an auger of a certain size. Similarly one can calculate how much soil is required to detect a single nematode or cyst with a certain probability, or how much soil is required to get a reliable estimation of the population density in that area. The aggregated distribution of nematodes, whether cyst nematodes or (semi) free living nematodes, is best described by the negative binomial distribution.
One of the problems in field experiments is the size of the plots to be sampled. In field trials of resistant cultivars, pesticides, or other nematode control measures, agronomists estimated crop yield at harvest by collecting the produce, e.g. tubers, from plots ranging from several square meters up to 100 m^2 or more per plot. If the same area is used for collecting the corresponding soil sample to estimate the nematode population density of the plot, this will result in erroneous correlations between nematode density and crop yield. Figure 2.2 shows the results of the scientific sampling method for *G. pallida*, employed on a row of square meter plots in the direction of cultivation. Log nematode densities are plotted and linear regression is applied to model the correlation. There is a clear trend of increasing population densities with increasing distance. This trend is even stronger at right angles to the direction of cultivation. When a soil sample is taken from an area covering up to 100 m2, an average over all encountered population densities in that area will be acquired. In fact, Figure 2.2 visualizes a part of the second distribution pattern encountered in the field - the medium scale distribution. For most nematodes, the area of the small scale distribution is confined to only a couple of m^2. For example, for potato cyst nematodes it was established that the optimum size for that distribution is 1 m^2 (1.33 m by 0.75 m, keeping in mind the spacing of the rows and the between row distance) and that an upper limit of 4 m^2 is acceptable if necessary.

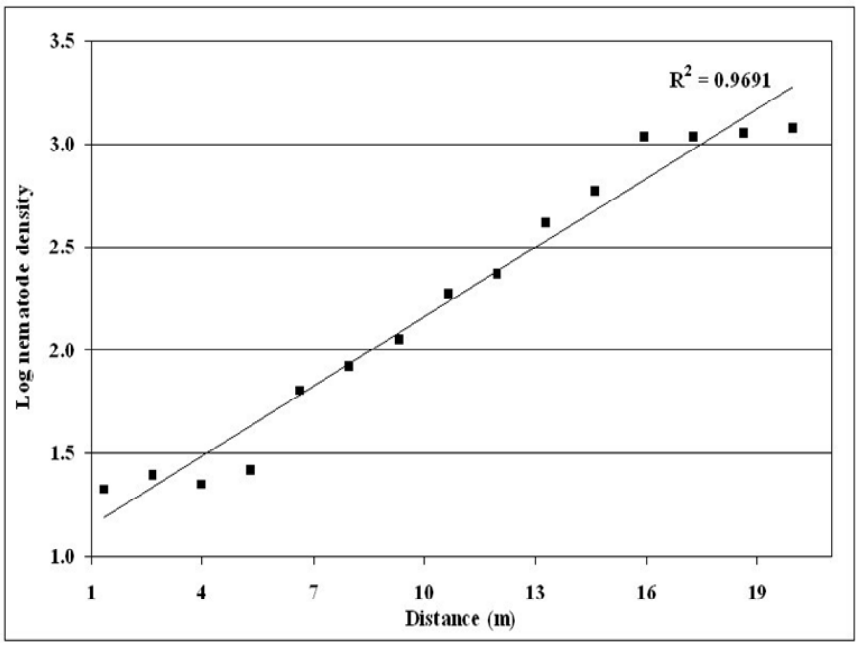

Figure 2.2. Densities of Globodera pallida (log numbers per 1.5 kg soil) in a row of square meter plots along the direction of cultivation including linear regression fit.

During the DWK339 research program, the small scale distribution pattern of *Meloidogyne chitwoodi* and *M. fallax* was established in the field in 1 square meter plots. The primary goal was the parameterization of the negative binomial distribution in this small area and the consequent development of a sampling method for scientific research. The obtained data, including data supplied by the PPO-agv, originate from different sites in the Netherlands and amount to 20 different plots. In all cases each plot was sampled 10 times. Each time a bulk soil sample of approximately 1.5 kg was collected by taking up to 60 single cores in a rectangular grid patter from an area of 1.33 by 0.75 m with the longest side in the direction of cultivation. Bulk samples were gently mixed and a sub sample of 500 g soil was taken and processed. Both the mineral and the organic fraction were screened for the presence of *Meloidogyne* spp. The mineral fraction was elutriated using Seinhorst's elutriator (Seinhorst, 1988). The organic fraction was incubated for 2 or 4 weeks, depending on the speed of emergence from the roots, using a mist chamber (Seinhorst, 1988). In Table 2.1 a short representation is given of the locations and some characteristics.

Table 2.1. Available data-sets used for the small scale distribution of Meloidogyne spp.

	Location	Year	Plot size (m^2)	Bulk sample size (g)	Sample processed (g)	Density	Soil type	Prior Crop
1	Smakt 251	2000	1	1500	500	542	sand	Black Salsify
2	Smakt 256	2000	1	1500	500	660	sand	Black Salsify
3	Vr99/1	2000	1	1500	500	5	sand	Potato
4	Vr99/2	2000	1	1500	500	34	sand	Potato
5	PAV-Zon/1	2000	1	>10kg	500	1202	sand	Black Salsify
6	PAV-Zon/2	2000	1	>10kg	500	172	sand	Potato
7	PAV-Baexem 55	1993	4	2500	100	38	sand	Barley
8	PAV-Baexem 55	1993	4	2500	100	598	sand	Barley
9	PAV-Baexem 95	1993	4	2500	100	473	sand	Potato
10	PAV-Baexem 95	1993	4	2500	100	571	sand	Potato
11	PAV-Baexem 262	1993	4	2500	100	2	sand	Boon
12	PAV-Baexem 262	1993	4	2500	100	17	sand	Boon
13	Vredepeel field 14/1	2000	1	1500	500	8	sand	Maize
14	Vredepeel field 14/2	2000	1	1500	500	444	sand	Maize
15	Smakt/Dream/1	2001	1	1500	500	139	sand	Potato
16	Smakt/Dream/2	2001	1	1500	500	3276	Sand	Potato
17	Smakt/Nijverheidsweg/1	2001	1	1500	500	543	Sand	Winter wheat
18	Smakt/Nijverheidsweg/2	2001	1	1500	500	150	Sand	Winter wheat
19	Kooienburg/1	2001	1	1500	500	243	Silty loam	Potato
20	Kooienburg/2	2001	1	1500	500	263	Silty loam	Potato

Generally, *Meloidogyne* spp. is highly aggregated within the square meter. At low nematode densities the coefficient of variation exceeds 100% (100% = 1 in graph 2.3). With increasing numbers counted – higher density in the soil sample – the reliability of the density estimation increases. Nevertheless, the coefficient of variation remains high, more than 25%, for the total number of juveniles (mineral and organic fraction) even at high population densities. The aggregation factor k of the negative binomial distribution – a measure of aggregation of the organism is 5 for the total number of juveniles. This is the lowest value, and therefore the highest aggregation – ever found up to now for a (semi) free living nematode (trichodorids: 45; *Pratylenchus* spp: 90). When the data of the mineral and the organic fraction are evaluated separately K is 25 and 2 respectively, when at least 200 juveniles are counted. This indicates that the highest variation can be subscribed to the organic fraction. One possible explanation is the presence of egg masses in the organic fraction. One egg mass included in the organic soil fraction will yield 200 up to 500 more juveniles in the suspension.

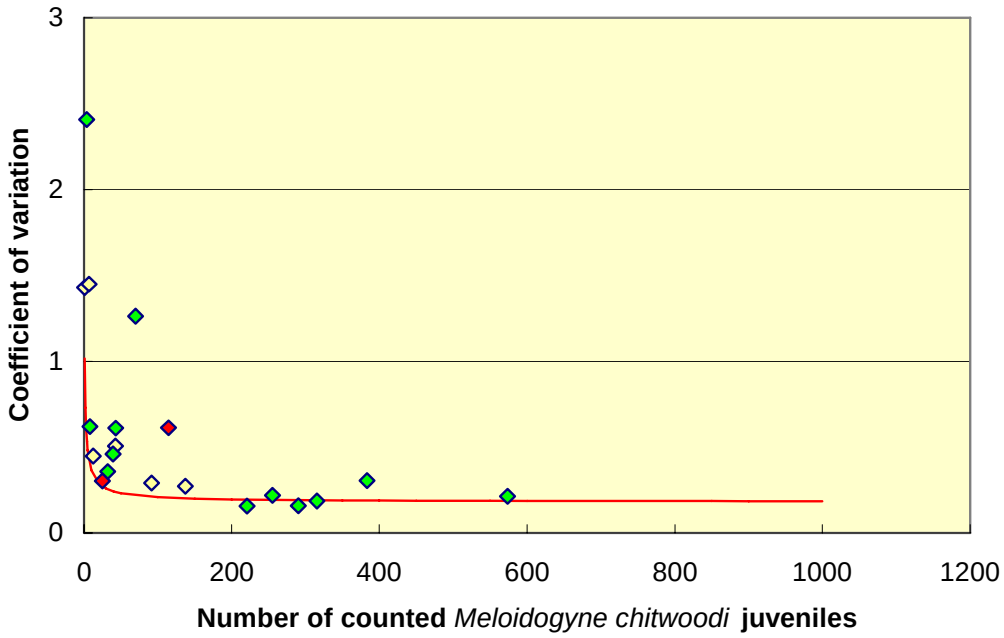

Figure 2.3. *The relation between counted numbers of nematodes and the coefficient of variation of the mineral fraction. Line: coefficient of variation according to a negative binomial distribution with a factor aggregation of 25.*

2.1.2 Medium scale distribution

The medium scale distribution describes the change of population densities over larger areas than those covered by the small scale distribution. It is the pattern that results when active and mechanical redistribution and spread acts on the small scale distribution pattern. Soil will be lifted by machinery, mixed and displaced, either in the direction of cultivation (cultivation, ploughing and harvesting) or at right angles to it (ploughing, winter-ploughing). This redistribution results in the horizontal growth of the primary point infestation over adjacent areas. Quite distinct shapes can result, such as the development of the so-called infestation focus or hotspot. Within the area covered by the medium scale distribution there are different population densities at different locations that are related to each other. As most farmers in a growing area or country use the same kind of machinery, and sometimes even the same cropping frequency, the resulting infestation foci of a nematode species tend to be of the same shape at any given location. An oval shaped spot (lens) in the field will appear where plant growth is retarded or, in extreme cases, completely inhibited. When the infestation focus is subject to intensive crop rotation with a host, population densities in the centre will reach a maximum (carrying capacity of host/crop rotation) over an increasing area. It depends on the speed of redistribution of the nematode species researched what kind of magnitude of size of the medium scale distribution pattern can be expected.

As this kind of infestation foci are also known to occur with other cyst and (semi) free living nematodes it is reasonable to assume this will also be the cause with *Meloidogyne* spp. Therefore, as a first exploratory step towards the development of detection methods for his species, data from field experiments can be used to obtain some insight. Nine data-sets, most of them provided by the PPO-agv were analyzed to determine whether the same model as used for the description of infestation foci of the potato cyst nematode could be applied to Meloidogyne. The data-sets originated from research on host-suitability and the effectiveness of nematicides and nematostatics. Only data from the original mapping of these fields were used to obtain an impression of the unspoiled situation. The population decline per meter, the length and width gradient and the maximum population densities were estimated. The analysis was only partially successful.

It was indeed possible to estimate a length gradient (direction of cultivation) for most of the mapped infestations and to obtain a maximum density in spring. However, the information in the data-sets was insufficient to estimate a width gradient (at right angles to the direction of cultivation).

Table 2.2. Data-sets used to explore the medium scale distribution.

Field	Location	Year	Max. density/ 100 ml	90% of densities <	Average length gradient	Prior Crop
1	Klijndijk	1996	2500	610	*	Sugar beet
2	Smakt	1996	1370	520	0.95	Potato
3	Smakt a	2000	2605	1220	0.94	Beans/Barley
4	Smakt b	2000	1634	990	0.96	Beans/Barley
5	Smakt	2001	2527	840	0.9	Barley
6	Rips	1989	240	75	*	Divers
7	Vredepeel	1999	385	245	0.97	Sugar beet
8	Baexem	1992	1865	1140	0.98	Evening primrose
9	Dream	2001	3225	1740	0.9	Potato/Barley

** = Impossible to estimate from data.*
Gradient = Decline of population density per meter.

Table 2.2 presents an overview of the nine fields and the corresponding parameter estimations. The derived length gradients were all higher than 0.94 indicating that the decrease of population densities is very slow. Much slower than those derived for the potato cyst nematode, were a decrease of 0.83 was found per m in the direction of cultivation and 0.64 at right angles. Figure 2.4 illustrates a section through such an infestation (Table 2.2, field 5).

When we assume that the difference between the length and width gradient is the sole result of passive redistribution by agricultural machinery, we can use the results from the potato cyst nematode analysis (Schomaker & Been, 1999) to estimate the width gradient. We therefore will use the ratio between both gradients established in that analysis, which was 0.77. The average length gradient for *Meloidogyne chitwoodi* (and *fallax*) was 0.94. This implies that the width gradient should have a value of 0.72. On this basis an estimate can be made concerning the shape and size of a *Meloidogyne* spp. infestation focus. What is needed is the maximum density for the centre of the focus. When a density of 500 or 1000 juveniles/100 ml is supposed, as well as no local variations of soil type in the field causing higher or lower multiplication and spread, then an infestation focus with a central population density of 500 juveniles/100 ml will be 150 meter long and 28 meter wide with a total area of 4200 m2. An infestation focus with a central population density of 1000 juveniles/100 ml an area of 300 (l) x 60 (b) = 36000 m^2.

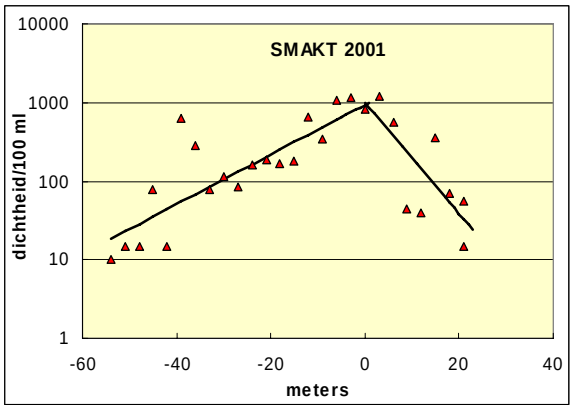

Figure 2.4. Gradients from the test site in the Smakt 2001 field.

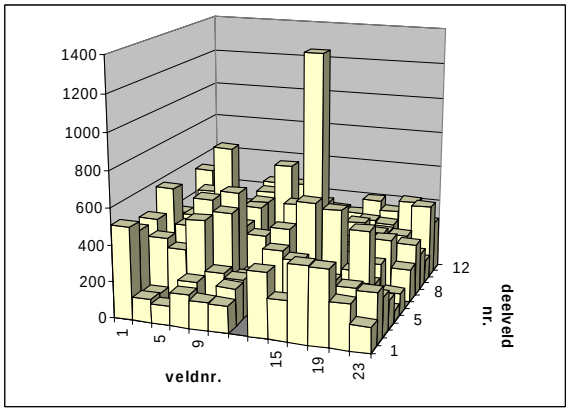

Figure 2.5. Population densities Smakt 1996. Each square is 6x6 meter.

The resulting infestation foci of *Meloidogyne* spp. are huge and numbers of detectable individuals high (when compared to cysts as detectable unit) and detection therefore easy. In Figure 2.6 the detection probabilities are presented for foci starting with 500 up to 20000 juveniles/kg of soil using the standard AMEX200cc soil sampling method, which requires collection of 200 cc per 1/3 ha. The two data series represent the fraction of suspension which is actually counted; either 100% or, as commonly used by statutory soil sampling agencies, 20%. Based on the maximum densities as presented in Table 2.2 (juveniles/100ml), the detection probability for all the fields presented in Table 2.2 is high at the date of sampling.

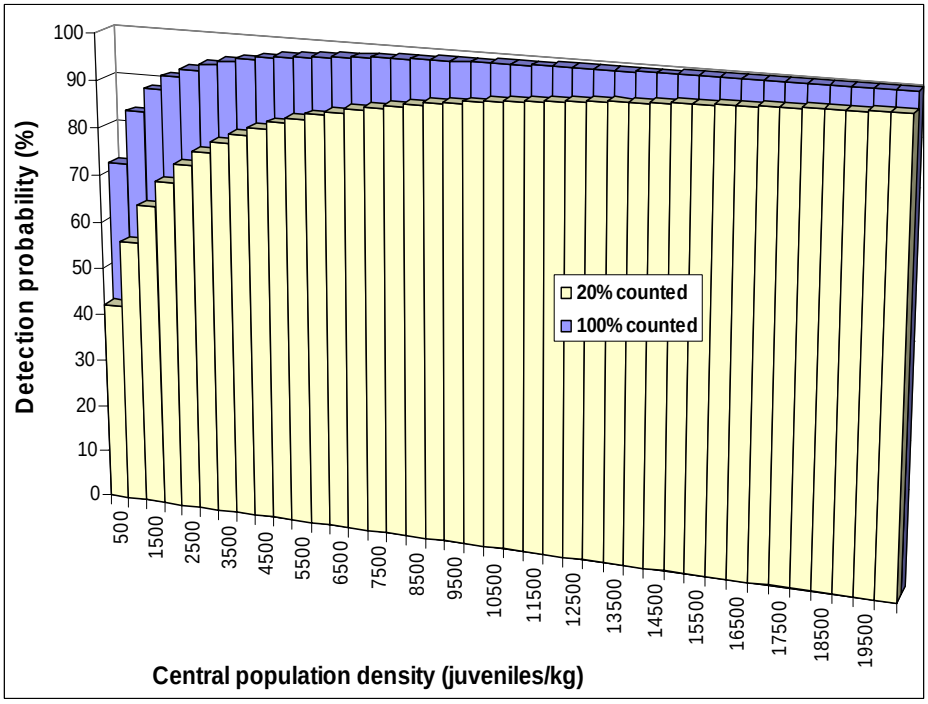

Figure 2.6. Detection probabilities of the AMEX200cc soil sampling method for potato cyst nematodes applied on foci of increasing size (defined by the central population density) of Meloidogyne spp. Foci dimensions according to parameters established from data from field experiments (Table 3.2). 100% and 20% of the suspension counted.

2.1.3 Large scale distribution

Once the first hotspot is established, passive redistribution by machinery will not only cause the focus to grow, but will also, by way of clods of infested soil adhering to the machinery, result in secondary infestations when these clods are deposited further along the row. The secondary infestations will be found primarily in the direction of cultivation and less frequently at right angles to that direction. As long as susceptible hosts are grown, these infestations will grow and finally merge and result in completely infested fields, with maximum population densities defined by the carrying capacity of the host. The intermediate distribution pattern can be regarded as the combination of several medium scale patterns. It depends on the speed of the various dispersion vectors of the organism how long it will take to reach the final stadium. With potato cyst nematodes this was only the case in the short rotations used in the starch potato growing areas. When the data presented in Table 2.2 regarding *Meloidogyne* spp. are considered two possibilities seem to be feasible in explaining the huge infestations encountered.

1. *Meloidogyne* spp. spreads much faster in both directions than other nematodes (as can be derived from the average length gradient of 0.94). This could be due to the fact that almost every arable or vegetable crop and even green manure and weeds are host of this nematode. Furthermore as the dispersed unit can be an egg or a juvenile (instead of cysts for PCN) with up to 10000 juveniles per kg after almost all crops (Table 2.2) and up to 500000 juveniles per kg after potatoes, there are far more separate units that can be dispersed than with a species like PCN.
2. The results of these data-sets are biased as the fields providing this information were pre-selected in order to make them useful as an experimental site. Therefore, only old and large infestations were available for analysis.

In order to investigate which of these possibilities is true, research has to be focused on mapping infestations of *Meloidogyne* spp. when they are still restricted to a small area, preferably shortly after introduction in the field. If the focus model is applicable, then the data can be used to estimate both gradients including the width gradient, which until now is completely unknown. If the results indicate that in a very short period the whole field is infested, the mapped large scale distribution can be used to calculate detection probabilities.

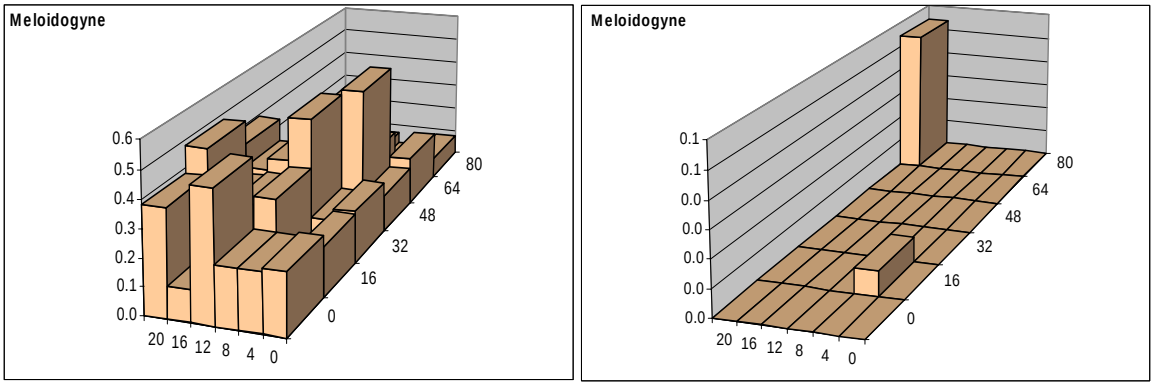

Figure 2.7. Example of two pre-sampled fields. Each square measures 8 x 3 m – with the longest distance in the direction of cultivation. The central square m2 is sampled.

2.2 Material & Methods

2.2.1 Selection of fields

Starting in 2002 and up to 2004, fields found to be infested with either *Meloidogyne chitwoodi* or *M. fallax* were provided by the PPO-agv, the IRS or local sampling agencies. More than 10 fields were provided in 2002 from which a selection was made, excluding those infestations which were treated by nematicides or nematostatics by the grower owning that field or which would have, by way of previous or current crops grown, a too low population densities to obtain reliable nematode counts in the autumn when the actual mapping would be carried out. Selected fields were pre-sampled in spring, to gain insight in the location, size, severity and shape of the infestation. In particular an effort was made to obtain fields which still contained distinct infestation foci which could present a young primary infestation of *Meloidogyne* spp. All fields were sampled using a course grid pattern. Table 2.3 provides an overview of all fields pre-sampled

*Table 2.3. List of pre-sampled fields: The central square meter of each block with an area of **L** x **B** was sampled by collecting approximately 1 kg of soil. Subsequent blocks were situated according to the number of rows and cols in column 3 and 4, respectively.*

	Year	Rows	Cols	Nr. of samples	L (m)	B (m)	Sampled	Current crop
1	2002	6	6	36	16	4	Square	Potato
2	2002	6	6	36	12	6	Square	Italian rye grass
3	2002	6	6	36	12	6	Square	rye grass
4	2003	6	6	36	12	6	Central m^2	Maize
5	2003	5	6	30	12	6	Central m^2	Maize
6	2003	6	6	36	12	6	Central m^2	Beets
7	2003	4	5	20	20	12	Central m^2	English rye grass
8	2004	6	5	30	20	8	Central m^2	Wheat
9	2004	6	6	36	20	8	Central m^2	Potato
10	2004	5	4	20	20	8	Central m^2	Wheat

Figure 2.6 displays two of the pre-sampled fields. In some cases population densities were extremely low, prohibiting the use of these infestations for more intensive mapping (Fig 2.6 B). From the fields pre-sampled in 2002 only one was suitable for mapping. The same field was used in 2003 to monitor population decline in time (chapter 3). The same procedure was applied in later years. In 2003 four fields were pre-sampled and three proved to be suitable for mapping in autumn; in 2004 only one out of the three pre-sampled fields was mapped. Together with two fields supplied by Nicole Viaene from the Agricultural Research Centre, Department of Crop Protection, Merelbeke, Belgium, a total of 7 infestations were mapped.
All intensive mappings were carried out as soon as possible after harvest of the crop. The mapped areas covered a part or the whole op the pre-sampled area. As it was financially impossible to sample and process each square meter of the targeted area, the area was subdivided in smaller areas, from which the central square meter was intensively sampled. Consequently, the mapping consisted of a grid of 8 by 3 square meters. In order to obtain some information concerning the variation of population densities within the area of the grid represented by a square meter sample, 11 of these 'represented areas' were completely mapped by sampling each square meter within the area (block sampling).

Table 2.4. Some characteristics of the intensively mapped fields.

	Year	Country	Soil type	Harvest data	Sampling		Sampled			Nr. of samples	Bulk Size [2]	Prior Crop
					Date	Grid [1]	Rows	Cols	Area			
1	2002	Bel.	sand	15/09/01	23/04/02	8 x 3	10	13	3120	130	2097	Potato
2	2002	Bel.	sand	??/08/01	11/04/02	8 x 3	10	13	3120	130	1949	Onions
3	2002	Neth.	sandy loam	27/09/02	04/10/02	8 x 3	12	10	2880	120	1676	Potato
4	2003	Neth.	sand	05/09/03	13/11/03	4 x 3	12	11	1584	132	1775	Maize
5	2003	Neth.	sand	02/09/03	02/10/03	8 x 3	8	15	2880	120	1761	English rye grass
6	2003	Neth.	sand	11/09/03	24/10/03	3 x 3	11	7	693	77	1830	Sugar beets
7	2004	Neth.	sand	15/07/04	25/11/04	2.6 x 1.5	16	12	766	192	1594	Wheat + fodder reddish*

[1] *Meter.*

[2] *Sample size in g fresh weight.*

* *As resistant green manure.*

2.2.2 Sample processing

Samples were collected from plots of one square meter (1.33 x 0.75m) using a 25 cm long, 1.5 cm core diameter, auger. Generally, when using this core size and collecting 60 to 80 cores in a grid pattern, a soil sample of approximately 1.5 kg can be collected easily and quickly for almost all relevant nematode species. Samples were stored in plastic bags at 4 degrees prior to processing. Soil samples were gently mixed and a volume of 200cc soil was sub sampled using a container with the correct dimensions. Samples were split by sieving into a mineral fraction and an organic fraction which were processed separately. For the mineral fraction the Oostenbrink elutriator was used; the organic fraction was stored in a mist chamber for two up to four weeks depending on the root system of the previous host crop. Both the mineral and the organic suspensions were brought to 100 ml and from each fraction four aliquots of 10 ml were counted. Each tenth sample was checked to species level.

2.3 Results and Simulation studies

SAMPLE IV (Been & Schomaker, 2000) developed for simulating sampling procedures for potato cyst nematodes was adapted for *Meloidogyne* spp. Two kinds of simulation studies were carried out:

1. One according to the focus model which was parameterized for *Meloidogyne* spp. using the results of the small scale distribution and the length and width gradient parameters.
2. One using the full field infestations using the maps of the fields and the combined variation of the small scale distribution and the block sampling data. (Table 2.5).

Table 2.5. Some characteristics of the intensively mapped fields.

Field	Year	Country	Population density (juveniles/kg)			CoV (%)	Detection probability AMEX200cc method [1]		Type of infestation
			Min	Max	Mean		100% counted	20% counted	
1	2002	Belgium	100	10111	2059	87.4	72.5	65.5	Full field
2	2002	Belgium	0	2020	259	121.1	59.9	45.7	Full field
3	2002	Netherl.	0	5633	1238	87.6	67.8	56.9	Full field
4	2003	Netherl.	0	2509	308	168.8	30.3	23.3	Focus
5	2003	Netherl.	154	7529	1784	69	70.4	62.5	Full field
6	2003	Netherl.	33	3367	578	115.7	20.6	15.2	Focus
7	2004	Netherl.	0	968	97	187.3	11.5	8.8	Focus

[1] *Detection probability at sampling date.*

Table 2.5 presents some characteristics of the 7 fields mapped in the course of this project. Four of these fields can be considered as full field infestations although; in some of them still the primary focus can be detected. The other three fields were definitely infestations foci in different states of development, mostly with adjacent secondary foci.

2.3.1 Infestation foci

In general, the visible shape of these foci is less distinct as that of potato cyst nematodes, mainly caused by the larger variation of measured population densities. This is both due to the added variability of the organic fraction and the variability of the Oostenbrink elutriator for this nematode. Analyzing these foci resulted in a length gradient of 0.85 and a width gradient of 0.73. These are different then those calculated using the data-sets provided by the PPO-agv. In Figure 2.8 the resulting difference in detection probability is visualized. However, these new gradients are based on only three infestation foci. The range of length/width gradients for the potato cyst nematode was 0.77/0.55 up to 0.91/0.76 for both the lower and upper 10% kwantile of the bivariate normal distribution. This means that the gradients found for the three foci of *Meloidogyne* spp. could fit well into this frequency distribution and cannot as yet be regarded as significantly different from PCN.

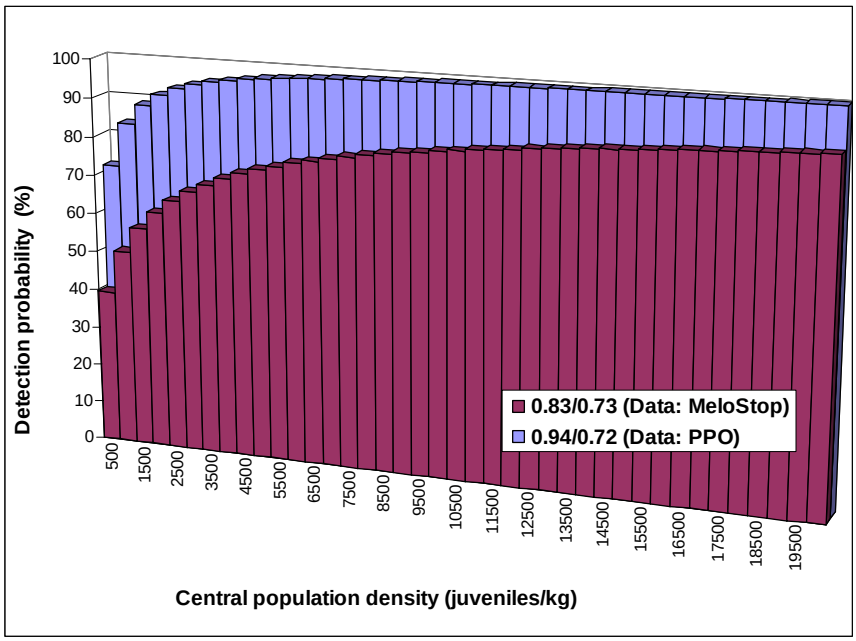

Figure 2.8. Differences in detection probability using the length and width gradient derived from data provided by PPO-agv (test site data with width gradient estimated using the ratio between both gradients found in Globodera. spp) and according to the three mapped foci in this research project.

Figure 2.9 presents an analysis of the old and the two new sampling methods for potato cyst nematodes as prescribed by AMEX200cc applied on the *Meloidogyne* infestation focus model for central population densities of 500 up to 20000 juveniles per kg of soil. The analysis presumes that the whole suspension is counted. Of course, when less soil is collected, the detection probability per focus of a certain size decreases. Note that all maximum population densities as presented in Table 2.2 and interpreted as central population density would yield high detection probabilities.

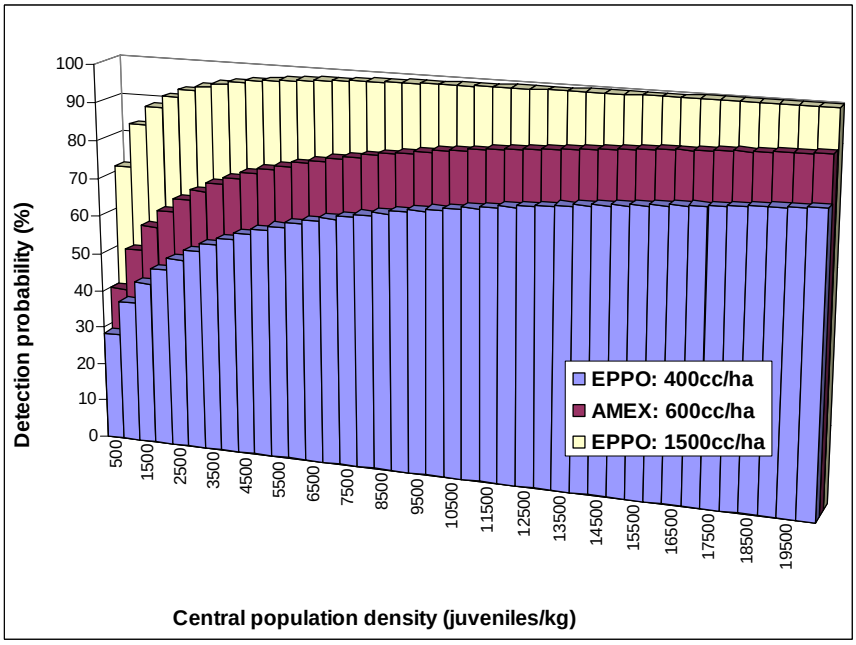

Figure 2.9. Detection probabilities of three different soil sampling methods: 400cc, 600cc and 1500 cc per ha. Sampling grid used 7.5 x 7.5 m. Core size: 2.22, 3.33 and 8.33 cc.

2.3.2 Full Field infestations

Table 2.6 presents some information concerning the variation of the area presented by each square meter of the mapped fields. This can be regarded as a kind of medium scale distribution required when the focus model does not apply and actual mapping data are used to calculate detection probabilities when applying a certain sampling method. The variation within the blocks is estimated and added to the variation within a square meter (small scale distribution). When SAMPLE IV simulates the sampling process for detection of a mapped field, the expected population density at a certain point in the field will be based on the population density of the actually sampled square meter including the variation of the population densities in the block containing that square meter. This population densitiy varies according to the averaged estimated variation within the sampled blocks in Table 2.6. Using this full field infestation sampling approach, detection probabilities for all mapped fields were calculated using the standard AMEX200cc soil sampling method for PCN when the whole or 20% of the suspension is counted. The data are also presented in Table 2.5. Figure 2.10 presents an example of one of the full field infestations encountered.

Table 2.6. Some characteristics of the medium scale distribution within blocks blocks based on the number of counted nematodes (CV = coefficient of variation).

Block	Year	Country	Mean density	Min	Max	Variation	cv
1	2002	Belgium	7.9	0.0	62.0	184.2	1.7
2	2002	Belgium	100.0	26.0	242.0	2895.5	0.5
3	2002	Netherlands	135.8	49.0	326.0	4311.0	0.5
4	2002	Netherlands	67.8	9.0	234.0	2624.3	0.8
5	2002	Netherlands	271.3	35.0	973.0	75099.0	1.0
6	2003	Netherlands	9.9	0.0	51.0	110.6	1.1
7	2003	Netherlands	266.2	94.0	606.0	22614.9	0.6
8	2003	Netherlands	336.1	82.0	1424.0	53131.0	0.7
9	2003	Netherlands	101.7	16.0	1990.0	4778.5	0.7
10	2003	Netherlands	49.9	18.0	119.0	804.5	0.6
11	2004	Netherlands	58.8	2.0	202.0	2964.2	0.9

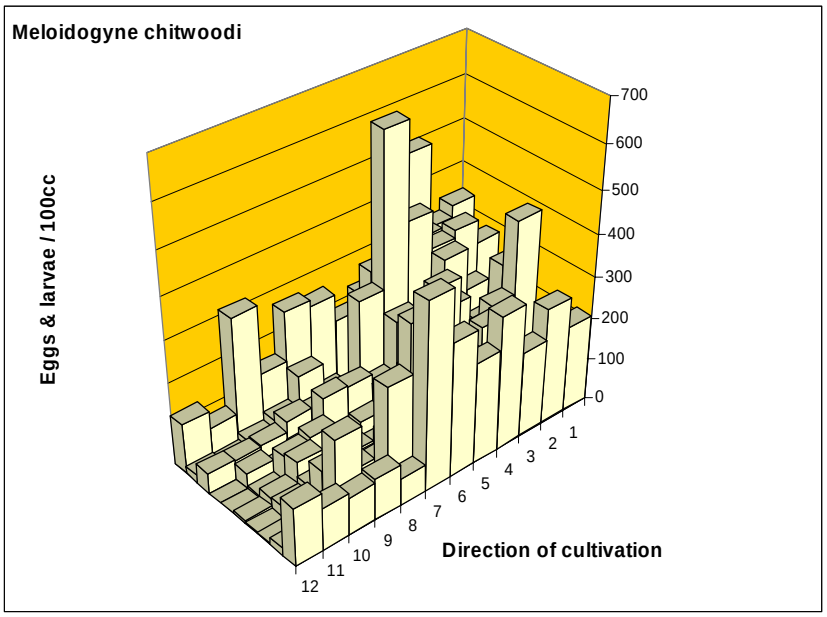

Figure 2.10. Large scale distribution pattern of Meloidogyne chitwoodi after potato harvest. Area sampled: 03 ha. Each block represents an area of 8 by 3 meters (8 m in the direction of cultivation). Central square meter of block (1.33 x 0.75 = 1m^2) sampled.

As the infestation focus model applies for *Meloidogyne* spp. and detection is the primary goal of the current research project all further analyses were carried out using the focus model. In Table 2.4 the percentage population decline between harvest of the host crop and the date that a new host would be sown or planted is presented. In general, we can conclude that roughly about 80% of the population of *Meloidogyne* spp. is died during this period. In Figure 2.11 an example of the relation between time after harvest and nematode numbers is presented for the seed potato data of the Smakt test-site and modelled using an exponential equation. A factor of decrease can be calculated revealing that per week a fraction of 0.945 of the population was lost. As the decline is exponentially, actual numbers decrease only slightly after several month and population decline seems to vary little at the end of the non-host period during winter.

Table 2.4. Natural decline of population densities between harvest of host crop and planting or sowing of next crop.

Field	Country	Host	Decline	First Date	Last Date
1	Belgium	Fodder beets	80%	10/11/04	16/03/05
2	Belgium	Summer barley	90%	18/08/04	16/04/05
3	Netherlands	Potato (Consumption)	70%	week 41 '02	week 17 '03
	Netherlands	Potato (Seed)	60%	week 37 '02	week 17 '03
4	Netherlands	Potato (Consumption)	95%	week 42 '03	week 18 '04
	Netherlands	Potato (Seed)	94%	week 37 '03	week 18 '04

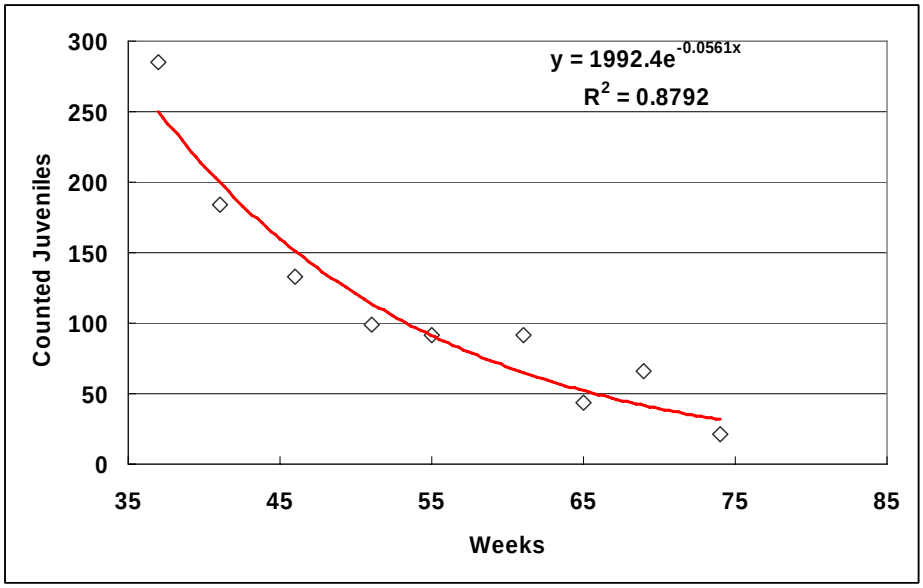

Figure 2.11. Decline of population densities expressed as numbers of Meloidogyne chitwoodi per unit of soil in time after harvest of seed potatoes. Decline according to a negative exponential curve. Remaining fraction after one week of decline: 0.945

Using a population decline of 80% and the above used and parameterized infestation model, detection probabilities after harvest can be compared to those at sowing or planting of the new host. The same range of central population densities was used as before and the results are presented in Figure 2.12.

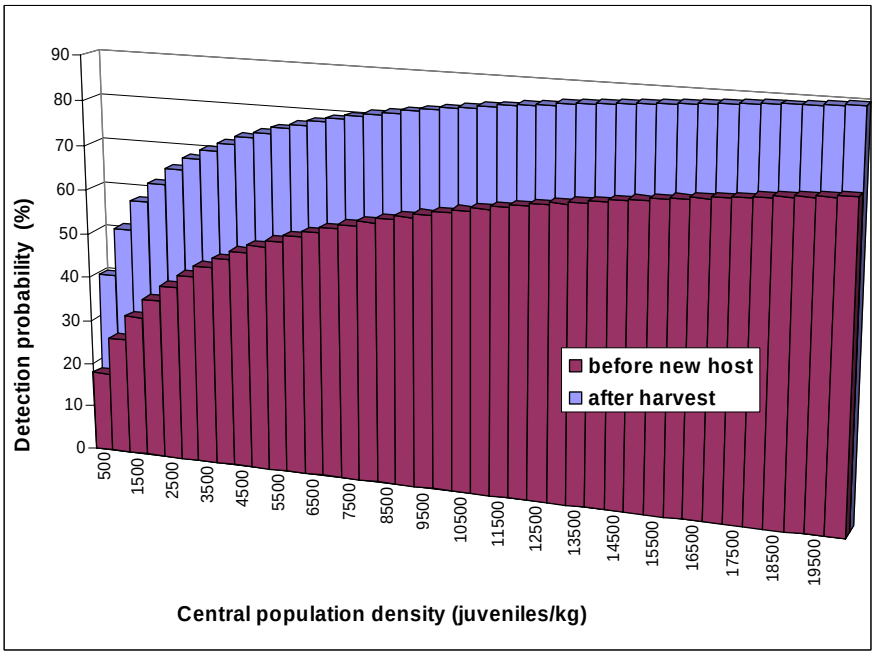

Figure 2.12. Detection probabilities when applying the AMEX200cc: 1/3 ha sampling method on infestation foci of Meloidogyne spp., with central population densities ranging from 500 up to 20000 juveniles/kg of soil, directly after harvest and prior to a new host after population decline during winter.

Generally speaking, the expected decline in detection probability is not as severe as was expected from an 80% reduction of population densities. It should be noted that densities as expressed as juveniles/kg of soil are high with *Meloidogyne* spp. and that the mayor problem in accurate detection is the high aggregation factor of the small scale distribution. Again, when taking into account the maximum population densities in Table 2.2 and the population densities measured when samples were taken after potato and sugar beets for the population decline data sets, the detection probability, when using the standard AMEX200cc soil sampling method, of an infestation focus of *Meloidogyne* spp. is high when compared to the detection probability of the potato cyst nematode.

As the highest variation of population densities is encountered in the organic fraction, causing also a huge difference in the parameter *k* of the negative binomial distribution it was suggested to collect more soil, but only to process the mineral fraction thereby enhancing the detection probability. The procedure was implemented in some exploratory research in the Netherlands. Results from this research project revealed a dramatic decline of the number of juveniles in the organic fraction after potato. Within 4 weeks after harvest the organic fraction only contributed about 20% of the total number of juveniles in the soil sample. Taking a larger soil sample and only processing the mineral fraction (*k* of 25 for the negative binomial distribution) will further increase detection probability. However, some further data mining is required to validate this approach for other host crops.

2.4 Discussion

The mapping of fields revealed that infestation foci of *Meloidogyne* spp. do exist and can be found, although with much more effort than was necessary for finding potato cyst nematode infestations in the field. Frequently, a newly detected infestation covered the whole area sampled and could be regarded as a full field infestation. However, the general shape of the infestation foci found is similar to that of an PCN infestation, although parameter values slightly differ. On the basis of the information available it seems likely that the development time from an infestation focus to a full field infestation is shorter than that of potato cyst nematodes. Probably as a result of the higher number of units that can be dispersed.
Mapping infestation foci of *Meloidogyne* spp. did not reveal the precisely defined shape as described by Schomaker & Been (1999) for potato cyst nematodes, but a more irregular shaped infestation focus. Whether this is caused by a more irregular spread of the nematodes (juveniles, eggs and egg masses compared to cysts) or due to the sampling method can be discussed. However, that the error suffered when sampling and estimating *Meloidogyne* population densities is significantly higher than with a species like *Globodera* is obvious. Both mapping of infestations and the estimation of the aggregation factor suffer from the inherent error of the methods for elutriation, currently available.
There are two mayor problems which have to be solved before accurate population density estimation of the same order as currently possible for cyst nematodes is feasible:

- The machinery used to elutriate *Meloidogyne* spp., the Oostenbrink elutriator and the zonal or Hendricks centrifuge, is of insufficient quality to obtain reliable results. A coefficient of variation of the population density estimation of 50% was deduced (Van Beers *et al.*, 2002). The authors present suggestions on possible sources of error.
- The organic fraction of the sample introduces a large additional error to the population density estimation. Contrary to the mineral fraction, where mixing of the soil suffices to obtain a Poisson distribution of individuals throughout the bulk sample this is not the case for the organic fraction. Here the probability of collecting a part of the root system with no, one or several egg masses causes large variations in the number of nematodes in the organic fraction.

The result of these insufficiencies is that the aggregation within a square meter is overestimated. Not only the error of sampling an aggregated distribution (small scale distribution), but also the laboratory error adds to the variability which is used to estimate the degree of aggregation. This results in a very small k value which in fact is the product of the real aggregation coefficient and the additional methodological error. In the simulations this results in a lower detectability at a certain core size and the need of a larger bulk sample to compensate for this. When a field is mapped the same insufficiencies result in a higher heterogeneity of the population densities of the map and of the medium scale distribution as calculated within the 8 by 3 meter blocks.

This implies that the actual aggregation factor of the negative binomial distribution for *Meloidogyne* spp. is higher than used in the calculations, that the densities within the foci and full field infestations mapped are actually less heterogenic and that as a result we underestimate the detection probability and overestimate the variability of population density estimations. As soon as better elutriation methods become available the same soil sample size will yield better results.
Another difference with the well researched *Globodera* species is the number of pathogens per unit of soil. These are definitely higher than for cyst nematodes with up to 500000 juveniles/kg of soil measured directly after harvest of a potato crop. This implies that detection, even though the aggregation coefficient *k* is overestimated, will be higher than with PCN. However, the aggregation factor will seriously hamper precise estimation of the population density within a field.

The most appropriate time of sampling is directly after harvest of the host crop. Population densities are then highest while the nematode numbers are divided between the mineral and organic fraction. The numbers of nematodes decreases exponentially in time and detection probabilities decline also but with less extend. If potatoes are the target crop one has to choose between sampling prior to planting of the tubers – sampling after the last crop – or sampling after harvest when a possible infestation has been boosted up in densities by the potato crop. Sampling after harvest of potatoes will yield a higher detection probability but will also cause severe economic losses by the farmer. To further increase the detection visual inspection could be added – however, this will only yield extra information when contamination is expressed in visual symptoms.

3. Population dynamics of *Meloidogyne chitwoodi* in relation with potatoes

3.1 Introduction

One of the consequences of the quarantine status is that potatoes are surveyed for the presence of *Meloidogyne*. This is carried out by the Plant Protection Service, by taking test samples on approximately 400 farms throughout the Netherlands. Each individual test sample consists of 200 potatoes which are kept at 18C till the amount of 2150 degree day's is reached, after which the tubers are checked visually for symptoms caused by *Meloidogyne*. This procedure is not very accurate, especially since it is known that some cultivars are not very sensitive in showing symptoms. Some of these cultivars may not show visual symptoms on the outside, but may contain *Meloidogyne* on the inside. This project was started to investigate the relationship between nematode numbers in the soil and presence of *Meloidogyne* in tubers. To find out the best moment for sampling of *Meloidogyne*, population dynamics in soil during and after growing potatoes was assessed. Two field trials were conducted in soil with a natural infestation of *M. chitwoodi*. Potatoes were grown in plots of 6 by 6 meters. Each plot was sampled in March (Pi) before planting. After harvest plots were kept free from any plants and sampled monthly during a year. From some plots potatoes were collected to investigate their quality by scoring visual symptoms internally and externally and by different molecular methods.

3.2 Materials and methods

3.2.1 Site description

During 2001-2005 two field sites with a natural infestation of *M. chitwoodi* were selected. One in the south-east of the Netherlands, called SMAKT (SM), qualified as a sandy soil (95% sand, <1% clay, 4.6% silt content) with an organic matter content of 2.3%, pH-KCl of 6.7, CaCO3 < 0.1% and a P-total of 183. The second experimental field is located in the north-west of the Netherlands, called WIERINGERMEER (WM), qualified as sandy soil with an organic matter content of 1.4%, pH-KCL 7.4 and CaCO3 3.7%. The experimental field was divided into 20 plots of 6*6 m each. The plots were arranged in 4 blocks with each 5 plots of one treatment. Two treatments were started in week 19 with the sensitive (for visual symptoms) potato cultivar Asterix: cultivation of seed-potatoes and cultivation of warehouse potatoes. The potatoes were grown according to good agricultural practice for both fertilization and crop protection against pests and diseases. Organic manure was applied, containing 100 kg P, 175 kg K and 140 kg effective N per ha, supplemented by 75 kg N from mineral fertilizer. Plots were weeded by hand or chemically. The plots with seed potatoes were harvested around week 37 and the warehouse potatoes around week 41. Two to three weeks prior to harvest, plant growth was stopped mechanically and chemically. In each net plot of 3 m by 2 m all potatoes were harvested and stored at 4C. After harvest plots were kept free from any plants and sampled monthly during a year. A fraction of these potatoes were analyzed for internal and external symptoms of *Meloidogyne chitwoodi*. In order to have an objective standard for establishing the quality of *Meloidogyne* infested potatoes, a tuber damage index (PPO-Knot-Index) was developed. To calculate this PPO-Knot-Index (PKI), five different classes were defined according to the amount of symptoms on the skin and Meloidogyne eggs inside the tubers. Tuber damage ratings, so called PPO-Knot-Index (PKI) ratings were done using a scale from 0 to 4 as follows: 0 = no symptoms outside or inside. 1 = no symptoms outside but females an/or eggs visible inside; 2 = just visible symptoms outside; 3 = clearly visible symptoms outside; 4 = abundantly present symptoms outside (Table 3.1). In general 30 randomly selected potatoes per plot are used to establish the PKI. Each individual potato was first analyzed for external symptoms, after which three pieces of skin were removed with a knife. These cuttings give the possibilities to find Meloidogyne females and egg masses inside the tubers. The newly developed PKI proved to be a reliable index to quantify the amount of damage. After this first inspection tubers were stored at 18 C in order to permit root-knot nematode development. After +/- 8 weeks, t = 8, final PKI's were determined.

Table 3.1. Classification of potato tubers infected by M. chitwoodi.

Group	Symptoms, galls	Eggs, visible after peeling
0	No galls	No
1	No galls	Yes
2	< 30% of tuber galled	Yes
3	30 – 100% of tuber galled	Yes
4	Tuber extremely galled	Yes

With these classifications the PKI-index per plot was calculated by the formula:

PKI-index = (((# tubers group 0 + 1) x 0) + (# tubers group2 x 10) + (# tubers group3 x 33) + (# tubers group 4 x 100)) / Number of tubers observed

Supplementary to the field experiments, tubers from cultivar Kondor, Arinda and Arnova were collected in 2005 and used for molecular analyses. From each cultivar, potatoes were collected from a field infested with *Meloidogyne*, and from heavy marine clay soils in which no *Meloidogyne* infestation was present.

3.2.2 Nematode sampling

All plots were sampled before planting (March-April) and after harvesting the potatoes. In the centre of each plot (2 m x 3 m) 35 soil cores (diameter 12 mm) from the top 0-5 cm and from the 5-25 cm were taken in a regular pattern. Of each soil layer a sub sample of 100 ml of soil served to extract nematodes. This soil sample was first sieved over a 180 mµ sieve with water, after which nematodes in the suspension were collected by using an Oostenbrink elutriator. The remaining organic matter fraction with mainly root debris was incubated at 20C for 28 days to allow egg hatching and emergence of motile endoparasitic stages. The total number of nematodes was established by counting approximately 20% of the total sample under a dissecting microscope.

3.2.3 Statistical analysis

A factorial design with completely randomized blocks was used. Data were transformed (log10[x+1]) to meet assumptions of normality and homogeneity of variances. Object means were calculated and further analyzed for differences among treatments [ANOVA, Genstat]. Subsequently object means were transformed back. These object means are called 'median' and are less influenced by extreme data than normal means. For the ease of interpretation, medians were expressed in total numbers per 100 ml of soil.

3.3 Results

In this chapter first the number of *M. chitwoodi* nematodes (presented as 'medians') per location, cultivation (seed- or ware potato) and soil layer (0 to 5 cm, 5 to 25 cm or 0 to 25 cm below the surface) are presented. Subsequently the development of the population in time is discussed.

3.3.1 Population dynamics of *M. chitwoodi*

At location Smakt seed potatoes were harvested in week number 37 of 2002. After harvest, another 10 soil samples were taken to measure the development of the final population (Pf). The first soil sample was taken in the week of harvest, the last one more than forty weeks later (in the year 2003, week number 26). Ware potatoes were harvested four weeks after seed potato, in week number 41. After harvest, another 9 soil samples were taken to measure Pf until week number 26 in 2003. Results from Smakt are presented in Table 3.2 and in Figure 3.1.

Table 3.2. Number of Meloidogyne chitwoodi in 100 ml soil, mineral and organic soil fraction combined. Smakt 2002-2003.

	Seed potato				Ware potato			
soil sample/ weeknr [1]	0 – 5 cm	5 – 25 cm	0 – 25 cm	soil sample/ weeknr [1]	0 – 5 cm	5 – 25 cm	0 – 25 cm
Pi 19	2	2	2	Pi 19	4	4	4
Pf1 37 (0)	39 e	207 f	175 f	-	-	-	-
Pf2 41 (4)	17 de	93 e	78 e	Pf1 41 (0)	106 e	213 ef	197 ef
Pf3 46 (9)	9 cd	80 de	67 de	Pf2 46 (5)	84 e	321 f	279 f
Pf4 51 (14)	3 bc	58 cde	47 cde	Pf3 51 (10)	15 d	144 de	119 de
Pf5 3 (18)	7 cd	47 cde	42 cde	Pf4 3 (14)	20 d	187 def	156 def
Pf6 9 (24)	5 bc	45 cd	38 cd	Pf5 9 (20)	12 cd	119 cde	102 d
Pf7 13 (28)	2 ab	35 c	28 c	Pf6 13 (24)	12 cd	98 cd	83 cd
Pf8 17 (32)	1 ab	48 cde	39 cde	Pf7 17 (28)	4 bc	65 bc	53 bc
Pf9 22 (37)	2 ab	9 b	9 b	Pf8 22 (33)	2 b	44 b	36 b
Pf10 26 (41)	0 a	0 a	0 a	Pf9 26 (37)	0 a	0 a	0 a
F prob.	< 0.001	< 0.001	< 0.001	F prob.	< 0.001	< 0.001	< 0.001

[1] *Between brackets the number of weeks after harvest (week of harvest being week 0).*

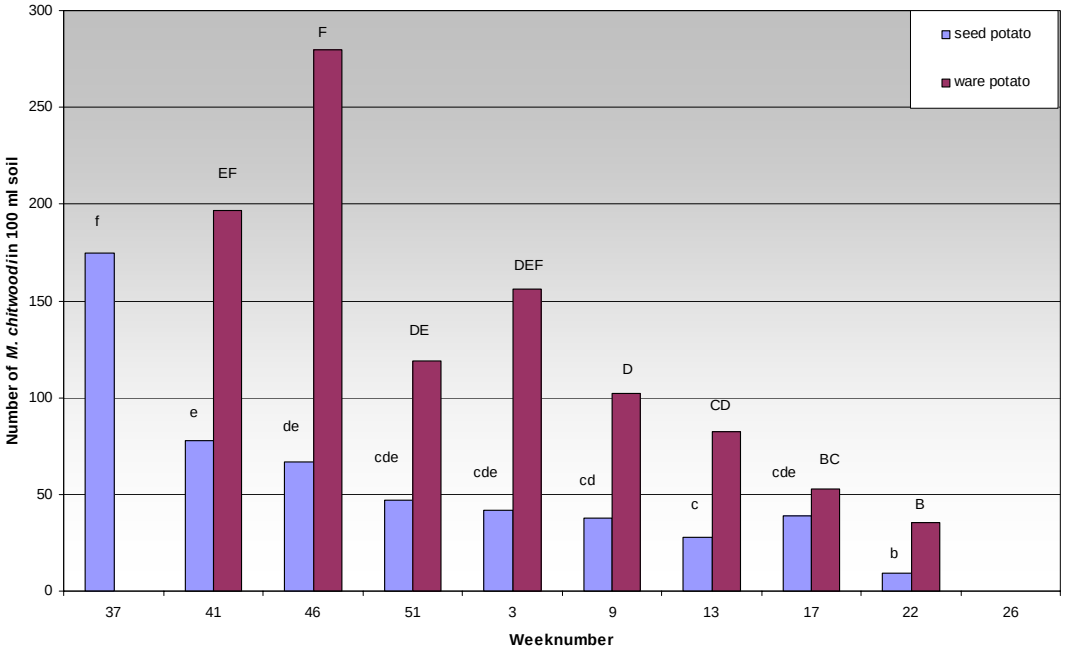

Figure 3.1. Average number of M. chitwoodi in total soil layer (0 – 25 cm), Smakt 2002-2003 (same letter means no significant difference between medians, at P = 0.05).

At location Wieringermeer seed potatoes were harvested in week number 37 of 2003. After harvest 13 soil samples were taken from week 37 in 2003 until more than one year later (week 43, 2004). Ware potatoes were harvested five weeks after seed potato in week number 42 in 2003. After harvest 12 soil samples were taken until week number 43 in 2004. Results from Wieringermeer are presented in Table 3.3 and in Figure 3.2.

Table 3.3. Number of Meloidogyne chitwoodi in 100 ml soil, mineral and organic soil fraction combined. Wieringermeer 2003-2004

	Seed potato				Ware potato			
soil sample/ weeknr [1]	0 – 5 cm	5 – 25 cm	0 – 25 cm		soil sample/ weeknr [1]	0 – 5 cm	5 – 25 cm	0 – 25 cm
Pi 18	38 c	38 b	38 B		Pi 18	22 c	22 b	22 b
Pf1 37 (0)	3271 i	12197 i	10564 I		-	-	-	-
Pf2 42 (5)	636 gh	2401 h	2058 H		Pf1 42 (0)	2955 g	4435 e	4233 f
Pf3 46 (9)	1033 h	1510 gh	1415 gh		Pf2 46 (4)	1907 fg	2023 de	2187 ef
Pf4 51 (14)	350 f	1121 efg	968 fg		Pf3 51 (9)	737 ef	911 d	908 d
Pf5 2 (17)	433 fg	1348 fg	1178 G		Pf4 2 (12)	1082 efg	1222 d	1367 de
Pf6 6 (21)	322 ef	814 def	727 ef		Pf5 6 (16)	832 ef	1350 d	1276 de
Pf7 10 (25)	184 de	504 d	476 de		Pf6 10 (20)	403 e	855 d	789 d
Pf8 14 (29)	352 f	1093 efg	957 fg		Pf7 14 (24)	548 e	1394 d	1265 de
Pf9 18 (33)	126 d	714 de	600 de		Pf8 18 (28)	102 d	316 c	277 c
Pf10 22 (37)	109 d	491 d	417 D		Pf9 22 (32)	75 d	257 c	222 c
Pf11 33 (48)	7 b	80 c	66 C		Pf10 33 (43)	5 b	31 b	26 b
Pf12 38 (53)	2 a	14 a	12 A		Pf11 38 (48)	1 ab	7 A	6 a
Pf13 43 (58)	1 a	15 a	12 A		Pf12 43 (53)	1 a	3 a	3 a

[1] *Between brackets the number of weeks after harvest (week of harvest being week 0).*

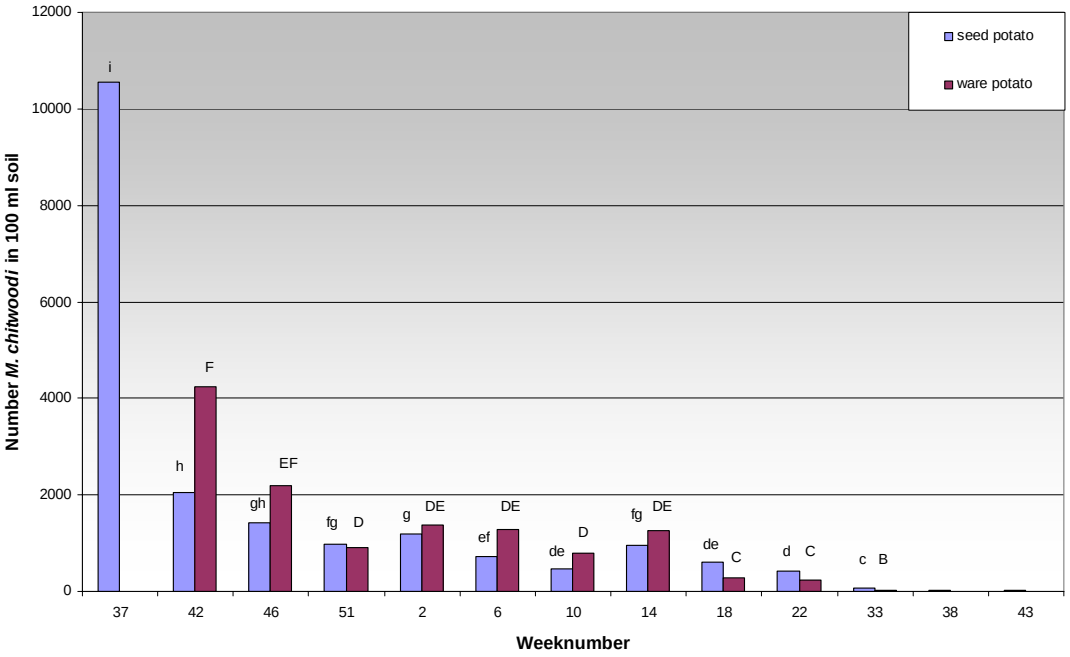

Figure 3.2. Average number of M. chitwoodi in total soil layer (0 – 25 cm), Wieringermeer 2003-2004 (same letter means no significant difference between medians at P = 0.05).

According to the results presented in Table 3.2 and 3.3 the population of *M. chitwoodi* nematodes increased strongly during potato cultivation. At Wieringermeer the size of the population before the potato cultivation (initial population or Pi) was already higher than at Smakt and during crop growth the number of nematodes at Wieringermeer increased enormously, leading to an extreme high final population densities shortly after harvest.

A likely explanation seems that the year 2003 was quit warm and dry, at which Meloidogyne had a higher multiplication. One other possible explanation could be that the local Meloidogyne population found in the Wieringermeer has a higher reproduction capacity.

As expected the total number of Meloidogyne found at both sites and in most sampling weeks, were higher after ware-house potatoes than after seed-potatoes. This is the consequence of the longer growing period of ware-house potatoes, in which more Meloidogyne could feed and multiply.

At both locations and for both cultivations population density of *M. chitwoodi* decreased in time. During this period mature Meloidogyne eggs hatch spontaneously if soil temperature is higher than 5C. These infectious juveniles (J2's) largely depend on their energy reserves, since they do not feed between hatching and penetration of the host plant (Christophers, 1995). If there is no host the J2 juveniles will die, resulting in declining numbers during a period of fallow. It seems that the rate of decline decrease at the end. At Smakt halfway the next year some population levels were below detection limit. At the Wieringermeer site the nematode numbers were an order of magnitude higher than at the Smakt site after harvest, and a consequently more time was required before the population densities decreased below the detection limit of the used sampling method. At Wieringermeer more than one year after harvest *M. chitwoodi* could still be detected, although the number of nematodes especially in the soil layer between 0 and 5 cm then became very low.
At both locations more *M. chitwoodi* were found in the soil layer from 5 to 25 cm than in the soil layer from 0 to 5 cm. This is probably a consequence of a combination of fewer roots growing in the 0-5 cm soil layer and a higher natural decline due to higher fluctuations in temperature and moisture.

3.3.2 Modeling the population development of *M. chitwoodi*

The population decline was modeled with an exponential curve using Genstat (8th edition), with a Generalised Lineair Model (GLM, with link = logarithm and distribution is negative binomial). The number of *M. chitwoodi* for ware and seed potato at both soil layers (0 to 5 cm and 5 to 25 cm depth) were use as input. The course of the *M. chitwoodi* population in time can be described using equation 1.

$$Pf = A * e^{-B * w} \quad (1)$$

Description of parameters in equation (1)

- Pf = number of *M. chitwoodi* nematodes in 100 ml soil
- w = time (number of weeks after harvest)
- A = size of the population at harvest in 100 ml soil
- B = rate of decline of the population in a week

The results of these analyses at location Smakt are presented in Figure 3.3.

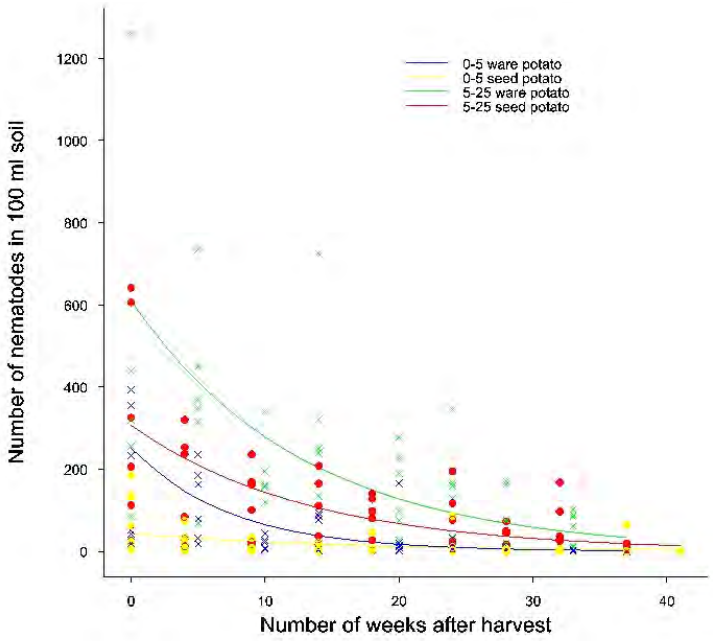

Figure 3.3. Development of M. chitwoodi population in time, Smakt 2002-2003.

With this model only 50% of variance could be explained. Especially data from ware potatoes in the soil layer 5 to 25 cm deviated considerably from the calculated population size. To a lesser degree this applied also for seed potatoes in the same soil layer. To get better results and a higher degree of explained variance, an adapted model to predict population development is used in the next paragraph.
Data from Wieringermeer were treated in the same way and are presented in Figure 3.4.

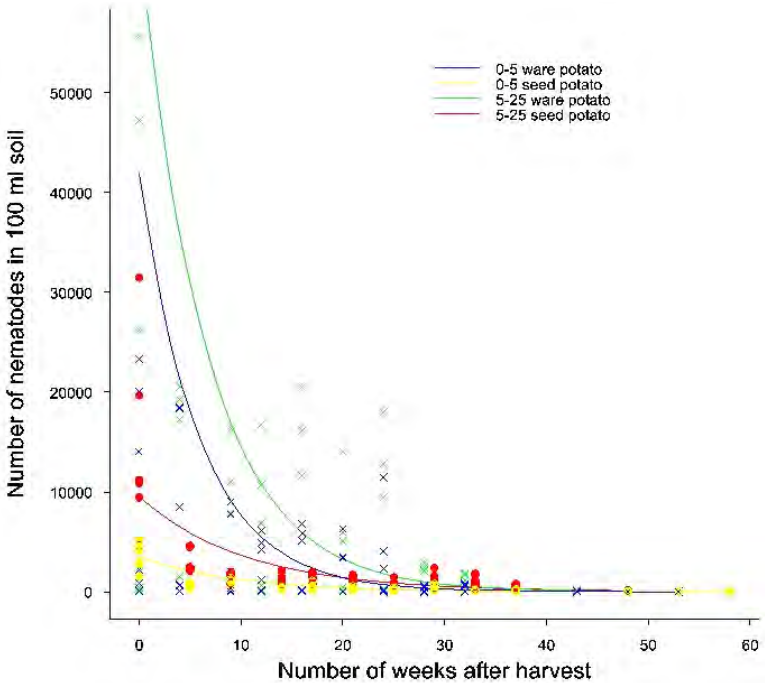

Figure 3.4. Development of M. chitwoodi population in time, Wieringermeer 2003-2004.

At Wieringermeer the predicted population density directly after harvest was very high for both ware and seed potatoes. But the model could only explain 57 percent of the variance. Especially in the period between 10 and 25 weeks after harvest (during winter) for ware potatoes in the soil layer from 5 to 25 cm, quite a number of observations deviated strongly from the population size which was predicted by the model. Decrease of the population density seems to be less fast than predicted by this (simple) negative binomial model. In Table 3.4 and 3.5 estimates for the parameters A and B for both cultivations are presented; separate per soil layer and for the topsoil as a whole.

Table 3.4. Parameters of the GLM analysis of M. chitwoodi densities, Smakt 2002-2003.

Cultivation	Soil layer (depth in cm)	Log factor A	Factor A		Factor B	
Seed potato	0 – 5	3.74	42	a	- 0.06	b
Seed potato	5 – 25	5.73	307	b	- 0.08	b
Ware potato	0 – 5	5.52	251	b	- 0.14	a
Ware potato	5 – 25	6.41	608	b	- 0.08	b
Seed potato	0 – 25	5.53	252	a [1]	- 0.075	a
Ware potato	0 – 25	6.27	529	a	- 0.080	a

[1] *Analysis of the topsoil (0 to 25 cm) was done separately from those of the two soil layers. Letters of significance of the topsoil therefore can not be compared with those of the soil layers.*

At Smakt with seed potatoes there were no significant differences in the rate of decrease of the population between the two soil layers. For ware potatoes however factor B in soil layer 0 to 5 cm was significantly higher than for the other rate parameters. In the topsoil as a whole the initial population for ware potatoes was higher than for seed potatoes and same holds for the rate of decrease of the population. Differences between cultivations were not significant.

Table 3.5. Parameters of the GLM analysis of M. chitwoodi densities, Wieringermeer 2003-2004.

Cultivation	Soil layer (depth in cm)	Log Factor A	Factor A		Factor B	
Seed potato	0 – 5	8.15	3457	a	- 0.11	b
Seed potato	5 – 25	9.16	9494	b	- 0.10	b
Ware potato	0 – 5	10.64	41928	c	- 0.17	a
Ware potato	5 – 25	11.08	65000	c	- 0.15	a
Seed potato	0 – 25	9.02	8248	a [1]	- 0.096	a
Ware potato	0 – 25	10.99	58972	b	- 0.151	b

[1] *Analysis of the topsoil (0 to 25 cm) was done separately from those of the two soil layers. Letters of significance for the topsoil therefore can not be compared with those of the soil layers.*

At Wieringermeer the predicted population size (factor A) directly after harvest, was significant higher for ware potatoes than for seed potatoes. Concerning the rate of decrease (factor B) of the population there was no significant difference between soil layers (0 to 5 cm and 5 to 25 cm), but there was a significant difference between cultivations because the decreasing factor was much higher for ware than for seed potatoes.

Also for the topsoil as a whole the size of the population at harvest was significant higher for ware potatoes than for seed potatoes, as was the rate of decrease. Although the population size at harvest for ware potatoes was much higher than for seed potatoes, due to the higher decreasing rate for ware potatoes, half a year after harvest both populations had a comparable size.

3.3.3 Adapted models in population development of *M. chitwoodi*

To reach a better fit of the data than in the preceding paragraph, in this paragraph for Smakt and for Wieringermeer an adapted model is proposed. For both locations distinction is made between the number of *M. chitwoodi* in the mineral fraction, the organic fraction and for the total number (mineral and organic fraction combined). In this paragraph population density of *M. chitwoodi* is always presented as numbers in 100 ml soil (unless otherwise is indicated). The numbers were statistically analyzed using Genstat and subsequently incorporated in the models (equations) which are presented.
The number of *M. chitwoodi* decreased in time. When population density was expressed as the (natural) logarithm of the number of *M. chitwoodi*, the population decreased shortly after harvest and also during a relatively long period after harvest. This decline followed more or less a straight line. During an intermediary period however the decrease was very slow and in this (winter)period there even seems to be more or less a 'standstill' in population density.
The course of (the logarithm) of the population size also depended on the cultivation (ware of seed potato) and the fraction analyzed (mineral, organic or total fraction). For both locations this pattern of the logarithm of the population density was manifest, but unfortunately it could not be described for both locations with the same equation. For Wieringermeer a 'linear – sigmoid' equation could be applied. But this could not be used for the trial at Smakt (because the statistical model using this equation didn't converge). Therefore for Smakt a less sophisticated 'polynomial' model was used to describe population decline in time.

3.3.3.1 Location Smakt

The decrease of the population density of *M. chitwoodi* in time at Smakt is described by a 'third grade polynomial' equation, described in equation 2 below:

$$\log Pf = \beta_0 + \beta_1 * w + \beta_2 * w^2 + \beta_3 * w^3 \qquad (2)$$

Description of the variables in equation 2

- log Pf: natural logarithm of the number of *M. chitwoodi* (in 100 ml soil)
- w: time in number of weeks after harvest
- $ß_0$, $ß_1$, $ß_2$, $ß_3$: parameters

In Table 3.6 parameters for this equation are presented for each cultivation (ware or seed potato), soil layer and fraction of nematodes (mineral fraction, incubated or total). Parameter B_0 indicates the size of the 'initial' population at time zero that is shortly after harvest. According to this model, the course of the (natural logarithm of the) population at Smakt is visualized in the graphics 1 to 12 of the Figures 5, 6 and 7.

Table 3.6. Parameters B0, B1, B2 and B3 as estimates for ß0, ß1, ß2 and ß3 for each cultivation, soil layer and fraction, Smakt 2002-2003.

Graphic nr	Potato cultivation	Soil-layer	Fraction	Parameter				Explained variance (%)
				B0	B1	B2	B3	
1	ware	0 – 5	mineral	3.36	- 0.17	0.009	- 0.00018	77
2	seed	0 – 5	mineral	3.20	- 0.18	0.005	- 0.00008	73
3	ware	5 – 25	mineral	5.05	- 0.15	0.013	- 0.00032	83
4	seed	5 – 25	mineral	4.91	- 0.21	0.013	- 0.00027	81
5	ware	0 – 5	organic	4.34	- 0.21	0.004	- 0.00005	59
6	seed	0 – 5	organic	2.21	- 0.16	0.006	- 0.00007	25
7	ware	5 – 25	organic	4.34	- 0.03	0.002	- 0.00010	65
8	seed	5 – 25	organic	3.74	- 0.26	0.013	- 0.00021	72
9	ware	0 – 5	total	4.84	- 0.23	0.009	- 0.00019	74
10	seed	0 – 5	total	3.65	- 0.21	0.007	- 0.00010	57
11	ware	5 – 25	total	5.65	- 0.15	0.013	- 0.00034	83
12	seed	5 – 25	total	5.25	- 0.24	0.014	- 0.00029	86

In all graphs of Figures 3.5, 3.6 and 3.7 a decrease of the population density is visible. Compared to the results of the preceding paragraph, the percentage explained variance of the population density is clearly higher (Table 3.6). With this adapted model the course of the population in time can be explained much better than with the exponential model of paragraph 1.3. However this doesn't hold for the organic fraction of seed potatoes in the soil layer between 0 and 5 cm where the percentage explained variance was very low. It seems that this was mainly caused by the very high number of *M. chitwoodi* in one of the plots of this treatment (plot number 140) during winter. The sigmoid curve of the course of the population is visible for both cultivations especially in the mineral fraction and in the total fraction. In the organic fraction the sigmoid character of the curve only becomes visible for seed potatoes in the soil layer from 5 to 25 cm.

For ware potatoes the period of a standstill of the population seems to be between week 5 and 25 after harvest. Roughly this is the period between half of November and half of March. For seed potatoes this period starts a little bit later between week10 and 30 after harvest. However because seed potatoes at Smakt were harvested 4 weeks earlier than ware potatoes, for seed potatoes this 'period of stabilization' lies also between half of November and half of March. It is very likely that this phenomenon is caused by the lower soil temperature during winter.

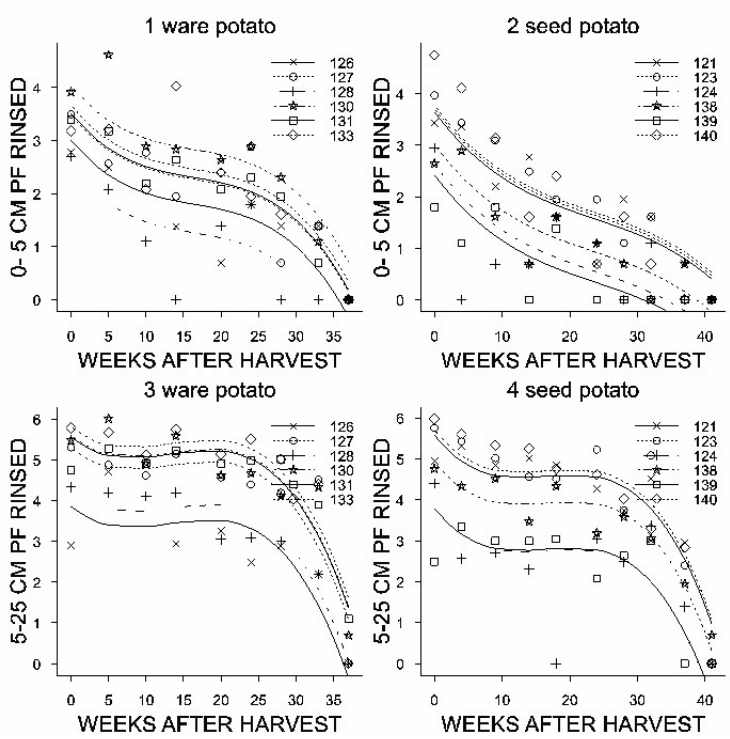

Figure 3.5. Development of the log number of M. chitwoodi in time for the mineral fraction, Smakt 2003-2004.

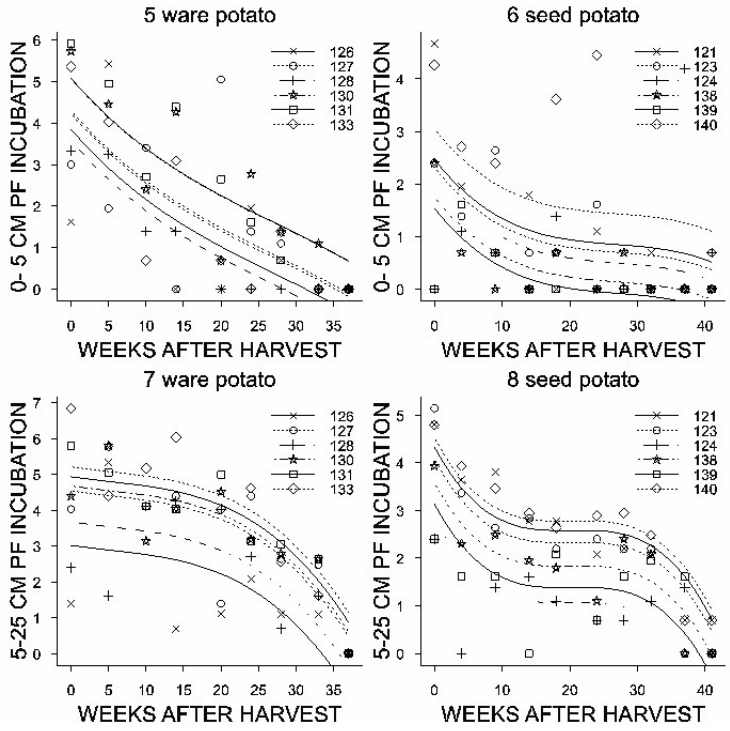

Figure 3.6. Development of the log number of M. chitwoodi in time for the organic fraction, Smakt 2003-2004.

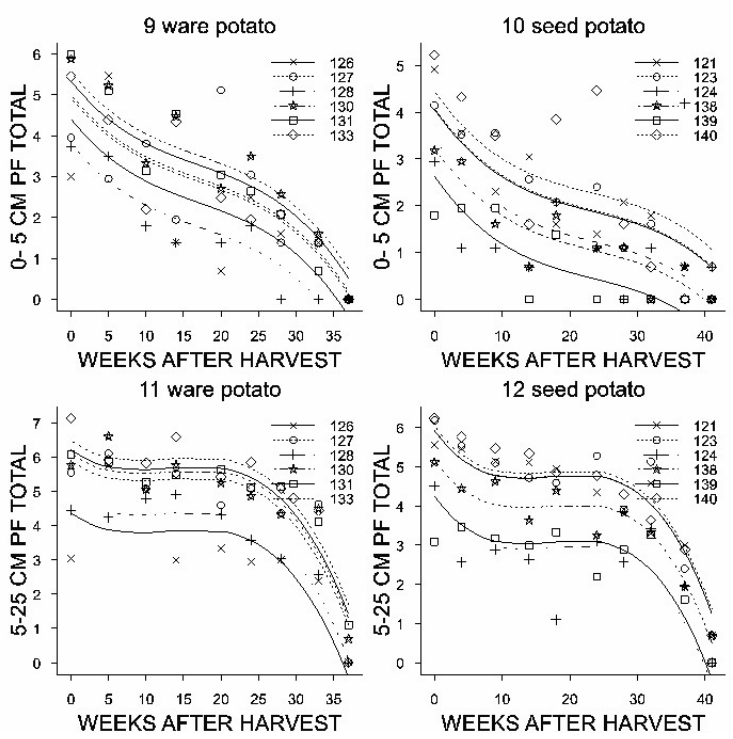

Figure 3.7. Development of the log number of M. chitwoodi in time for the total fraction, Smakt 2003-2004.

3.3.3.2 Location Wieringermeer

For location Wieringermeer the linear–sigmoid equation could be applied, which is presented below:

$$\log Pf = \alpha + \beta * w + \frac{\gamma}{1 + e^{-\lambda(w-\mu)}} \qquad (3)$$

Description of the variables in equation 3

- log Pf = natural logarithm of the number of *M. chitwoodi* (in 100 ml soil)
- α = the (natural logarithm of the) size of the population at harvest (in 100 ml soil)
- ß = rate of decline of the population in a week
- w = time as number of weeks after harvest
- γ = asymptote of the sigmoid term
- λ = rate parameter of the sigmoid term
- μ = bending point of the sigmoid term

The first two terms of equation 3 are describing a straight line with intercept α en slope ß. The third term describes an s-shaped curve (sigmoid curve) that, while time increases, causes an increase of the intercept from α to (α+γ). For ware potatoes there was a huge difference between plots in numbers of *M. chitwoodi* shortly after harvest. This varied from approximately tens to tens of thousands of specimen in 100 ml soil.
When the data of all plots of ware potatoes where incorporated in the model, the model couldn't converge and didn't deliver any results. A population size of tens of *M. chitwoodi* in 100 ml soil after cultivation of a good host, doesn't seem to be very representative for a practical field situation. For further analysis therefore the data of ware potatoes ware restricted to plots with a (very) high population size (plots 13, 14 and 15 of the dataset). With this restriction the model indeed did converge and delivered results (see the Figures 3.8, 3.9 and 3.10). By using this restriction the applicability of the model is of course decreased.

The parameters of the equation are presented in Table 3.7 for each cultivation, soil layer and fraction of *M. chitwoodi*. According to this model the course of the (natural logarithm of the) population size at Wieringermeer, is reflected in the graphs 1 to 12 of the Figures 3.8, 3.9 and 3.10.

Table 3.7. Parameters A, L, M. G and B as estimates for α, λ, μ, γ and ß for each cultivation, soil layer and fraction of M. chitwoodi, Wieringermeer 2003-2004.

Graphic nr	Potato cultivation	Soil-layer	Fraction	Parameter					Explained variance (%)
				A	L	M	G	B	
1	ware	0 – 5	mineral	9.20	0.27	18.48	5.44	- 0.27	96
2	seed	0 – 5	mineral	7.21	0.14	20.06	5.09	- 0.21	93
3	ware	5 – 25	mineral	9.72	0.26	19.22	5.96	- 0.26	99
4	seed	5 – 25	mineral	8.39	0.15	22.57	5.82	- 0.21	93
5	ware	0 – 5	organic	9.43	0.40	10.65	3.46	- 0.26	94
6	seed	0 – 5	organic	*	*	*	*	*	*
7	ware	5 – 25	organic	9.98	0.39	10.55	3.79	- 0.25	94
8	seed	5 – 25	organic	7.70	0.22	23.58	3.26	- 0.19	90
9	ware	0 – 5	total	9.87	0.23	13.79	5.16	- 0.28	97
10	seed	0 – 5	total	7.54	0.19	20.23	4.35	- 0.20	82
11	ware	5 – 25	total	10.43	0.24	14.04	4.70	- 0.24	98
12	seed	5 – 25	total	8.95	0.16	23.80	5.36	- 0.21	92

Equation 3 couldn't be adapted for the data of the incubation fraction of seed potatoes in soil layer 0 to 5 cm (in this case the model didn't converge). So for this case a third degree polynomial model (the same as for Smakt, see paragraph 1.4.1) was used to describe development of the population in time. The values for the parameters of this model were respectively: B_0: 6.276, B_1: - 0.18, B_2: 0.003 and B_3: - 0.003 and the model could explain 77 percent of the variance of the data.

For the organic fraction of seed potatoes in the soil layer 5 to 25 cm, the linear-sigmoid model initially also couldn't be applied. This was caused by the plots 19 and 20 who showed a deviating, capriciously number of *M. chitwoodi*. When these two plots were not incorporated in the analysis, the linear-sigmoid model could be applied. The results presented in Table 6 only refer to the four remaining plots of this object (see also graphic number 8 of Figure 9). By this restriction of data the applicability of the linear-sigmoid model is of course decreased.

At Wieringermeer in most cases more than 90 percent of the variance of the data could be explained by the linear-sigmoid model. This was higher than with the polynomial model at Smakt (see Table 5). Only for the total fraction of seed potatoes in the soil layer 0 to 5 cm, 82 percent of the variance of the data could be explained. This exception can be clarified because the organic fraction is incorporated in the total fraction and as described above the linear-sigmoid model could not fit the organic fraction. For ware potatoes the rate of decrease of the population size (described by parameter B in the equation) was higher than for seed potatoes. The differences in the rate of decrease between the fractions of *M. chitwoodi* (mineral, incubation, total) were very small, as were the differences between the soil layers. Parameter M indicates the centre of the 'stabilization' period. For seed potatoes this is some weeks earlier than for ware potatoes, which is explained by the earlier date of harvest of the previous cultivation.

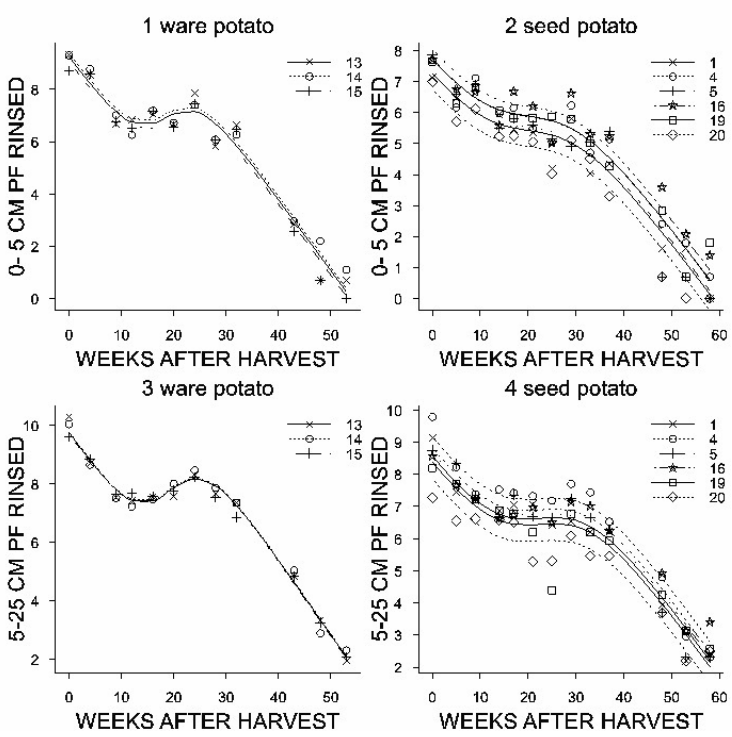

Figure 3.8. Development of the log number of M. chitwoodi in time for the mineral fraction, Wieringermeer 2003-2004.

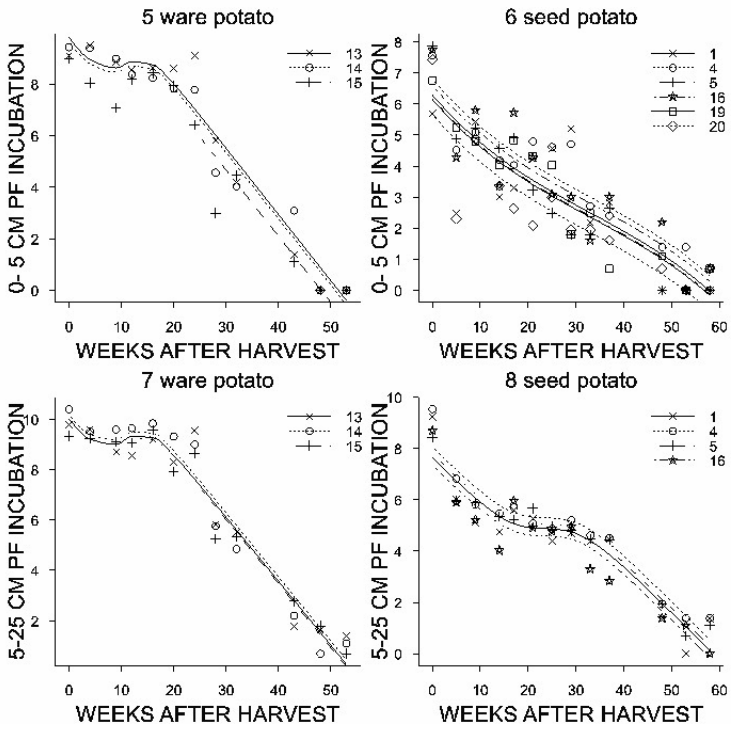

Figure 3.9. Development of the log number of M. chitwoodi in time for the organic fraction, Wieringermeer 2003-2004.

In almost all graphs of Figures 3.8, 3.9 and 3.10 a sigmoid curve is visible. But this is clearly not the case for the organic fraction of seed potatoes in the soil layer between 0 and 5 cm (graphic number 6, Figure 9). So once again this situation (seed potato, organic fraction, soil layer 0 – 5 cm) deviated from the other ones, for the data of this object where analyzed using another model as well.

For ware potatoes the period of a very slight alteration of the population size seems to be between week 10 and 25 after harvest. Approximately this is between half of December and half of March. For seed potatoes the period of (very) slow decrease of the population size appears a little bit later between week 15 and 30 after harvest. While at Wieringermeer seed potatoes were harvested 5 weeks earlier than ware potatoes, the 'stabilization' period also seems to be between half of December and half of March for seed potatoes.

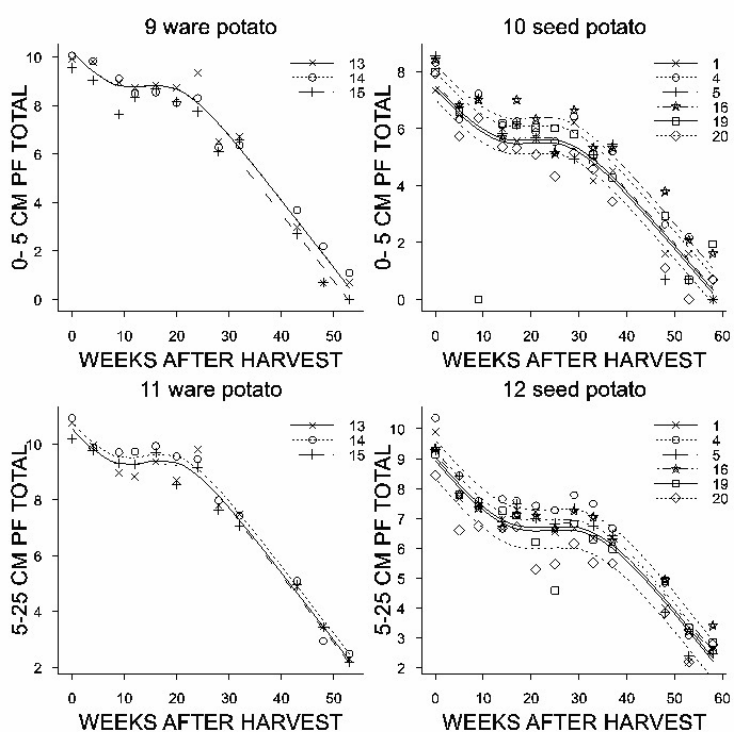

Figure 3.10. Development of the log number of M. chitwoodi in time for the total fraction, Wieringermeer 2003-2004.

3.3.4 Quality of potatoes

Data on the quality of potatoes, based on visual symptoms outside and inside of the potato tuber, are summarized in Tables 3.8, 3.9 and 3.10.

Table 3.8. Population densities of M. chitwoodi before and after harvest of the potato crop and symptoms found in Asterix seed potatoes. Smakt 2002.

Plotnr.	Pi (n/100 ml)	Pf (n/100 ml)	Class 0+1	Class 0	Class 1	Class 2	Class 3	Class 4	% Visual symptoms outside
124	0	94	97	Nd.	Nd.	41	12	0	35,33
121	65	287	8	Nd.	Nd.	31	109	2	94,67
123	6	497	12	Nd.	Nd.	56	82	0	92,00
139	1	22	71	Nd.	Nd.	74	5	0	52,67
140	0	550	25	Nd.	Nd.	44	78	3	83,33

Table 3.9. Population densities of M. chitwoodi before and after harvest of the potato crop and symptoms found in Asterix seed potatoes. Wieringermeer 2003.

Plotnr.	Pi (n/100 ml)	Pf (n/100 ml)	Class 0+1	Class 0	Class 1	Class 2	Class 3	Class 4
1	32	16063		32	2	6	0	0
4	22	26008		25	1	14	0	0
16	114	9872		31	2	7	0	0
19	45	8137		37	0	3	0	0
20	13	4246		33	2	5	0	0

Table 3.10. Population densities of M. chitwoodi before and after harvest of the potato crop and symptoms found in Asterix seed potatoes. Per plot 30 tubers were tested. Smakt 2004.

Plotnr.	Pi * (n/100 ml)	Pf * (n/100 ml)	Class 0+1	Class 0	Class 1	Class 2	Class 3	Class 4	% visual symptoms outside
3	0	407	0	Nd.	Nd.	10	18	2	100,00
4	0	15	0	Nd.	Nd.	20	6	4	100,00
5	0	2	6	Nd.	Nd.	19	5	0	80,00
15	0	5	0	Nd.	Nd.	19	7	4	100,00
17	1	57	3	Nd.	Nd.	23	4	0	90,00
20	1	162	4	Nd.	Nd.	19	7	0	86,67
24	0	155	11	Nd.	Nd.	17	1	1	63,33
26	1	1	14	Nd.	Nd.	13	3	0	53,33
31	0	9	1	Nd.	Nd.	20	8	1	96,67
34	1	4	7	Nd.	Nd.	18	5	0	76,67
36	0	353	13	Nd.	Nd.	12	4	1	56,67
	0	1	13	Nd.	Nd.	16	1	0	56,67

* *Both Pi and Pf are from 2003, potatoes are grown in 2004.*

In the data of 2002 Smakt it can be seen that irrespective of the Pf densities found in soil, the tubers had always a relatively high percentage of symptoms. Even without a distinction between class 0 and 1, more than 50% of the tubers were placed in class 2, 3 or 4. A similar result was found for the potatoes from Smakt 2004. For the WM field experiment in 2003 the opposite was found: very high densities of *Meloidogyne* in soil and very low number of symptoms found for the potatoes.

3.4 Conclusion and discussion

3.4.1. Modeling population dynamics of *M. chitwoodi*

At Wieringermeer the population density of *M. chitwoodi* was, with tens of thousands of specimens in 100 ml soil a few days after harvest, extremely high. At Smakt the population density was several hundreds of specimen in 100 ml of soil a few days after harvest, which is quite common for the (final) population density of *M. chitwoodi* after a good host.
At both locations for ware potatoes the number of *M. chitwoodi* was higher than for seed potatoes, which corresponds with the expectations while the former cultivation has a longer growing period and therefore allows more time for reproduction to nematodes.
For both trials it appears that *M. chitwoodi* occurs in high numbers in both soil layers (0 to 5 cm and 5 to 25 cm). Although the number of *M. chitwoodi* is lower in the top layer than in the deeper one, for quite a long time *M. chitwoodi* is also detectable in the top layer. At Smakt *M. chitwoodi* could still be detected in the top layer more than 30 weeks after harvest and at Wieringermeer *M. chitwoodi* could be detected one year after harvest.
If population density of *M. chitwoodi* is described with a rather (simple) negative exponential equation, the size of the population in time can be characterized with two parameters (in this report these parameters are indicated as A and B respectively). The first one, parameter A, describes the estimated size of the population at time of harvest. For ware potatoes this parameter is (much) higher than for seed potatoes. The second parameter B quantifies the decline of the population size in time and indicates that population size decreases in time with a fixed factor. In absolute numbers a high initial population therefore declines shortly after harvest much faster than a low initial population. At Smakt parameter B was relatively low and only for ware potatoes in the soil layer between 0 and 5 cm this factor was significantly higher than for the other situations.
Concerning the topsoil as a whole at Smakt factor B seemed to be higher for ware than for seed potatoes, but this difference was not significant. At Wieringermeer, where population size shortly after harvest was much higher than at Smakt, 'decline' factor B was higher than at Smakt and there was no difference between both soil layers at this location. As in Smakt, the 'decline' factor was higher for ware than for seed potatoes, at Wieringermeer this difference was significant.

With an adapted model the numbers of *M. chitwoodi* in the mineral fraction and in the organic fraction can be distinguished from one another. When the natural logarithm of the number of nematodes is plotted against time (expressed as weeks after harvest), in many cases a sigmoid curve appears. The middle part of this sigmoid curve is more or less flat, which means that during this period the number of *M. chitwoodi* hardly decreases. At Smakt this 'stable' period roughly lies between half of November and half of March and at Wieringermeer between mid of December and mid March. This implies that the population during winter hardly or not declines and that this period of 'stabilization' at Smakt approximately starts a few weeks earlier than at Wieringermeer (probably while soil temperature in the sandy soils Smakt sooner drops below a certain 'threshold' temperature).

The number of *M. chitwoodi* in the mineral fraction appears to be a good indication of the total numbers (mineral and incubated fraction) of this nematode. At Wieringermeer there was also a good relation between the course of the population size in the soil layer between 0 and 5 cm and that in the soil layer between 5 and 25 cm. At Smakt this relation was less good than at Wieringermeer. The number of *M. chitwoodi* nematodes in the mineral fraction of a soil sample in layer between 0 and 5 cm shortly after harvest, appears to be a rather good indication of the total number of this nematode in the topsoil. However to increase reliability and reach a higher chance of detection of *M. chitwoodi*, a soil sample of the topsoil (0 to 25 cm) as a whole remains the best.

4. Multiplex Real-time PCR for detection of *Meloidogyne chitwoodi* and *M. fallax* and practical applications

4.1 Introduction

Meloidogyne chitwoodi and *M. fallax* have acquired importance as quarantine pests. Identification and early detection of these species is necessary for inspection of harvested crops or soil samples, for design of crop rotation systems and for research purposes. The aim of this project was to develop a reliable method to identify *M. chitwoodi* and *M. fallax* in harvested products of the hosts of these nematodes, e.g. tubers, bulbs, roots of plants and use this method to evaluate the EU-method that uses visual inspection of symptoms to detect these nematodes.

4.2 Detection of *Meloidogyne chitwoodi* and *M. fallax*

4.2.1 Methods to detect *M. chitwoodi* and *M. fallax*

Considerable effort has been put in the development of detection assays for *M. chitwoodi* and *M. fallax*. Protein based assays have been described as well as numerous DNA based methods. Most of these DNA based assays use PCR-amplification followed by gel-electrophoresis to visualize the sizes of the amplified products.
To limit the number of detection assays required, some approaches enabling simultaneous detection of *M. chitwoodi* and *M. fallax* in a single test have been developed. However, all of them have their specific disadvantages. Some multiplex PCR tests require for every species to be detected a species specific primer set in the reaction. This enables the multiplex detection of both *M. chitwoodi* and *M. fallax* but from our experience the sensitivity of such multiplex assays is decreased compared to the simplex assays using only one species specific primer set. The occurrence of primer interactions is attributed to this decreased PCR efficiency.
Alternatively, PCR assays that can detect *M. chitwoodi* and *M. fallax* in a single assay using only two primers are hypothetically more sensitive. However, size differences of the amplicons can be too small for routine testing and confusing DNA banding patterns can be caused by heteroduplex formation.
A different approach that can be used for multiplex detection is real-time PCR.

4.2.2 Real-time PCR

Real-time PCR takes advantage of the 5' -> 3' nuclease activity of *Taq* DNA polymerase in conjunction with one or more fluorogenic DNA probes. The real-time PCR TaqMan system uses TaqMan probes that are labelled at each end with a reporter and a quencher dye. The probes hybridize to an internal region of a real-time PCR product during the annealing stage. While the probe is intact, fluorescence emitted by the reporter is absorbed by the quencher. During PCR amplification, the hybridized TaqMan probe is cleaved by *Taq* DNA polymerase during strand elongation, resulting in an increase in reporter fluorescence. By using multiple specific probes multiple species can be detected in a single assay. The measurement of fluorescence throughout the reaction by a fluorometer eliminates the need for post-PCR processing steps, easing automation of the technique and large-scale sample processing. The reaction tubes remain closed throughout the assay, so there is a reduced risk for contamination of the PCR mixture with target DNA, originating from previous reactions. Carryover contamination is also prevented by the use of uracil N-glycosylase (UNG) and the use of dNTP's with additional UTP in all PCRs. The incorporation of a sequence-specific probe in combination with increased efficiency due to the short amplicons enhances sensitivity and specificity.
Real-time PCR enables quantification of initial target DNA. This is done by determining the Cycle Threshold (Ct)-value, which is the number of PCR cycles necessary to increase fluorescence above background. The more template DNA is present, the sooner fluorescence arises, the lower the Ct value.

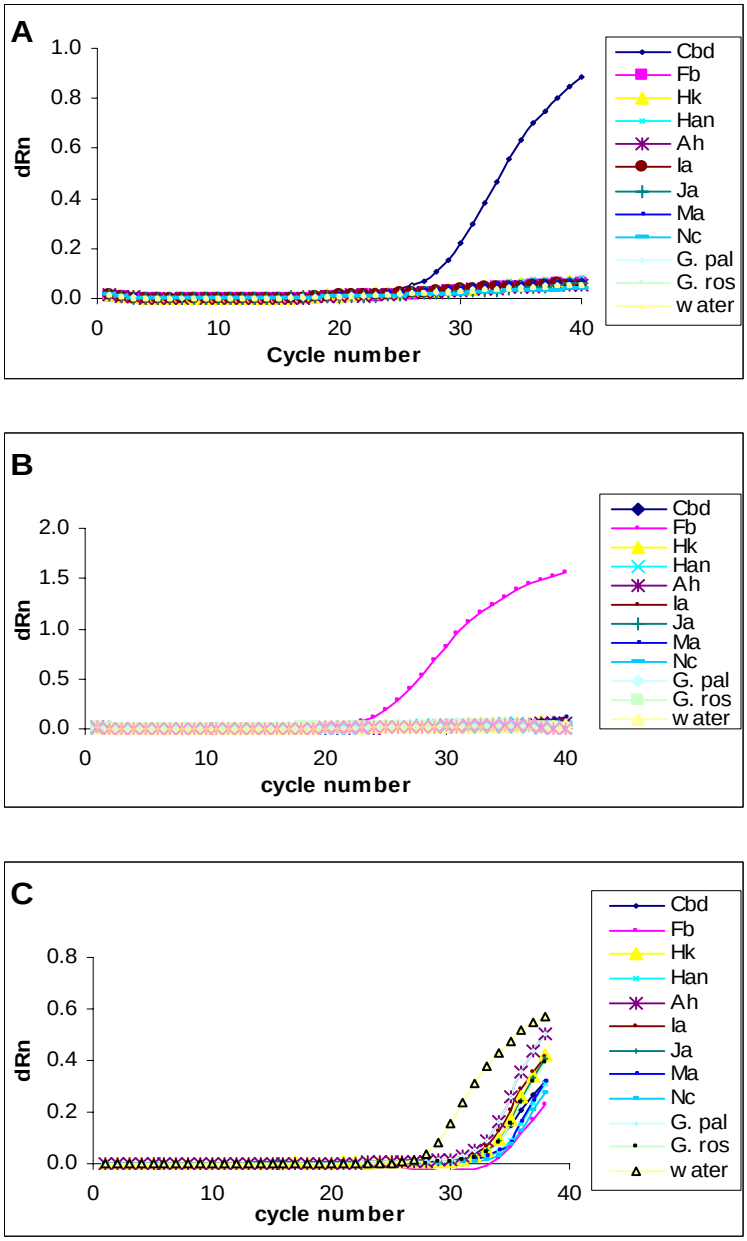

Figure 4.1. *Specificity of the multiplex real-time PCR for M. chitwoodi and M. fallax. Typical results of amplification plots showing the testing of 3 ng amounts of genomic DNA of isolates of eight Meloidogyne species and G. pallida and G. rostochiensis using the multiplex real-time PCR. A: detection of M. chitwoodi measuring FAM signal; B: detection of M. fallax measuring VIC signal; C: detection of IAC-DNA measuring NED signal. Cbd: M. chitwoodi; Fb: M. fallax; Hk and Han: M. hapla; Ah: M. arenaria; Ia: M. incognita; Ja: M. javanica; Ma: M. minor; Nc: M. naasi; G.pal: G. pallida; G.ros: G. rostochiensis.*

4.2.3 Multiplex real-time PCR test for *M. chitwoodi* and *M. fallax*

A multiplex real-time PCR approach was developed for the simultaneous detection of *Meloidogyne chitwoodi* and *M. fallax* in a single assay that offers many advantages over current tests for multiplex detection of these quarantine organisms with respect to speed, sensitivity, quantification and reliability, (Zijlstra & Van Hoof, 2006). It utilizes three fluorogenic Minor Groove Binding (MGB) TaqMan probes: one FAM labelled to detect *M. chitwoodi*, one VIC labelled to detect *M. fallax* and one NED labelled to detect the internal amplification control (IAC) which enables the elimination of scoring false negative results. One common primer set is used for the amplification of part of the ITS region of *M. chitwoodi* and *M. fallax* and one primer set for the amplification of the IAC. In this multiplex real-time PCR the NED signal of the IAC should always be produced even when no *Meloidogyne* target is available. When no NED signals are measured for amplification of the internal control, this indicates that the test conditions (incorrect PCR mixture, failure of the thermal cycler, presence of inhibitory substrates in the sample) did not allow PCR during the assay and that the results of the test can not be trusted. This prevents the scoring of false negative results. The test is specific. FAM respectively VIC fluorescence can only be measured when the assay contains DNA of *M. chitwoodi* (Figure 4.1A) respectively *M. fallax* (Figure 4.1B). No FAM or VIC signals are obtained when DNA isolated from *M. hapla*, *M. naasi*, *M. minor*, *M. incognita*, *M. javanica*, *M. arenaria, G. pallida* or *G. rostochiensis* is used as template DNA. The NED signal of the IAC is observed in every reaction (Figure 4.1C).

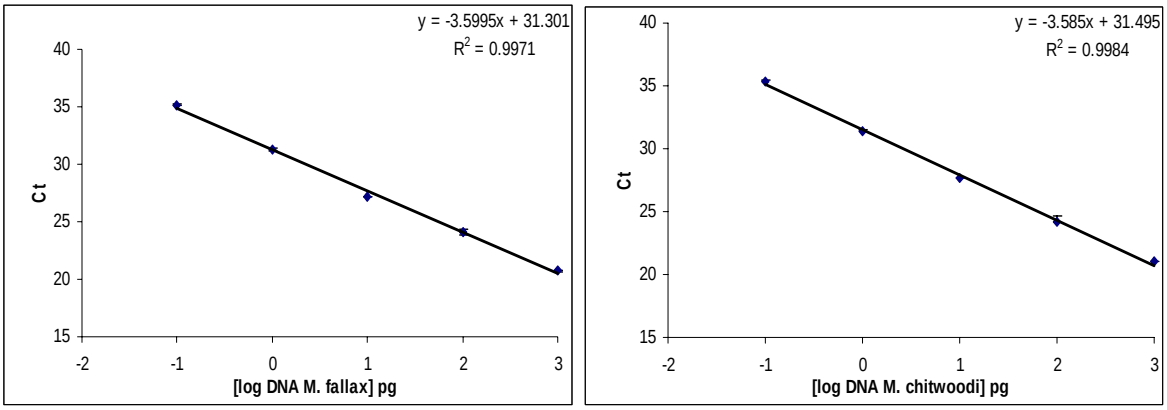

Figure 4.2. Standard curves demonstrating the quantification of genomic DNA of M. chitwoodi (A) and M. fallax (B) using the multiplex real-time PCR. Ct values were plotted against the log of DNA amounts of M. chitwoodi (A) or M. fallax (B). The equation of the regression line and its R^2-value are displayed in the graph.

Genomic DNA amounts of *M. chitwoodi* and *M. fallax* as low as 100 fg could still easily be detected when the test was performed on purified genomic DNA extracted from *Meloidogyne* juveniles (Figure 4.2). Taking into account that a nematode consists of approximately 1000 cells this is the equivalent of approximately 0.001 *Meloidogyne* juvenile. By comparing the level of sensitivity of the multiplex real-time PCR assay with that of a regular PCR also targeting the ITS sequence, the multiplex real-time PCR turned out to be at least ten times more sensitive than the tested agarose gel electrophoresis based detection. Amplicons originating from 1 pg DNA of *M. chitwoodi* or *M. fallax* could occasionally be observed on the agarose gel whereas template DNA amounts of 10 pg or more always resulted in visible amplicons. Template amounts of less than 1 pg DNA never resulted in visible amplicons, whereas the presented multiplex real-time PCR could still easily detect DNA amounts of 100 fg.

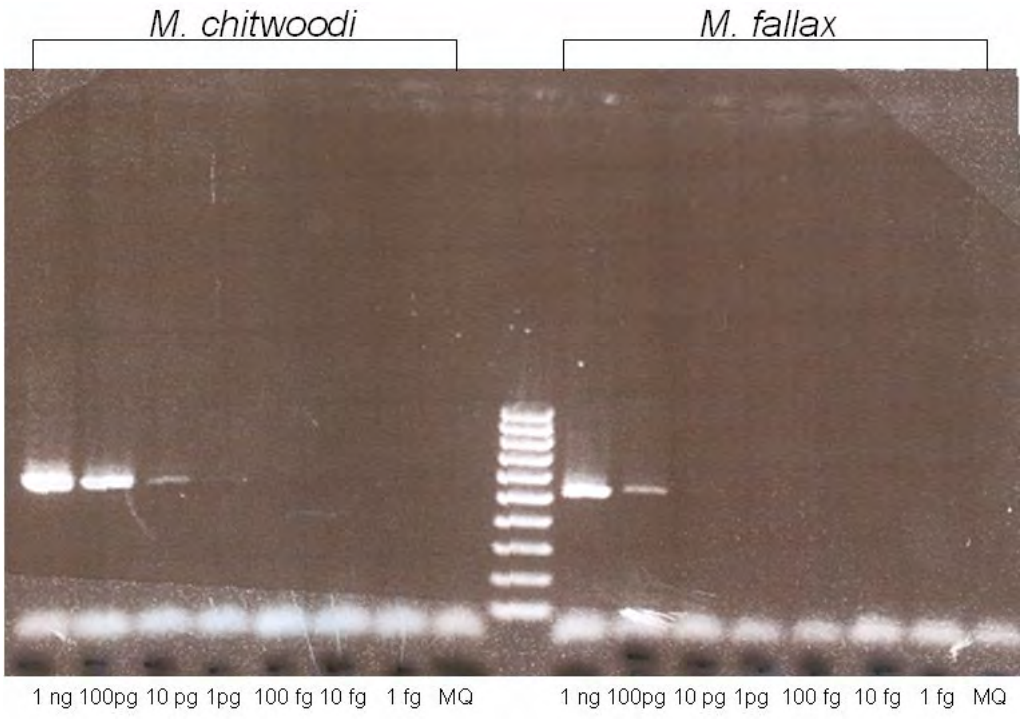

Figure 4.3. Agarose gel showing amplification products of PCR reactions using ITS primers CF-ITS and HCFI-28S (Zijlstra, 1997) using different template amounts (1 fg-1 ng) of genomic DNA of M. chitwoodi (left panel) and M. fallax (right panel) as indicated below the picture. MQ: water.

4.3 Practical applications of the multiplex real-time PCR test for *M. chitwoodi* and *M. fallax*

4.3.1 Detection of *M. chitwoodi* and *M. fallax* in roots of samples of infested bulbs

Samples of bulbs had been harvested from fields that were known to be infected with *M. chitwoodi* or *M. fallax*. DNA was extracted from these samples of roots of the following bulbs: Crocus 'Geel', Iris 'Blue magic', Iris 'Telstar' and Tulipa 'Monte Carlo'. Table 4.1 shows the species that had been identified morphologically in samples from soil from which the plants had been harvested. Figure 4.4 shows pictures of the material prior to DNA extraction, from which can be seen that the material to be analyzed was in poor condition.

Figure 4.4. Part of root of Iris 'Telstar' (A), bulbs with roots of Iris 'Telstar' (B) and Crocus 'Geel' (C); and bulb with roots of Tulipa 'Monte Carlo' (D).

For each DNA sample, multiplex real-time PCRs were done twice. Ct values of FAM, VIC and NED are shown in Table 4.1. The higher the NED Ct values, the worse PCR conditions were. The reactions with Crocus 'Geel' A2 and Tulipa Monte Carlo, that showed for both succeeding experiments Ct values of 40 for FAM and VIC, indicating that no *M. chitwoodi* and *M. fallax* amplification took place, also showed high NED Ct values. This means that PCR conditions were poor so that we can not conclude whether *M. chitwoodi* and/or *M. fallax* were present in the samples tested. The FAM and VIC Ct values of Iris Telstar E8 were relatively high at 071005 indicating that both nematode species were not present or only at very low amounts. The FAM and VIC Ct values at 311005 were 40 and the NED Ct value was high, indicating that amplification conditions were poor so that from these Ct values nothing can be concluded. In all other reactions PCR conditions are supposed to be favourable enough according to their NED Ct values of 31.5 to 35.15. The real-time PCR analysis of 071005 of Crocus 'Geel'G2 indicated the presence of *M. chitwoodi* due to the FAM Ct of 27.13. Surprisingly the experiment of 311005 showed FAM and VIC Ct values of 40 whereas the NED Ct value was 32.14. It is assumed that despite of the fact that amplification conditions were fine, no amplification of the target DNA took place due to break down of the target DNA. Results of the experiments performed on the other samples led to similar conclusions in both repetitions. With respect to interpretation of the FAM and VIC Ct values it is difficult at this moment to define a cut-off value, since not enough data for detection in bulbs are available, especially not in material in such poor condition. The lower the Ct value, the more certain one can be that *M. chitwoodi* or *M. fallax* is present. Predictions of diagnosis by real-time PCR are shown in Table 4.1. These are not in contradiction with the results of identification of *M. chitwoodi* and *M. fallax* in soil based on morphological characters.

This experiment has shown that it is possible to detect *M. chitwoodi* and/or *M. fallax* in roots of bulbs. When material of a better quality is used, and cut-off values are determined for this type of material, results will most likely easier to be interpreted.

Table 4.1. FAM, VIC and NED Ct values of the multiplex real-time PCR assays for M. chitwoodi and M. fallax on roots of bulbs. Tests were performed on 07/10/05, 31/10/05 and 10/11/05. Mc: M. chitwoodi present; Mf: M. fallax present; Mf?: M. fallax perhaps present; ?: not clear due to unreliable results; ND: no data due to temporary breakdown in power supply. The last column shows the results of the morphological identification of juveniles present in the soils where the bulbs have been grown.

Date	071005			311005			Diagnosis by	Morphology
Material tested	FAM	VIC	NED	FAM	VIC	NED	real time PCR	in soil
Crocus 'Geel' A2	40.00	40.00	38.14	40.00	40.00	38.97	?	Mf
Crocus 'Geel', G2	27.13	38.98	32.83	40.00	40.00	32.14	Mc	Mc
Iris 'Blue magic', A7	36.34	33.54	35.15	40.00	30.30	32.10	Mf	Mf
Iris 'Blue magic', F7	33.79	40.00	32.47	26.60	40.00	33.45	Mc	Mc
Iris 'Telstar', D8	40.00	37.20	33.86	40.00	32.20	32.46	Mf	Mf
Iris 'Telstar', E8	38.00	36.90	33.54	40.00	40.00	36.78	?	Mc
Tulipa 'Monte Carlo'	40.00	40.00	37.45	40.00	40.00	36.50	?	Mf
Water	40.00	40.00	31.60	40.00	40.00	31.50	-	

4.3.2 Relation between the presence of Meloidogyne symptoms on potato tubers and the presence of *M. chitwoodi* in these tubers measured by the real time PCR assay

Potato tubers of cultivar Asterix were harvested from experimental field sites in 2002, 2003 and 2004. From the field sites of 2002 and 2003 soil samples were analyzed for the presence of *M. chitwoodi*. Soil samples for assays of *M. chitwoodi* nematodes were collected before planting in March or April (resulting in the mean preplant population density for the field (Pi)) and after tuber harvest in September (resulting in the final nematode density (Pf)). In the centre of each plot (2 m x 3 m) 35 soil cores (diameter 12 mm) from the top 0-5 cm and from the 5-25 cm were taken in a regular pattern. Of each soil layer a sub sample of 100 cc of soil served to extract nematodes. This soil sample was first sieved over a 180 mμ sieve with water, after which nematodes in the suspension were collected by using an Oostenbrink elutriator. The remaining organic matter fraction with mainly root debris was incubated at 20°C for 28 days to allow egg hatching and emergence of motile endoparasitic stages. The total number of nematodes was established by counting approximately 20% of the total sample under a dissecting microscope. Results of Pi and Pf of the field sites of 2002 and 2003 are shown in Table 4.2.

Table 4.2. Initial population densities (Pi) and final population density (Pf) of M. chitwoodi on potato cultivar Asterix on different test sites with different soil textures, and number of harvested tubers per class in 2002 and 2003. Numbers of tubers per class analysed by TaqMan analysis are between brackets.

Year	Test site	Field site and soil texture	Pi[a]	Pf[b]	No. per class[c]						Total analysed
					0/1	0	1	2	3	4	
2002	121	Smakt: sandy soil	65	235	8(8)	Nd	Nd	31(9)	109(30)	2(2)	150(49)
	123	(95% sand, <1%	6	400.4	12(12)	Nd	Nd	56(20)	82(25)	0(0)	159(57)
	124	lutum, 3.1% silt	0	75.6	97(96)	Nd	Nd	41(15)	12(12)	0(0)	150(123)
	139	content) organic	1	17.8	71(69)	Nd	Nd	74(25)	5(5)	0(0)	150(99)
	140	matter: 2.3%, pH 6.7	0	447.7	25(25)	Nd	Nd	44(15)	78(25)	3(3)	150(68)
2003	1	Wieringermeer: sandy	32	16063	34(34)	32(32)	2(2)	6(6)			40(40)
	4	soil (90% sand, <2%	22	26008	26(26)	25(25)	1(1)	14(14)			40(40)
	16	lutum, 5.9% silt	114	9872	33(33)	31(31)	2(2)	7(7)			40(40)
	19	content) organic	45	8136.7	37(37)	37(37)	0(0)	3(3)			40(40)
	20	matter content: 1.4, pH 7.4	13	4246	35(35)	33(33)	2(2)	5(5)			40(40)

[a] *Pi = number of nematodes per 100 cc of soil at planting.*

[b] *Pf = number of nematodes per 100 cc of soil after harvest.*

[c] *Number of harvested tubers in class; 0/1: no outside symptoms; 0: no symptoms outside or inside; 1: symptoms inside but no symptoms outside; 2: just visible symptoms outside; 3: clearly visible symptoms outside; 4: abundantly present symptoms outside.*

Nd: Not determined.

Each year after harvest the tubers were stored at 4C until the first visual examination for symptoms at t = 0. Tuber damage ratings, so called PAV-Knot-Index (PKI) ratings were done using a scale from 0 to 4 as follows: 0 = no symptoms outside or inside. 1 = no symptoms outside but females an/or eggs visible inside; 2 = just visible symptoms outside; 3 = clearly visible symptoms outside; 4 = abundantly present symptoms outside. Each potato was first analyzed for external symptoms. Subsequently tubers were peeled at three random spots to see whether eggs and/or females were present inside. In general PKI's were determined for 30 randomly selected potatoes per plot. After this first inspection tubers were stored at 18 C in order to permit root-knot nematode development. After 8 weeks, t = 8, the temperature sum of 2150 degree days had been reached and final PKI's were determined.

In 2002 750 tubers were harvested and divided in classes by visual inspection of symptoms (Table4.2). From these 750 tubers 396 tubers were selected. These tubers were peeled and DNA was extracted from the peel. A precursor of the multiplex real-time PCR for *M. chitwoodi* and *M. fallax* (including a pre-amplification step) was performed on the resulting 396 DNA samples. Results are summarized in Table 4.3. In none of the tubers *M. fallax* was detected. The tubers of class 3 and 4 with clearly visible symptoms were all shown to contain *M. chitwoodi* by the real-time PCR assay. In 3.6% of the class 2 tubers, those which showed some symptoms, the real-time PCR did not detect *M. chitwoodi* or *M. fallax*. In 57.6% of the class 0/1 tubers, those tubers without symptoms on the outside, the real-time PCR detected *M. chitwoodi*. This seems to be an alarming number. However, no distinction was made between class 1 tubers, tubers in which eggs or females were visible after cutting 3 pieces of potato peel, and class 0 tubers, in which no eggs or females were visible after cutting 3 pieces of potato peel. In other words, it is not clear how many of these class 0/1 tubers would have been classified as class 1. In the succeeding experiments visual examination had divided class 0 from class 1 tubers.
In 2003 200 tubers, of classes 0, 1, and 2, were analyzed by the multiplex real-time PCR for *M. chitwoodi* and *M. fallax*, (Table 4.2). In Figure 4.5A can be seen that not all class 0 tubers from the field trial of 2003 gave a FAM Ct of 40 as would have been the case when they would not contain *M. chitwoodi*. A certain percentage gave FAM Ct values lower than 40, varying from 22.1 to 39.6.

Table 4.3. *Number of tubers of field trial in 2002 per class based on PKI, categorized based on results of the multiplex real-time PCR test. Percentages are shown between brackets. M.c: M. chitwoodi; M.f: M. fallax.*

Results real-time PCR	Class			
	0/1	2	3	4
M. chitwoodi present	121 (57.6)	81 (96.4)	97 (100)	5 (100)
No M.c or M.f present	62 (29.5)	3 (3.6)	0	0
Result not reliable	27 (12.9)	0 (0)	0	0
Total number tested	210	84	97	5

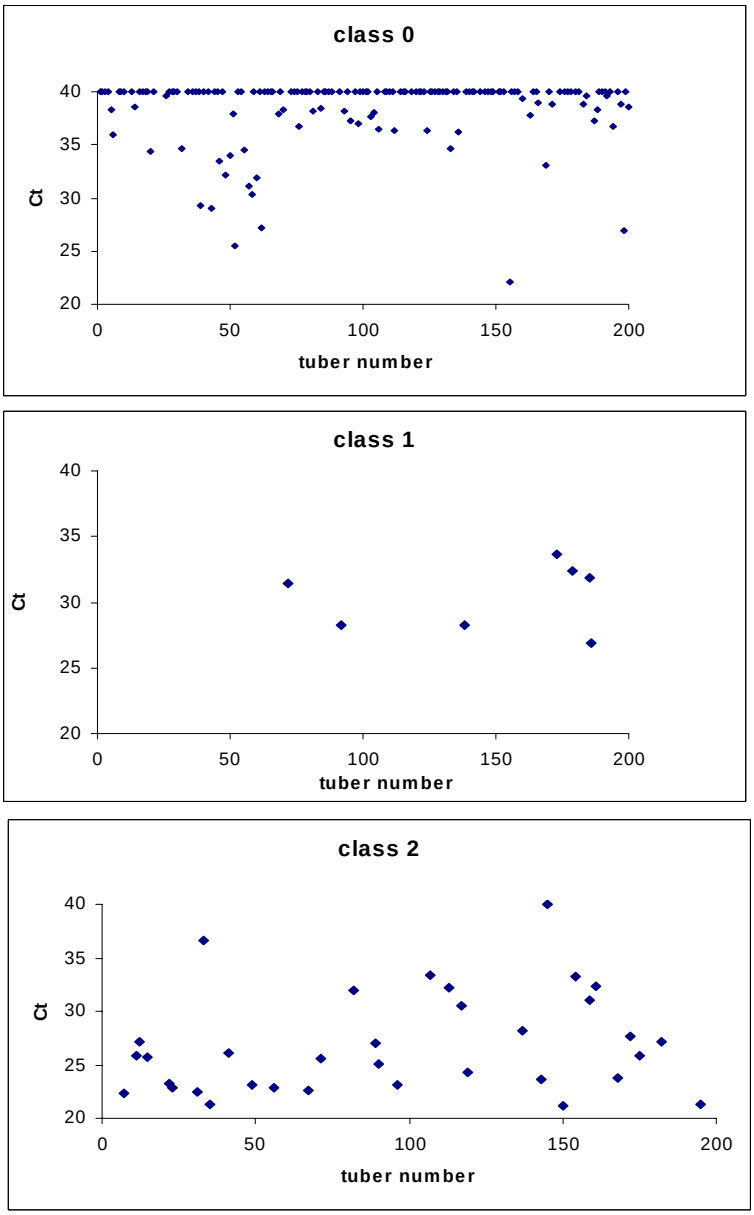

Figure 4.5. *Ct values of real-time PCR analyses performed on DNA samples extracted from peels of potato tubers per class, harvested in the field trial from 2003.*

To test until which infection level of potato's the real time PCR assay could still reliably detect the presence of *M. chitwoodi* in potato tubers, a reconstruction experiment was set up that resembled the testing of potatoes that were infested in the range of 0.1 egg/cm2 of potato peel to 1000 eggs/cm2 potato peel. Results (Figure 4.6) revealed that a FAM Ct value of 35, corresponding to detection of an infection level of approximately 1 egg/cm2 of potato peel (approximately 1 egg mass per tuber), could be seen as a cut-off value: all tubers tested giving a FAM Ct value of 35 or lower were supposed to contain *M. chitwoodi*. However, the reconstruction experiment indicated that a

FAM Ct value of 38.25, corresponding to detection of an infection level of 0.1 egg mass per tuber, has a SD of 1.06, which might be acceptably low enough for reliable detection.
Moreover, since it would have too serious consequences to qualify an infected tuber as healthy, we would consider tubers with a FAM Ct between 35 and 40 as suspicious. This classification based on FAM Ct values enabled us to determine which percentage of classes of tubers studied was considered to either contain *M. chitwoodi*, to not contain *M. chitwoodi* or to be suspected to contain *M. chitwoodi*. In combination with the Ct values for the IAC, reliability of the results could also be distinguished. Concluding results for the tubers tested in 2003 are summarized in Table 4.4. Interestingly, in 10.8% of the tubers without noticed symptoms outside or inside, the test clearly detected *M. chitwoodi,* whereas in another 18.3% of the class 0 tubers the presence of *M. chitwoodi* was suspected. In all class 1 tubers, those without outer symptoms but with symptoms inside, *M. chitwoodi* was detected by the test. The total percentage of infected tubers including the suspicious tubers of class 0/1 was 32.1%. This is lower than the percentage infected class 0/1 tubers that had been identified in the field trial of 2002 which was 57.6%. Interestingly the Pi's and Pf's were much lower in the field trial in 2002 compared to the field trial in 2003 whereas you would have expected it to be the other way around. More research is needed to explain this phenomenon.

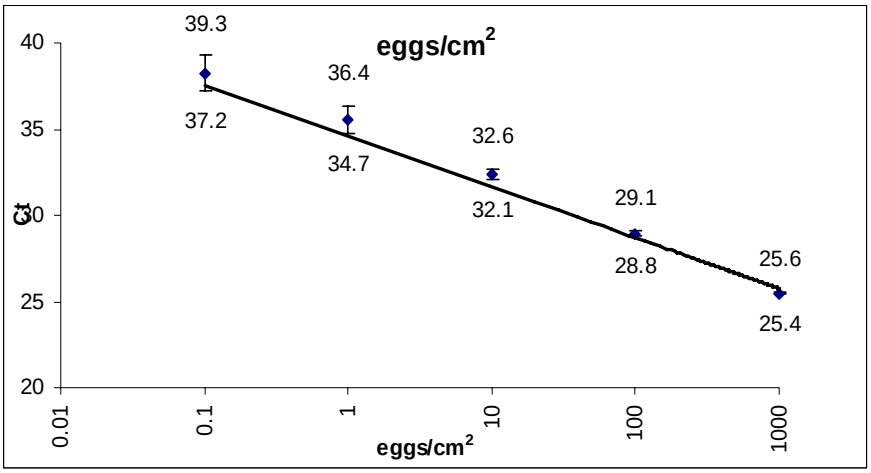

Figure 4.6. Standard curve demonstrating the results of multiplex real-time PCR assays resembling the testing of potato tubers that were infested with known amounts of eggs/cm2 of potato peel. Ct values were plotted against number of M. chitwoodi eggs/cm2 present in the tuber tested.

4.3.3 Testing whether real-time PCR analysis can save time with diagnosis of tubers

According to the council directive 2000/29/EG the visual inspection of tubers to determine whether the tubers are infected with *M. chitwoodi* or *M. fallax* is done at time at harvest and at 8 weeks after time of harvest as described in paragraph 4.3.2. To test whether there would be a difference in TaqMan results performed on DNA from tubers extracted at time of harvest (t=0) and those performed on DNA from tubers that were stored for another 8 weeks (t=8), 150 potato tubers from the field trial in Smakt in 2004 were selected in such a way that at time of harvest 60

tubers were class 0/1, 40 class 2, 40 class 3 and 10 class 4. From half of the amount of tubers of each class DNA was extracted at time of harvest (t=0). Upon peeling, from the 30 class 0/1 tubers 3 were classified as class 0 and 27 as class 1. Numbers per class analyzed are shown in Table 4.5. The remaining tubers were visually classified 8 weeks after storage at 18C (t=8). Subsequently DNA was extracted and Real-time analyses were performed on all t=0 and t=8 tubers. Results of the Real-time analyses are shown in Table 4.5. A two-way ANOVA was preformed to test whether there are differences between classes and/or time after harvest for the FAM Ct value and the NED Ct value. Interaction between time and class was not significant and there were no significant differences between t=0 and t=8 for each class. The FAM Ct value showed a significant decrease from class 0 to 4 at both times. For the NED Ct value the overall F-test showed no significant difference between classes.
This means that the multiplex real-time PCR assay can be successfully assessed at time at harvest whereas diagnosis by the visual inspection is finalized only 8 weeks after harvest. Using the multiplex real-time test for *M. chitwoodi* and *M. fallax* this saves 8 weeks in time, eliminates the need for storage of tubers at 18 C (for those that have to be examined for symptoms) and at 5-8 C (for those that need to be processed or marketed), resulting in lower costs.

Table 4.4. Number of tubers (Asterix) of field trial in 2003 per class, categorized based on the Ct values obtained with the multiplex real-time PCR analysis. According to the real-time PCR analysis the tuber is considered to contain M. chitwoodi when CtFAM<35; the tuber is suspected to contain M. chitwoodi when 35<Ct<40 and CtNED<35; the tuber is considered to not contain M. chitwoodi when Ct FAM=40 and Ct NED<35; test results are considered not reliable when CtFAM=40 and CtNED>35.

		Class			
Ct FAM	Ct NED	0/1	0	1	2
<35	≤40	24	17	7	33
35<Ct<40	<35	29	29	0	1
40	<35	109	109	0	0
40	>35	3	3	0	1
Percentage not infected		66.1%	69.0%	0%	0%
Percentage infected		14.5%	10.8%	100%	94.3%
Percentage infected incl. suspicious tubers		32.1%	29.1%	100%	97.1%
Percentage unidentified		1.8%	1.9%	0%	2.8%

Table 4.5. FAM Ct values of the Real-time PCR analysis on the 150 potato tubers harvested from the field trial in 2004.

Tubers	Class	Total numbers analyzed	FAM Ct values *M. chitwoodi*				NED Ct values IAC			
			Mean	SD	Min	Max	Mean	SD	Min	Max
T=0	0	3	38.10a[1]	2.59	35.15	40	31.17a[1]	0.59	30.5	31.6
	1	27	30.52b	2.45	25.78	36.63	32.58a	1.62	29.1	36.1
	2	20	28.24c	3.06	25.25	40	32.85a	1.92	29.7	38.3
	3	20	25.70d	1.22	23.41	27.71	32.72a	1.28	30.4	34.83
	4	5	23.99d	1.26	22.38	25.31	32.96a	2.77	29.7	37
T=8	0	3	35.31a	8.13	25.95	40	31.67a	1.51	30.6	33.4
	1	27	29.55b	2.79	25.89	35.63	32.8a	2.07	29.8	40
	2	20	27.88c	2.36	24.78	35.18	33.33a	2.84	29.7	40
	3	20	26.68c	2.07	23.80	30.90	33.81a	3.65	29.1	40
	4	5	23.12d	0.79	22.54	24.38	33.56a	3.64	29.1	37

[1] *Displays the significant differences between all analyses.*

4.3.4 Sensitivity of the real time PCR assay when testing bulks of tubers

To find out how many tubers can be tested in a single real-time PCR assay in such a way that one tuber that is slightly infected will still be detected when all other tubers tested in the sample are not infected the following experiment was done.
Samples of freeze dried powder of tubers without *M. chitwoodi* and of tubers without inner and outer symptoms (class 0) but in which the multiplex real-time PCR assay had detected *M. chitwoodi*, were selected. Subsequently amounts of these powder samples (of slightly infected tubers) were mixed with 0, 4 or 9 equal amounts of powder of samples without *M. chitwoodi*, resembling powder extracts of the testing of samples of respectively 1, 5 or 10 tubers with only one slightly infected class 0 tuber. DNA was extracted and multiplex real-time PCR tests for *M. chitwoodi* and *M. fallax* were performed. FAM Ct values are shown in Table 4.6.

Table 4.6. FAM Ct values of multiplex real-time PCR analysis on mixtures of freeze dried powder samples from potato tubers that were slightly infected with M. chitwoodi and tubers that did not contain M. chitwoodi.

Tuber	Mixed with 0 parts of not infected potato powder	Mixed with 4 equal parts of not infected potato powder	Mixed with 9 equal parts of not infected potato powder
1	31.8	31.6	39.6
2	28	31.2	31.7
3	33.9	37.3	40
4	31.4	35.3	36

These preliminary results indicate that real-time PCR testing of bulks of 5 tubers or less allow the detection of one slightly infected tuber. When bulk samples of 10 tubers are used there is a risk that a single slightly infected tuber present in the bulk will not be detected anymore (see tubers 1 and 3 in Table 4.6 with FAM Ct values of 39.6 and 40). One should realise that this way of testing is much more sensitive than the generally used way by visual inspection, since the visual inspection would not determine the slightly infected tuber at all. When it should be necessary to scale up the number of tubers in a bulk sample, one could decide to accept a less sensitive detection. In that case the above mentioned experiment could be repeated with class 2 tubers to find out how many tubers a bulk can contain so that one tuber with minor symptoms in it can still be detected.
Alternatively one could put more effort in optimizing the assay in terms of sensitivity.

4.3.5 Detection in potato tubers of different cultivars

Potato tubers of cultivars Arinda, Arnova and Kondor of class 0 to 3 were selected for analysis with the multiplex real-time PCR assay for *M. chitwoodi* and *M. fallax*. Table 4.7 shows the tested samples with their Ct values for FAM. VIC and NED from which easily can be concluded that all tubers of class 1, 2 and 3 tested contained *M. chitwoodi*, whereas no *M. chitwoodi* was detected in symptom less class 0 tubers. This means that the multiplex real-time PCR test for *M. chitwoodi* and *M. fallax* also enables detection of *M. chitwoodi* in potato tubers of other cultivars than Asterix.

Table 4.7. Ct values for FAM, VIC and NED of the multiplex real-time PCR analysis on tubers of potato cultivars Arinda, Arnova and Kondor, divided in classes.

Cultivar	Class	Ct values FAM	VIC	NED
Arinda	0	40	40	31.76
Arinda	1	32.6	40	33.12
Arnova	0	40	40	33.01
Arnova	2	27.78	40	33.51
Arnova	3	27.49	40	33.33
Kondor	2	32.7	40	32.31
Kondor	3	26.43	40	34.44

4.4 Conclusions

- A multiplex real-time PCR test for simultaneous detection of *M. chitwoodi* and *M. fallax* has been developed.
- The test was shown to be specific for detection of *M. chitwoodi* and/or *M. fallax*.
- The test can still easily detect DNA amounts of *M. chitwoodi* or *M. fallax* of 100 fg which is the equivalent of approximately 0.001 *Meloidogyne* juvenile.
- The test was shown to be at least 10 times more sensitive than a comparable conventional PCR assay. The test enable detection of 100 fg of genomic DNA whereas the comparable conventional PCR assay showed a detection limit of 1-10 pg genomic DNA.
- The test enables detection of *M. chitwoodi* and/or *M. fallax* in DNA extracted from nematodes of *M. chitwoodi* or *M. fallax*, in infected samples of roots of bulbs that were in poor condition (Iris, Crocus), as well as in tubers of different potato cultivars (Arinda, Arnova, Asterix, Kondor).
- The test is more sensitive in detecting *M. chitwoodi* in tubers than visual inspection of tubers for presence of symptoms. Generally the test always detected *M. chitwoodi* in tubers with outside symptoms. Test results with the multiplex real-time PCR test for *M. chitwoodi* and *M. fallax* indicated that, depending on the field site and year of harvest, 32.5% to 57.6% of the tubers without outer symptoms were infected with *M. chitwoodi*, which had not been noticed by visual inspection.
- The test can successfully diagnose at time at harvest whereas diagnosis by visual inspection is finalized only 8 weeks after harvest.
- Generally the test always detected *M. chitwoodi* in tubers with outside symptoms.
- Test results with the multiplex real-time PCR test for *M. chitwoodi* and *M. fallax* indicated that, depending on the field site and year of harvest, 32.5% to 57.6% of the tubers without outer symptoms were infected with *M. chitwoodi*.
- Testing of the tubers harvested in 2003 revealed that a considerable percentage of tubers without noticed symptoms outside or inside (class 0) may contain *M. chitwoodi*: in 10.8% of the class 0 tubers, the test clearly detected *M. chitwoodi*, whereas in another 18.3% of the class 0 tubers the presence of *M. chitwoodi* was suspected. In all class 1 tubers, those without outer symptoms but with symptoms inside, *M. chitwoodi* was detected by the test.
- Preliminary results indicate that testing of bulks of tubers with the multiplex real-time PCR assay allowing the detection of one slightly infected tuber of class 0 (without inner and outer symptoms after visual inspection) can be done when bulks consist of 5 tubers or less.

5. Synthesis

This document describes the research that was carried out during the years 2002-2006 (DWK-research program 397) to provide information and tools necessary for the detection of *Meloidogyne chitwoodi* and *M. fallax* in crop rotations with potatoes with special focus on: 1) the distribution patterns of *Meloidogyne* spp. to develop sampling strategies for detection, 2) population dynamics in time, especially the natural decline of *Meloidogyne* spp. after harvest and 3) methods to identify both species in harvested potatoes based on molecular techniques. This research was instigated with the ultimate goal of providing sound scientific information for a EU-recommended inspection method. At the end of this synthesis a method is proposed.

Distribution patterns of Meloidogyne spp. to develop sampling strategies for detection

During 2002 to 2006 research was carried out to map the medium and large scale distribution of *Meloidogyne chitwoodi* en *M. fallax* in 5 fields in the Netherlands and 2 in Belgium. Combined with data on the small scale distribution of both species from previous research it was possible to model both infestation foci and full field infestations and calculate detection probabilities using the simulation program SAMPLE IV. Calculations are based on 3 infestation foci and four full field infestations. There is still a need to obtain more datasets on mapped *Meloidogyne* infestations to acquire more reliable parameter sets for both models, especially the infestation focus model. As the infestation focus model applies for *Meloidogyne* spp. and detection was the primary goal of the research project all further analyses were carried out using the focus model.
Compared to infestations of the potato cyst nematode, from which cysts have to be collected when soil sampling for detection is carried out, Meloidogyne species yield 2nd stage juveniles, eggs and egg masses, which results in comparatively large numbers of detectable units when soil samples are collected and elutriated. Most elutriation methods will yield 2nd stage larvae and egg masses, but will loose the eggs. The proportion of the number of eggs to the total number is unknown. When the organic fraction is omitted a large fraction of the population, depending on the time after harvest of the host, will be disregarded. However, the number of detectable entities per unit of soil after a host crop is definitely higher for *Meloidogyne* spp. than for PCN. This will result in higher detection probabilities compared to cyst nematodes.
Research on elutriation techniques, comparing the Oostenbrink elutriator and the zonal or Hendricks centrifuge, revealed that laboratory error is extremely large (coefficient of variation of 50%) for this nematode. This seriously hampered a reliable population density estimation and measurement of the aggregation factor of the negative binomial distribution used to model the small scale distribution. It implies that the actual aggregation factor of the negative binomial distribution for *Meloidogyne* spp. is higher than that actually used in the calculations. It also implies that the densities measured within the foci and full field infestations mapped are actually less heterogenic and that, as a result, we will underestimate the detection probability in the calculations and overestimated the variability of population density estimations in the distribution pattern research. To compensate some of this error, larger samples have to be collected and processed as would be required if reliable methodology was available. As soon as better elutriation methods are available either the bulk sample size can be reduced or the same bulk sample size will yield better detection probabilities. Any method designed to detect an infestation focus of *Meloidogyne* spp. with a certain probability will perform even better when a full field infestation is encountered. Future research on elutriation methods has to be focused on improvement of the zonal centrifuge e.g. minimizing its variability or in upgrading proven concepts used in the past for contemporary use e.g. the Seinhorst elutriator for free living nematodes which can process up to 500cc of soil (Seinhorst, 1988).

Population dynamics in time, especially the natural decline of Meloidogyne spp. after harvest.

Population densities of *Meloidogyne* spp. decrease in winter when the soil is kept fallow. In several fields in both the Netherlands and in Belgium the population decline after harvest until sowing or planting of the next crop, and in most cases even beyond that period, has been established by periodic sampling. The information regarding the vertical distribution reveals that *Meloidogyne* spp. can be retrieved from the upper 5 cm of soil during the whole period between two crops. However, densities are lower in this layer compared to deeper layers and detection

would severely be hampered when only this soil horizon would be sampled. Two different models have been used to fit the data on population decline: a negative exponential function and a polynomial model. The latter cannot be used for predictive purposes. The former model fits with varying accuracy but has some severe drawbacks. If presumes that nematode densities will drop to zero in time. Although this seems a reasonable assumption, it has never been proven in the datasets analyzed, even when periodic sampling was prolonged throughout the year during absence of any host. Secondly, this is a statistical model which has to be parameterized for each host of *Meloidogyne* spp., which would require a tremendous amount of work to be carried out. However, the present data collected enable us to roughly estimate the decrease of the mapped infestations in time and the subsequent effect on the detection probability. In the current research project an average population decline of 80% could be established in the period between harvest and sowing or planting of the next crop. The effect on the detection probability of infestation foci was not as severe as expected; an average reduction of the detection probability of 28% within a range of central population densities of 500 up to 20000 juveniles per kg of soil was calculated. If a small infestation focus with a central population density of 500 juveniles/kg of soil (very young infection focus) is present after harvest detection will decline from 40% to 18% using the current AMEX200cc sampling method for PCN (whole suspension counted). An infestation focus with a central population density of 5000 juveniles/kg of soil will have a detection probability of 76% at harvest which will decease to 51% at the next crop. The larger the infestation (expressed as central population density) after harvest, the smaller the effect on the detection probability. In time, we find the largest drop in detection probabilities occurring during the first weeks after harvest as a result of an 'exponential' decline of population densities in the soil. Therefore, sampling directly after harvest of any host crop, but preferably a good host like will yield the highest detection probabilities.
The most important variable in this equation is the population density to be expected after different hosts if soil sampling prior to the next potato crop is envisaged. In terms of the focus model: what kind of central population densities are there to be expected? After potato, at the Wieringermeer site, population densities ranged from 1000 up to 500000 juveniles per kg of soil, the latter being an exception. After sugar beets population densities of 12000 juveniles/kg and after barley of 1200 juveniles/kg were found. Prior information collected by the PPO-agv (Table 2.2) also reveals high population densities. If these maximum densities can be regarded as estimates of the central population densities of a focus, detection probabilities are quite acceptable when compared to PCN. It will be necessary to compile a list of known maximum population densities at harvest as estimates of the 'maximum' population density for different host crops of *Meloidogyne chitwoodi* and *M. fallax*.

Methods to identify both species in harvested potatoes based on molecular techniques.

Laboratory research has been carried out to develop a new and rapid Multiplex Real-time PCR to establish the presence of *Meloidogyne chitwoodi* and *M. fallax* in potato tubers. This method proved to be more precise than visual inspection and was used to prove that large numbers of tubers (57.6% in 2002 and 10.8-31.1% in 2003) without visual symptoms but originating from an infested field were actually infected with *Meloidogyne chitwoodi* and *M. fallax*. The method can be applied directly after harvest, saving 8 weeks of incubation at 18 C. Up to 5 tubers could be processed as one lot in the laboratory, enabling the detection of slightly infected tubers without outer and inner symptoms. Thereafter, the method was released for practical application, which resulted in a scaled-up test which can process 100 tubers in one lot, enabling the possibility to use this test for statutory use.

A EU-recommended inspection method

Visual inspection of potato tubers is currently the official method for detecting the presence of *Meloidogyne chitwoodi* and *M. fallax*. The method is time consuming and unreliable, depending on the expression of the presence of the nematode in visual symptoms on the tuber, which extend is cultivar dependent. An alternative method would be soil sampling. The most appropriate time of sampling for detection is immediately after harvest of the host crop when population densities are highest. The nematode numbers then are divided between the mineral and organic fraction. If potatoes are the target crop, one has to choose between sampling prior to planting of the tubers – sampling after the last crop – or sampling after harvest when a possible infestation has been boosted up in densities by the potato crop. Although sampling after harvest of potatoes will yield a higher detection probability, it will also cause severe economic losses to the farmer. As an alternative one could choose to sample prior to cropping potatoes to establish whether the field if free of *Meloidogyne* spp. in order to obtain a 'licence to grow'.

A decision has to be made on what one wants to detect with known accuracy (e.g. what kind of focus or group of foci) if an infestation is present in the field under investigation. Analogous, with the potato cyst nematode, we can assume that when an infestation focus becomes detectable, approximately 3 secondary infestation foci will be present in the same strip. With potato cyst nematodes one infestation focus of 100 cysts/kg soil (central population density) and three infestation foci of 50 cyst/kg soil are presumed and the new proposed sampling method is targeted to detect this group of infestations. If we assume the same for *Meloidogyne* spp. we can calculate the combined detection probability for this event and modify the bulk sample size by adapting the core sample size until the same detection probability is obtained as for potato cyst nematodes. In the case of *Meloidogyne* spp. we target at 1 primary infestation, with a central population density of 500 juveniles/kg soil, and three secondary infestation foci, with a central population density of 250 juveniles/kg soil. The resulting method is presented in Table 5.1. To further increase detection the Real-time PCR method could be added.

Table 5.1. *The outline of a possible sampling method for Meloidogyne chitwoodi and M. fallax is presented. The method is analogous with the currently proposed sampling method for potato cyst nematodes, yielding approximately 90% detection probability for a conglomerate of 1 primary infestation focus (CPD 500 juveniles/kg) and three (CPD 250 juveniles/kg) secondary infestation foci. Length gradient: 0.85; width gradient: 0.73; aggregation factor: 5; Conversion cc to gram: 1,7. For comparison, the two new proposed sampling methods for potato cyst nematodes have also been accessed for the conglomerate of Meloidogyne foci.*

	Proposed method	400 cc/ha	1500 cc/ha
Area	1/3 ha	1 ha	1h
Sampling depth	25 cm	25 cm	25 cm
Sampling grid	7.5 x 7.5m	7.5 x 7.5m	7.5 x 7.5m
Number of cores	60	180	180
Core size	3.5 cc	2.22 cc	8.33 cc
Bulk sample size	210 cc	400 cc	1500 cc
Elutriated	210 cc	400 cc	1500 cc
Suspension counted	100%	100%	100%
Detection probability	90.1%	76.7%	99.6%

6. Samenvatting

Dit document geeft een overzicht van het onderzoek uitgevoerd in het kader van het DWK programma 397 in de jaren 2002 tot 2006. Het onderzoek was gericht op het verzamelen van de benodigde informatie en de ontwikkeling van gereedschappen, om de quarantaine aaltjes *Meloidogyne chitwoodi* en *M. fallax*, twee wortelknobbelaaltjes, op te sporen in rotaties met aardappel. Het onderzoek was gericht op 1) de ruimtelijke distributie patronen van *Meloidogyne* spp. t.b.v. de ontwikkeling van bemonsteringsmethoden; 2) de populatie-dynamica in de tijd, vooral de natuurlijke afname van de populatie na de oogst, en 3) het ontwikkelen van moleculaire methodieken om beide soorten te detecteren in geoogste aardappels. Het onderzoek had als doel voldoende wetenschappelijke informatie te verzamelen om richting de EU nieuwe inspectiemethoden te kunnen voorstellen.

Ruimtelijke distributiepatronen van Meloidogyne spp. t.b.v. de ontwikkeling van bemonsteringsstrategieën voor detectie.
Gedurende 2002 tot 2006 is er informatie verzameld om de midden- en grootschalige distributiepatronen (verdeling in het veld) van *Meloidogyne chitwoodi* en *M. fallax* in kaart te brengen. Het onderzoek is uitgevoerd op 5 percelen in Nederland en 2 in België. Gecombineerd met data van de kleinschalige verdeling van beide soorten, afkomstig uit voorafgaand onderzoek, was het mogelijk zowel besmettingshaarden als volvelds besmettingen te modelleren en detectiekansen te berekenen. Hiervoor is gebruik gemaakt van het simulatieprogramma Sample IV. Berekeningen zijn gebaseerd op 3 besmettingshaarden en 4 volveld besmettingen. Dit is een zeer smalle basis, en er bestaat dus nog een duidelijke noodzaak om meer datasets van in kaart gebrachte *Meloidogyne* besmettingen te verzamelen ten einde de betrouwbaarheid van de gebruikte parameters in beide modellen, vooral het besmettingshaard model, naar een acceptabel niveau te tillen. Daar het besmettingshaard model van toepassing blijkt voor *Meloidogyne* spp. en detectie het primaire doel van dit onderzoeksproject was, zijn alle verdere analyses uitgevoerd met gebruikmaking van dit model.
Vergeleken met besmettingen van het aardappelcysteaaltje, waarvan cysten verzameld moeten worden wanneer een bemonstering voor detectie plaatsvindt, leveren *Meloidogyne* soorten 2de stadia juvenielen, eieren en eimassa's, wat resulteert in een, in verhouding, groot aantal detectabele eenheden. De meeste spoelmethoden kunnen de juvenielen en de eimassa's vangen, maar de eieren gaan verloren. De proportie van het aantal eieren t.o.v. het totale aantal individuen is onbekend. Wanneer de organische fractie buiten beschouwing wordt gelaten zal een groot gedeelte van de populatie, afhankelijk van de tijd na de oogst en de stevigheid van de wortels, veronachtzaamd worden. Echter, het aantal detectabele eenheden per volume grond na een goed waardgewas is hoger voor *Meloidogyne* spp. dan voor het aardappelcysteaaltje. Dit resulteert in een hogere waarschijnlijkheid voor detectie.
Onderzoek naar opspoeltechnieken, een vergelijking van de Oostenbrink elutriator en de zonale, of Hendricks, centrifuge, heeft aangetoond dat de opspoelfout van beide apparaten extreem hoog is voor dit aaltje, met een variatiecoëfficiënt van 50%. Dit resulteert in zeer onbetrouwbare populatiedichtheidbepalingen van het aaltje, maar ook in een onbetrouwbare schatting van de aggregatiefactor van de negatief binomiale verdeling, die wordt gebruikt om de kleinschalige verdeling van het aaltje te beschrijven. Het betekend dat de werkelijke aggregatiefactor van de negatief binomiale verdeling voor *Meloidogyne* spp. hoger is dan die nu gebruikt is in de berekeningen. Het betekent ook dat de dichtheden, gemeten in de besmettingshaarden en volveldbesmettingen, in werkelijkheid minder heterogeen zijn. Hierdoor worden nu de detectiekansen onderschat en de variabiliteit van een populatiedichtheidsbepaling in het onderzoek naar distributiepatronen overschat. Om deze fout te compenseren moeten nu grotere bodemmonsters worden verzameld en verwerkt dan nodig wanneer een betrouwbare opspoelmethodieken beschikbaar waren. Zodra betere methoden beschikbaar komen (hier loopt momenteel hellas geen onderzoek) kan de grootte van het bulkmonster worden verkleind en op de kosten worden bespaard. Elke methode die is ontwikkeld voor de detectie van besmettingshaarden van *Meloidogyne* spp. zal een aanwezige volveld besmetting met nog grotere zekerheid kunnen aantonen. Toekomstig onderzoek naar opspoelmethoden zal gericht moeten worden op verbetering van de variatie van de zonale centrifuge of in het opschalen van bewezen concepten uit het verleden naar hedendaags gebruik. Bijvoorbeeld het Seinhorst opspoelapparaat voor vrijlevende aaltjes dat tot 500cc grond kan verwerken (Seinhorst, 1988).

Populatie dynamica in de tijd, gericht op de natuurlijke afname van Meloidogyne *spp. na de oogst.*
De populatie dichtheden van *Meloidogyne* spp. dalen in de herfst en winter wanneer de grond braak wordt gehouden. In verschillende velden, zowel in Nederland als in België is de populatiedichtheid op verschillende percelen gevolgd. Periodiek zijn monsters verzameld, beginnend direct na de oogst tot en met het tijdstip van zaaien of planten van het volgende gewas, en soms nog langer. De verticale verdeling door de bouwvoor is hierbij meegenomen. De informatie betreffende de verticale verdeling toont aan dat *Meloidogyne* spp. kan worden aangetroffen in de bovenste 5 cm van de bouwvoor gedurende de gehele periode tussen twee gewassen. Echter, de populatiedichtheden zijn in deze horizont lager vergeleken met de diepere lagen. Detectie zou bemoeilijkt worden als alleen deze horizont zou worden bemonsterd. Bouwvoor diep bemonsteren heeft daarom de voorkeur. Twee verschillende wiskundige functies zijn gebruikt om de data van de natuurlijke afname te beschrijven: een negatief exponentiele functie en een polynoom. De laatste kan niet worden gebruikt om populatiedichtheden te voorspellen. Het eerste model past met variërende nauwkeurigheid maar heeft enkele nadelen. Het neemt aan dat de dichtheden naar nul dalen in de tijd. Hoewel dit een redelijke aanname lijkt is deze niet bewezen met de datasets die zijn geanalyseerd. Zelfs wanneer de periodieke bemonstering gedurende een compleet teeltjaar, onder braak, is volgehouden. Ten tweede is dit een statistisch model dat voor elke waard van *Meloidogyne* spp. opnieuw zou moeten worden geparameteriseerd, wat betekent dat er een gigantische hoeveelheid werk moet worden verzet. Echter, de op dit moment verzamelde gegevens, maken het mogelijk om de afname van de populatiedichtheden, in de in kaart gebrachte besmettingen, ruwweg te voorspellen en het effect op de detectiekans te berekenen. Uit de verschillende datasets kan een gemiddelde afname van 80% van de populatie worden berekend tussen de oogst van het gewas en het tijdstip van zaaien of planten van het nieuwe gewas. Het effect op de detectiekans van besmettingshaarden bleek niet zo groot als was verwacht. Een gemiddelde reductie van de detectiekans van 28% binnen een range van centrale populatiedichtheden tussen de 500 en 20000 juvenielen per kg grond is berekend. Wanneer een kleine besmettingshard met een centrale populatiedichtheid van 500 juvenielen/kg grond (een zeer jonge besmettingshaard) aanwezig is na de oogst, zal de detectiekans afnemen van 40% naar 18% bij gebruik van de huidige AMEX200cc bemonsteringsmethode (hele suspensie geteld). Een besmettingshaard met een centrale populatiedichtheid van 5000 juvenielen/kg grond heeft een detectiekans van 76% na de oogst, en deze zal afnemen tot 51% tegen de tijd dat het nieuwe gewas de grond ingaat. Hoe groter de besmetting (uitgedrukt in de centrale populatie dichtheid) na de oogst, des te kleiner het effect van de natuurlijke afname op de detectiekans. In de tijd vinden we de grootste afname in populatiedichtheden in de eerste weken na de oogst. Daarom zal bemonstering direct na de oogst van de waardplant, vooral na een goede waard, zoals aardappel de hoogste detectiekansen opleveren. Wanneer bemonstering voor de volgende aardappelteelt is voorzien is de meest belangrijke variabele in elke berekening de populatiedichtheid die men kan verwachten na de teelt van verschillende waardgewassen. In termen van het besmettingshaard model: welke centrale populatiedichtheid kan men verwachten. Na aardappel, proefveld Wieringermeer, werden populatiedichtheden van 1000 tot 500000 juvenielen/kg grond aangetroffen, de laatste zijnde een uitzondering. Na suikerbieten werden populatiedichtheden tot 12000 juvenielen/kg, en na gerst tot 1200 juvenielen/kg aangetroffen. Eerdere informatie verzameld door PPO-agv (Tabel 2.2) laat ook hoge populatiedichtheden zien. Als deze maximale dichtheden beschouwd kunnen worden als schatters voor de centrale populatiedichtheid van een besmettingshaard zullen de detectiekansen vergeleken met het aardappelcysteaaltje zeer acceptabel zijn. Het zou wenselijk zijn een lijst samen te stellen van bekende ‘maximum’ populatiedichtheden die na de oogst van verschillende waardplanten van *Meloidogyne chitwoodi* and *M. fallax* zijn aangetroffen.

Methoden voor de identificatie van beide soorten in geoogste knollen gebaseerd op moleculaire technieken.
Laboratorium onderzoek is uitgevoerd voor de ontwikkeling van een nieuwe snelle Multiplex Real-time PCR voor het aantonen van *Meloidogyne chitwoodi* en *M. fallax* in aardappel knollen. Deze methode bleek gevoeliger dan visuele inspectie en is gebruikt om aan te tonen dat grootte aantallen knollen (57.6% in 2002 and 10.8-31.1% in 2003), zonder visuele symptomen, maar afkomstig van een besmet perceel, met *Meloidogyne chitwoodi* en *M. fallax* waren besmet. De methode kan direct na de oogst worden toegepast waardoor 8 weken incubatietijd bij 18 C kan worden bespaard. In het laboratorium konden tot 5 knollen tegelijk als een monster worden verwerkt en konden niet visuele besmettingen (binnen en buiten de aardappelknol) toch worden aangetoond. In de praktijk is de methode ondertussen verder ontwikkeld en kunnen nu 100 knollen tegelijkertijd per test worden gescreend. Hiermee is introductie van deze methode in regulier onderzoek mogelijk gemaakt.

Voorstel voor een EU- inspectie methode

Visuele inspectie van aardappelknollen is momenteel de officiële methode om de aanwezigheid van *Meloidogyne chitwoodi* en *M. fallax* in het geoogste product aan te tonen. De methode is tijdrovend en onbetrouwbaar; ze is afhankelijk van de expressie van de aanwezigheid van het aaltje in visuele symptomen op de knol, deze expressie is cultivar afhankelijk. Een alternatieve methode zou grondbemonstering kunnen zijn. Het meest gunstige moment voor de bemonstering voor detectie is onmiddellijk na de oogst van het gewas wanneer de populatiedichtheden in de grond het hoogst zijn. De aanwezige nematoden zijn dan verdeeld over de minerale en de organische fractie. Wanneer aardappel het doelgewas is moet er een keuze worden gemakt tussen bemonstering voor het poten van het gewas of na de oogst van de knollen wanneer een mogelijke besmetting door het aardappelgewas omhoog is gestuwd. Hoewel bemonstering na de oogst zal resulteren in een hogere detectiekans van een aanwezige besmetting zal deze keuze ook leiden tot hoge economische kosten voor de teler ten gevolge van partijafkeuringen. Een alternatief is de keuze om bemonstering voor de teelt uit te voeren, ten einde vast te stellen of het perceel vrij is van *Meloidogyne* en zodoende de teler een 'licence to grow' te verstrekken. Een beslissing moet dan worden genomen over wat men wil detecteren (welke grootte van de haard(en)) en met welke betrouwbaarheid de detectie moet plaatsvinden. Analoog met het aardappelcysteaaltje kunnen we aannemen dat wanneer een besmettingshaard aantoonbaar begint te worden er ongeveer 3 secondaire haarden aanwezig zijn in dezelfde strook. Bij het aardappelcysteaaltje wordt een primaire besmettingshaard van 100 cysten/kg grond (centrale populatie dichtheid) en drie secundaire besmettingshaarden van 50 cysten/kg grond verondersteld. De nieuw voorgestelde EU bemonsteringsmethode is gericht op detectie van dit agglomeraat met een detectiekans van 90%. Indien dezelfde aannames voor *Meloidogyne* spp. worden overgenomen kunnen we, met behulp van de in dit project vergaarde kennis, uitrekenen wat de benodigde steekgrootte, en dus de grootte van het bulkmonster, zal moeten zijn, om de vereiste detectiekans te verkrijgen. Voor *Meloidogyne* spp. is gekozen voor een primaire besmettingshaard met een centrale populatiedichtheid van 500 juvenielen/kg grond, en drie secondaire besmettingshaarden met centrale populatiedichtheden van 250 juvenielen/kg grond. De resulterende bemonsteringsmethode is weergegeven in Tabel 5.1. Voor een verdere veiligstelling van de export zou een extra test met de Real-time PCR methode van het geoogste product kunnen worden overwogen.

7. Remaining problems

- The number of infestation foci mapped is small and only from the south-eastern part of the country. Some more data sets are required to obtain a reliable parameterization of the infestation focus model of *Meloidogyne* spp. throughout the Netherlands,
- What are the maximum population densities of different host crops after harvest and can they be used as estimations of the central population density of the focus model.
- The elutriation methods currently used for detection and population density estimation show a large error. Research has to be focused on improvement of current methods to yield more reliable results (e.g. the possibility of minimizing the variability of the zonal centrifuge) or in updating past concepts for contemporary use (e.g. the Seinhorst elutriator for free living nematodes which can process up to 500cc (Seinhorst, 1988)).
- Densities of *Meloidogyne* spp. in the mineral and organic fraction directly after harvest and in time. Data are primarily focused on potato. Probably, data from past experiments with *Meloidogyne* spp. could be used to answer the first question.
- Assumption used as this moment: Losses of free eggs in soil and small J2's when sieving are negligible and constant.
- Research is needed to determine the maximum number of tubers that can be processed as a lot, enabling the detection of infected tubers answering the demands of the inspection services. This type of research can be done efficiently since the required reconstruction experiments can be performed with existing samples.

8. Acknowledgements

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