EUPHRESCO Final Report

For Non-Competitive research projects

Detection and management of the quarantine nematodes *Meloidogyne chitwoodi* and *Meloidogyne fallax* in the EU member states

Project duration:

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Project Coordination:

Geraldine Anthoine
French Agency for Food, Environment and Occupational Health and Safety (Anses)
Plant Health Laboratory
7 rue Jean Dixméras – F49044 Angers cedex, France
Phone : + 33 241207431, Fax: +33 241207430
Email: geraldine.anthoine@anses.fr

Loes den Nijs
NPPO The Netherlands
National Reference Center
Geertjesweg 15, 6706 EA Wageningen, Netherlands
Phone : + 31 317496840, Fax +31 317421701
Email: l.j.m.f.den.nijs@minlnv.nl

Nicole Viaene
Institute for Agricultural and Fisheries Research
Plant Sciences Unit - Crop protection
Burg. van Gansberghelaan 96 bus 2
9820 Merelbeke
Phone: +32 92722425, Fax +32 92722429
Email: nicole.viaene@ilvo.vlaanderen.be

Topic coordination (at the time of the project's initiation):

Paul H.J.F. van den Boogert
Department Plant Health Strategy & Development
15, Geertjesweg, 6706 EA, Wageningen, The Netherlands PO Box: 9102, 6700 HC Wageningen, The Netherlands
Phone: + 31 317 496694, Fax +31 317 421701
Email: p.h.j.f.van.den.boogert@minlnv.nl

David Caffier
Plant Health Laboratory
7 rue Jean Dixméras – F49044 Angers cedex, France
(no longer in this position)

Maurice Moens
Institute for Agricultural and Fisheries Research
Plant Sciences Unit - Crop protection
Burg. van Gansberghelaan 96 bus 2
9820 Merelbeke
(no longer in this position)

Research Consortium Partners

Country	Name Institute	Address Institute	EUPHRESCO Partner
Austria	Institute for Plant Health,	Spargelfeldstr. 191, A-1220 Wien, Österreich/AUSTRIA	AT-AGES
Belgium	ILVO	Burg. Van Gansberghelaan 96, B-9820 Merelbeke, Belgium	BE-ILVO
Bosnia-Herzegovina	University of Banjaluka	University of Banjaluka, Faculty of Agriculture, Bulevar vojvode, Petra Bojoirica 1A, 78000 Banja Luka	Bosnia Herzogovina
Bulgaria	Central laboratory for plant quarantine	120,N.Moushanov,1330, Sofia, Bulgaria	BG-NSPP
Bulgaria	Plant Protection Institute	35 P. Volov Str, 2230 Kostinbrod, Bulgaria	BG-NSPP
Czech Republic	State Phytosanitary Administration	Postal address: Ztracena 1099/10, 161 06 Praha 6, Czech Republic	CZ-SPA
Czech Republic	Diagnostic Laboratory Olomouc	Slechtitelu 23/773 77900 Olomouc Czech Republic,	
Denmark	Frø og Planter, Lab. for Diagnostik i Planter	Ministeriet for Fødevarer, Landbrug og Fiskeri Plantedirektoratet Skovbrynet 20, 2800 Kgs. Lyngby	DK
France	LNPV / LSV	LNPV Domaine de la Motte BP35327 LE RHEU cedex France	FR-DGAL / Anses
France	FN3PT	FN3PT-INRA UMR BiO3P Domaine de La Motte - B.P. 35327 -F- 35653 Le Rheu Cedex France	FR-FNPPPT
Germany	Julius Kühn-Institut/Plant Health Institute	Messeweg 11/12, 38104 Braunschweig, Germany	DE-JKI
Germany	Julius Kühn-Institut/Plant Health Institute	jToppheideweg 88 48161 Münster, Germany	DE-JKI
Netherlands	Plant Protection service	PO Box 9102, 6700 HC Wageningen The Netherlads	NL-PPS
Netherlands	NAK	NAK, Randweg 14, 8304 AS Emmeloord, Netherlands	NL-NAK
Portugal	IMAR-CMA	Dept. Life sciences, University of Coimbra, 3004-517, Coimbra, Portugal	IMAR-CMA
Serbia	Institute Tamis	PDS Institut Tamis, Novoseljanski put 33, 26000 Pancevo, Serbia	SERBIA
Slovenia	Agricultural Institute of Slovenia	Hacquetova 17, 1000 Ljubljana, Slovenia	SL_KIS
Switzerland	Agroscope Changins-Waedenswil ACW	Schloss, PO Box, 8820 Waedenswil Switzerland	CH-FOAG
Turkey	Plant Protection Research Institute	Gençlik cad. No:6 Bornova-İzmir/TURKEY	TR-GDAR
United Kingdom	The Food & Environment Research Agency	The Food & Environment Research Agency, Sand Hutton, York, YO41 1LZ, UK	UK-Fera
United Kingdom	AFBI	18a Newforge Lane, Belfast BT9 5PX, Northern Ireland UK	UK_AFBI

Introduction

This non competitive project was developed in the framework of Euphresco - round II of projects. As other Euphresco projects, it focuses on quarantine pests of interest. *Meloidogyne chitwoodi* and *M. fallax* are quarantine nematodes, responsible for quantitative and qualitative damage, including galling on roots and tubers of major crops such as potato, carrot, salsify. The nematodes have a limited distribution in Europe (Belgium, France, Germany, The Netherlands, Portugal and Turkey) with different frequency of occurrence varying from “detected but eradicated” to “present in several fields”. Consequently, conducting a reliable and sensitive survey is an efficient way to provide knowledge about the distribution of these pests at the European level and at the same time to prevent their dispersion.

This project aims at a comprehensive initiative for a better detection of *M. chitwoodi* and *M. fallax* by sharing knowledge, developing a network, identifying needs for research and comparing methods for a validated and harmonized approach for *M. chitwoodi* and *M. fallax* surveys.

This project is composed of 5 complementary topics:

- Topic 1: Ring test on the extraction of *Meloidogyne* juveniles from soil
- Topic 2: Ring test on detection and identification of *M. chitwoodi* and *M. fallax* by conventional and real time PCR assays
- Topic 3: Workshop on detection and management of the quarantine nematodes *M. chitwoodi* and *M. fallax*
- Topic 4: Treatment of waste contaminated by nematodes
- Topic 5: A European *Meloidogyne* research agenda

This report compiles the results of all topics.

EUPHRESCO project *Meloidogyne chitwoodi* and *Meloidogyne fallax*

Topic 1:

Ring test on the extraction of *Meloidogyne* juveniles from soil.

EUPHRESCO project *Meloidogyne chitwoodi* and *Meloidogyne fallax*

Topic 1:
Ring test on the extraction of *Meloidogyne* juveniles from soil.

PROJECT COORDINATOR: Loes den Nijs

PARTICIPANTS: Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Czech Republic (2x), England, France, Germany (2x), Portugal, The Netherlands (2x), Serbia, Slovenia, Switzerland, Turkey, N. Ireland (see topic 1 - appendix 1).

INTRODUCTION

In the EPPO diagnostic protocol PM 7/41 (Anonymous, 2009) various methods are described to extract the different nematode stages from soil, roots or plant parts. The extraction of *Meloidogyne* spp. can create great differences in outcome between laboratories as methods can be various and the nematodes can be present in different forms (eggs, juveniles, females). It is important to be aware of this when interpretations are given on data.
To gain insight into how different extraction processes operate and what effect these methods might have on the outcome, a ring test was conducted with two aspects; One of the basic techniques, Baermann funnel, was used as method performance test, the second element was to compare the standard technique used in the participating laboratories with the Baermann method (or modified one) as a reference method. The Baermann method was chosen as this method is used by many nematologists all over the world.
To avoid identification problems as a factor in the extraction results all mobile stages of all nematodes present in the extract were counted and distinction was only made between *Meloidogyne spp*, other plant parasitic nematodes and non plant parasitic nematodes. By counting nematodes, although only in three different groups, it will give information on the efficiency of the extraction method and on the identification skills on genus level of the laboratory personnel.

Material and methods

From a known naturally infested field in The Netherlands fifty liters of soil were collected. After thorough mixing of the soil two samples were taken to determine the infestation level of *Meloidogyne*. The soil type was sandy soil, pH 5.2 and organic content 3.3%. The soil samples were taken in November and processed with the Oostenbrink elutriator method with 4 weeks incubation. The mean initial density in the soil was 2025 second-stage ?? juveniles per 100 ml. Subsamples of 100 ml were taken and put into small plastic bags and stored at 4 ºC (30/11/2010).
Samples were sent to the participants in specially prepared boxes to maintain the low temperature (20/12/2010). Each participant received 10 samples per extraction method, method A, the Baermann funnel, and method B, the standard extraction method of their own laboratory.
Samples were stored or processed immediately, either way, the circumstances were noted and the extraction technique was described. A short description of the various methods can be found in topic 1 - appendix 2.
The results were scored on the specially prepared analysis report; distinction was made between *Meloidogyne* spp, other Plant Parasitic Nematodes (PPN) and saprophytic nematodes (Saprophytes). The data were analyzed using a Hierarchical Generalized linear Model (HGLM) with the extraction method as treatment. Use of the HGLM algorithm enabled the estimation of mean and variance of the counts per extraction method (Lee *et al.*, 2006). Only a small fraction of all combinations of institute and method were present (table 1) and the counts reported with the Baermann funnel varied strongly between institutes. Therefore, adding institute as a random blocking term to the statistical analysis did not make sense. This resulted in outlying median values for some of the methods B, the standard extraction methods of the laboratories. So institute was not added to the analysis. The counts were assumed to be gamma distributed and were

transformed with a logarithmic link function (Lee *et al*., 2006). The backtransformed means, also called medians, are reported.

Results

A total number of 19 participants joined the ring test of which twelve institutes performed two methods (A and B) and 7 performed only the standard method A, the Baermann funnel. The other methods, B, consisted of 7 different methods (table 1)

Table 1. Extraction methods and codes

Code	Extraction method
A	Baermann funnel
A?	Baermann funnel + (flotation sieving before Baermann)
C1	Oostenbrink elutriator
C2	Oostenbrink elutriator + 2 weeks incubation
C3	Oostenbrink elutriator + 4 weeks incubation
D	Automated zonal centrifugation
E	Centrifugation
F	Tray
G	Modified Cobbs & modified Baermann

The samples were prepared on the 29th of November, stored at 4 ºC, and were sent to the participants on the 20th of December 2010.
First samples were received by the participants on 22/12/2010 and the last on 20/01/2011. Analyses were started after receiving the samples or shortly after. Storage of the samples varied between 1 to 4 weeks at temperatures of 4 to 10^{o}C, with some outlying values for institute 22 and institute 4 with respectively 17 and 20^{o}C, the latter by mistake.
The analysis reports were received between the second week of January and the last week of March 2011.

Topic 1 - Appendix 3 shows the mean of the values found per extraction method and per institute, the extended data can be found in appendix 4.

In the following figures 1,2 and 3 the results are shown in diagrams that are modifications of box-and-whisker diagrams which display individual outlying points as well as the median in the box. The whiskers extend only to the most extreme data values (the minimum and maximum value), within the inner "fences", which are at a distance of 1.5 times the interquartile range beyond the quartiles, or the maximum value if that is smaller. Individual outliers are plotted with a cross by default, with labels specified by Number. "Far" outliers, beyond the outer "fences" which are at a distance of three times the interquartile range beyond the quartiles, are plotted with a different pen.

Figure 1. Boxplot of counts of *Meloidogyne*, using the standard extraction method, Baermann funnel. Numbers/100 ml. For institute 2 and 6, Meloidogyne juveniles were not found.

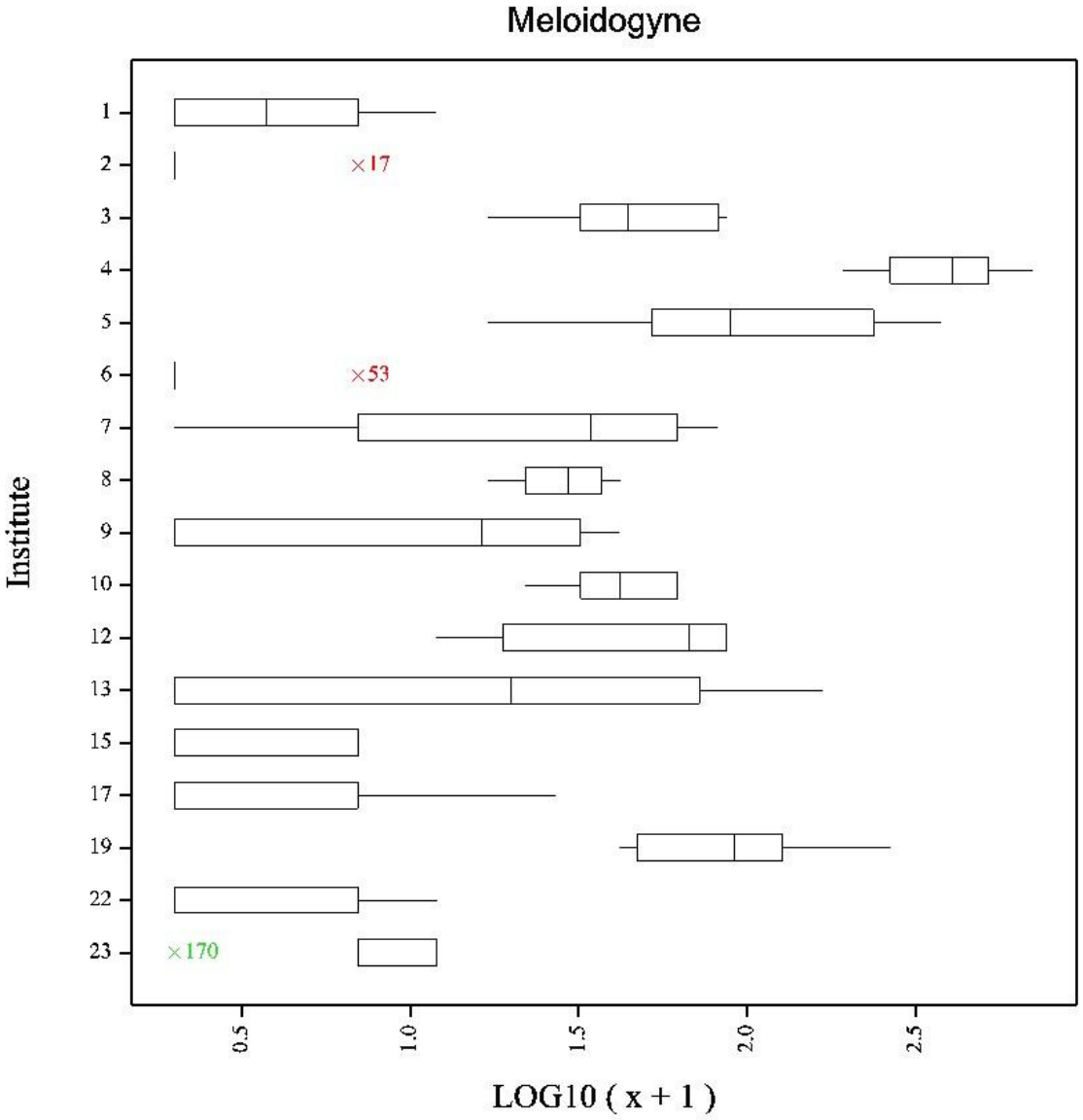


Figure 2. Boxplot of counts of other plant parasitic nematodes, using the standard extraction method Baermann funnel. Numbers/100 ml.

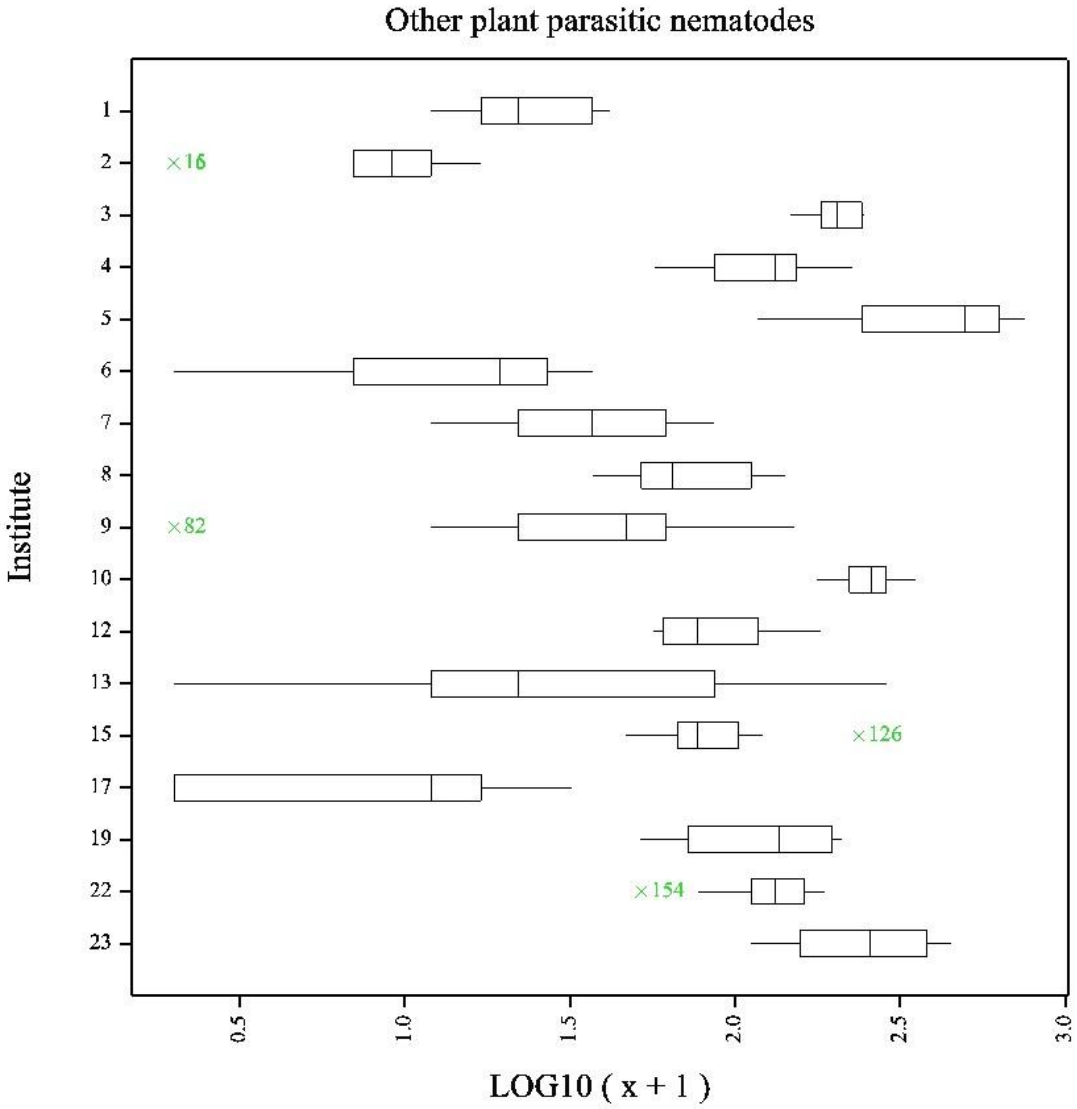
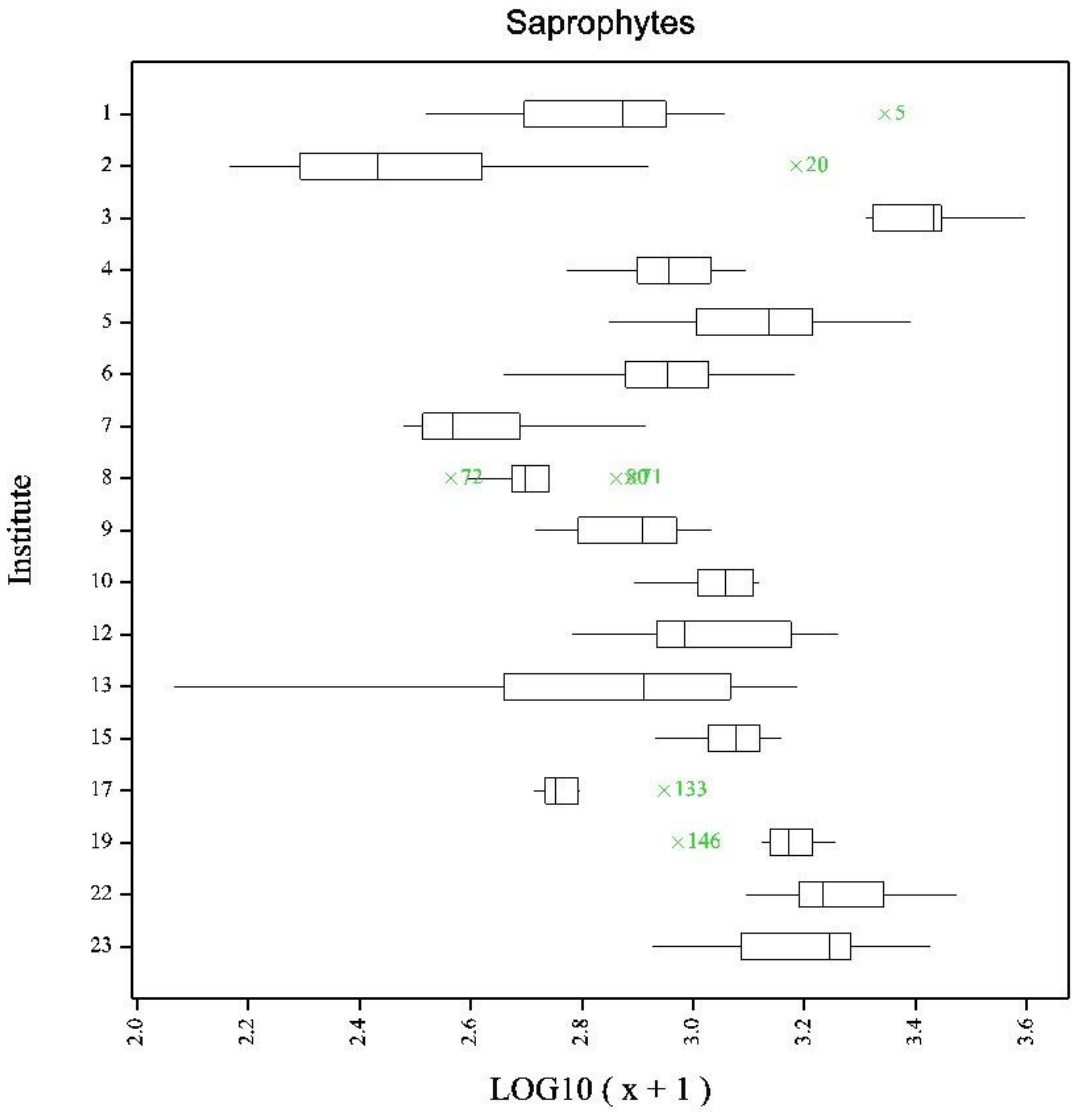


Figure 3. Boxplot of counts of saprophytic nematodes, using the standard extraction method Baermann funnel. Numbers/100 ml.



Analysing the data, the interest goes to the systematic differences among the extraction methods. Especially, the comparison of the other extraction methods with the standard Baermann method is the goal of this research. The interest is in the differences in the number counted per extraction method as well as the variance between the counts. The data are far from orthogonal because each institute, except 18 and 20, processed 10 samples with the standard extraction method but the 10 other (new) extraction methods were processed by only 1, 2 or 3 institutes. (table 2). Institute 5, 8, 9, 10, and 12 only tested the Baermann method.

Table 2. Number of samples processed per Institute andeExtraction method.

Extraction / Institute	A	A?	C1	C1+E	C2	C3	D	E	F	G
1	10			10						
2	10									10
3	10		10		10	10				
4	10						10			
5	10									
6	10								10	
7	10							10		
8	10									
9	10									
10	10									
12	10									
13	10									10
15	10			10						
17	10								10	
18			10		10					
19	10		10							
20		10								
22	10							10		
23	10							10		

The nematodes from a soil sample were collected in a 100 ml suspension. In most cases 2 subsamples of 10 ml were counted. The sum of these counts was multiplied by 5 and reported. The analysis was performed on the number/100 ml. This was the reason to assume the counts to be gamma distributed and apply a log link function. An alternative analysis assuming the counts are Poisson distributed should be based on the individual counts per sample. Next to the ability to analyse non-orthogonal data, a facility of the HGLM's is that the variances per extraction method can be obtained (Lee *et al.*, 2006). The median and variance per extraction method are shown in Tables 3 and 4. Institute 2 and 6 were discarded from the analysis because the *Meloidogyne* counts for the Baermann method were zero for Institute 2 and 6. Medians and variances without a common letter are significantly different according to Student's t-test.

Table 3. Median number of *Meloidogyne*, other PPN and saprophytes per extraction method, without Institutes 2 and 6. Medians without a common letter are significantly different according to Students t-test with probability 0.05.

Extraction method	*Meloidogyne*		Other PPN		Saprophytes	
Baermann funnel	61	a	137	ab	1170	b
Baermann +	34	a	122	a	136	a
Oostenbrink elutriator	167	b	475	d	3738	e
Oostenbrink elutriator+ centrifugation	81	a	135	ab	1533	cd
Oostenbrink elutriator+2 w incubation	2411	d	805	f	6048	f
Oostenbrink elutriator+4 w incubation	3733	d	942	g	7614	g
Automated zonal centrifugation	634	c	578	e	3527	e
Centrifugation	235	b	356	c	1987	d
Tray	287	b	178	ab	1227	bc
Modified Cobbs & modified Baermann	63	a	194	b	1476	bcd

Table 4. Variance of *Meloidogyne,* other PPN and saprophytes per extraction method, without Institutes 2 and 6. Medians without a common letter are significantly different according to Students t-test with probability 0.05.

Extraction method	*Meloidogyne*		Other PPN		Saprophytes	
Baermann funnel	2.12	d	1.07	d	0.32	bc
Baermann +	1.33	cd	0.16	bc	0.31	bc
Oostenbrink elutriator	0.27	b	0.08	b	0.29	bc
Oostenbrink elutriator+ centrifugation	1.24	cd	0.48	c	0.28	bc
Oostenbrink elutriator+2 w incubation	0.35	b	0.03	a	0.09	a
Oostenbrink elutriator+4 w incubation	0.26	b	0.03	a	0.04	a
Automated zonal centrifugation	0.04	a	0.01	a	0.05	a
Centrifugation	0.96	cd	0.38	c	0.48	c
Tray	0.58	bc	0.19	bc	0.15	ab
Modified Cobbs & modified Baermann	0.92	bcd	0.10	b	0.14	ab

This ring test was set up to find out the performance of the various extraction methods that are used in the European nematology laboratories, with emphasis on the extraction efficiency for *Meloidogyne* juveniles. It was not meant for quality assurance purposes and thus no negative controls were incorporated.

Tthe main objective was to test the performance of the standard method, the Baermann funnel, in various laboratories and compare this standard method with other extraction methods in use.
Although the moment of sending the samples to the participants was very inconvenient (one week before Christmas, some laboratories were closed between Christmas and New Year) the data clearly showed that results depend on the extraction method. Comparison of the Baermann method with the other method per laboratory showed, only once, the same amount of nematodes found (Institute 7, centrifugation method). In all other cases, the other method yielded higher numbers than the standard Baermann method (see topic 1 - appendix 3).
The number of *Meloidogyne* juveniles varied between zero and 6815. The initial density was more than 2000 juveniles/100 ml of soil. Some zero counts were excluded from the analysis because they were clearly outliers (frozen samples at arrival), some zero counts were found while with the same method nematodes were found in the other samples and thus stayed in the dataset.
The variance of methods can be very high (table 4), the standard extraction method, Baermann funnel, is clearly the most variable, the other similar methods have also high variances, the lowest variance was found with the automated zonal centrifugation which shows the advantage of using an automated method.
The results of the incubation method (2 weeks and 4 weeks incubation) make clear that incubation has an enormous effect on the efficiency of the method. In line with this, the storage of samples

needs much attention as storage can have the effect of incubation when temperatures are not adequate . An extraction method with incubation has the advantage of yielding higher numbers but the disadvantage of delayed results. Therefore, the purpose of the research might influence the choice of the extraction method.

The effect of the extraction method on the otherPPN and saprophytes is slightly different from that on *Meloidogyne,* it varies less between the other PPN and saprophytes. There are two aspects: 1) the incubation distorts the ratio of *Meloidogyne* and other PPN (ratio 3-400), because the incubation might have more effect on the *Meloidogyne*, as they produce egg masses which will be subjected to the incubation, than on the other PPN and saprophytes; 2) the relatively high number of *Meloidogyne* found in the Tray method when compared with the other PPN (ratio 160). In all other cases, the ratio is between 25 and 110. When identification was completely incorrect, the figures and the ratio would have been different . Based on these results, it seems right to conclude that identification of the *Meloidogyne* juveniles has been correct.

The (modified) Baermann method, Cobbs'method and Tray method are all based on more or less the same principle; wet soil on a surface to provide time for the nematodes to emerge from it. The elutriation is a completely different method,, where the nematodes are actively separated from the soil particles, by flowing water, and further separation takes place via a filter. In the third method, (the centrifugation),, an additional separation step is used in the Automated zonal centrifugation method, as in this method the nematodes get separated from the soil by centrifugation. Table 3 shows that the number of nematodes found with the (modified) Baermann method and the Cobbs'method were not significantly different from each other and clearly different from the centrifugation (normal and automated) and the elutriation (with or without incubation). It is however unclear why combination of the elutriation and centrifugation method (performed by one institute) yielded such low numbers.

The extraction method is important and the first step to determine the final numbers in the soil, and when the suspension is collected, the counting of the nematodes might be another aspect that influences the outcome. In the protocol for the standard Baermann method, it was therefore described how to determine the amount of nematodes in the suspension. For the non standard methods, however, it depends on the laboratory's procedures how the numbers of nematodes were determined. This could be a source of variance. Unfortunately, this component could not be separated from the final results per laboratory and therefore it could not be analysed. It should be considered as an integral part of the chosen method.

Conclusions

The used method influences clearly the yield of the nematodes. Variability in the outcome also depends on the used method. The variances detected on the other PPN and saprophytes are not consistent with that of *Meloidogyne*. The standard Baermann funnel produced the lowest number of *Meloidogyne* together with the methods based on this principle.
Incubation considerably influences the extraction efficiency, so storage of samples needs attention, especially for *Meloidogyne.*
Regarding the yield and variance, some methods are better than others: Ranking the methods on the basis of their efficiency, from low to high numbers, the results are: 1) (unclear) Baermann method; 2) modified Cobbs and Baermann funnel; 3) elutriation; 4) centrifugation; 5) Tray; 6) Automated zonal centrifugation; 7) elutriation with 2 weeks incubation; and 8) elutriation with 4 weeks incubation.
With this proficiency test, the advantage and disadvantage of the different extraction methods are made visible and the choice of the best method depends on the goal of the research. For survey purposes, it seems unadvisable to use the Baermann funnel.

Recommendations

Use a method that creates as little variability/variance as possible and perform it under stable conditions. Be aware that the extraction standard method, Baermann funnel, does not produce much yield and the nematode population densities might be well underestimated using this technique. Be aware that incubation strongly influences the results and take into consideration that storage circumstances can also affect the outcome.

References

Anonymous. 2009. *Meloidogyne chitwoodi* and *M. fallax*. Bulletin OEPP/EPPO Bulletin 39:5-17.
Lee, Y., Pawitan, Y., and Nelder, J.A. 2006. Generalized linear models with random effects: unified analysis via H-likelihood. CRC Press.

Note:
The results of this ring test were published as a manuscript:

den Nijs, L.and van den Berg,W. 2013. The added value of proficiency tests: choosing the proper method for extracting Meloidogyne second-stage juveniles from soil. Nematology 15:2, 143-151

Appendix 1

List of Subscribers EUPHRESCO Meloidogyne program: Ring tests

EUPHRESCO Partner	Name scientist	Email address scientist	Name Institute	Address Institute	Country
FR-DGAL	Geraldine Antoine	geraldine.anthoine@agriculture.gouv.fr	Plant Health Laboratory, Nematology Unit	LNPV 7 rue Jean Dixméras 49044 ANGERS CEDEX 01 France	France
TR-GDAR	Bilge Mısırlıoğlu	bilgemisirlioglu@yahoo.com	Plant Protection Research Institute	Gençlik cad. No:6 Bornova-İzmir/TURKEY	Turkey
NL-PPS	Loes den Nijs	L.J.M.F.den.Nijs@minlnv.nl	Plant Protection service	PO Box 9102, 6700 HC Wageningen The Netherlads	Netherlands
BE-ILVO	Nicole Viaene	nicole.viaene@ilvo.vlaanderen.be	ILVO	Burg. Van Gansberghelaan 96, B-9820 Merelbeke, Belgium	Belgium
BG-NSPP	Todorka Hristova	clkr@nsrz.government.bg	Central laboratory for plant quarantine	120,N.Moushanov,1330, Sofia, Bulgaria	Bulgaria
UK-Fera	Tom Prior	thomas.prior@fera.gsi.gov.uk	The Food & Environment Research Agency	The Food & Environment Research Agency, Sand Hutton, York, YO41 1LZ, UK	United Kingdom, England
CH-FOAG	Sebastian Kiewnick	sebastian.kiewnick@acw.admin.ch	Agroscope Changins-Waedenswil ACW	Schloss, PO Box, 8820 Waedenswil Switzerland	Switzerland
AT-AGES	Andreas Kahrer	andreas.kahrer@ages.at	Institute for Plant Health,	Spargelfeldstr. 191, A-1220 Wien, Österreich/AUSTRIA	Austria
DE-JKI	Björn Niere	bjoern.niere@jki.bund.de	Julius Kühn-Institut/Plant Health Institute	Messeweg 11/12, 38104 Braunschweig, Germany	Germany
SL_KIS	Sasa Sirca	gregor.urek@kis.si; sasa.sirca@kis.si	Agricultural Institute of Slovenia	Hacquetova 17, 1000 Ljubljana, Slovenia	Slovenia
CZ-SPA	Vladimir Gaar	vladimir.gaar@srs.cz;	State Phytosanitary Administration	Postal address: Ztracena 1099/10, 161 06 Praha 6, Czech Republic	Czech republic
UK_NI	Trevor Martin	Trevor.Martin@afbini.gov.uk	AFBI	18a Newforge Lane, Belfast BT9 5PX, Northern Ireland UK	United Kingdom, Northern Ireland
FR-FNPPPT	Anne-Claire Le Roux	anne-claire.leroux@rennes.inra.fr	FNPPPT	FNPPPT-INRA UMR BiO3P Domaine de La Motte - B.P. 35327 -F- 35653 Le Rheu Cedex France	France
Portugal	Isabel Abrantes	isabel.abrantes@zoo.uc.pt	IMAR-CMA	Dept. Life Sciences, University of Coimbra, 3004-517, Coimbra, Portugal	Portugal
NL-NAK	Ge van de Bovenkamp	gbovenkamp@nak.nl	NAK	NAK, Randweg 14, 8304 AS Emmeloord, Netherlands	Netherlands
Bosnia-herzegovina	Branimir Hjezic	branimirnjezic@yahoo.co.uk or branimir.njezic@agrofabl.org	Univerity of Banjaluka	Univerity of Banjaluka, Faculty of Agriculture, Bulevar vojvode, Petra Bojoirica 1A, 78 000 Banja Luka	Bosnia-Herzegovina
Serbia	Jasmina Bacic	jbacic2001@yahoo.com	Institute Tamis	PDS Institut "Tamis", Novoseljanski put 33, 26000 Pancevo, Serbia	Serbia
DE-JKI (EP)	Johannes Hallmann, Holger Heuer	johannes.hallmann@jki.bund.de; holger.heuer@jki.bund.de	Julius Kühn-Institut	Institute for Epidemiology and Pathogen Diagnostics, Toppheideweg 88, D-48161, Münster, Germany	Germany
CZ-SPA	Vaclav Cermak	vaclav.cermak@srs.cz	Diagnostic laboratory Olomouc	Diagnostic laboratory Olomouc State Phytosanitary Administration Slechtitelu 23/773 779 00 Olomouc Czech Republic	Czech republic

Appendix 2: Description of the extraction methods used in the proficiency test

Method	Principle	Description
Baermann funnel	Active movement of nematodes and gravity	Soil on filter paper in glass funnel. Soil is moist. 48 h for nematodes to emerge.
Baermann funnel, unclear	Flotation-sieving followed by gravity and active movement of nematodes	Soil was suspended in 10 L bucket. Stirr 10 sec, settle 45 sec, then pour over 3 sieves (50 µm). The soil remnants on the sieve were used further for Baermann.
Oostenbrink elutriator	Flotation, gravity, sieving, active movement of nematodes	Soil is sieved and mixed with water in device with water upstream. Outlet is poured over sieves. Soil residues with nematodes on cotton filter for 1 night
Oostenbrink elutriator with incubation	Flotation, gravity, sieving, active movement of nematodes	Soil is suspended and poured over 180 µm sieve. Then, same as before. The residues on 180 µm sieve incubates on a moist cotton filter for 2 or 4 weeks in a climate chamber
Automated zonal centrifugation	Sieving and gravity	Soil is suspended in water, through a 425 µm sieve. Material on sieve is mixed and added to solution, tot volume 1 L; 0.5 L is sucked up in centrifuge. $MgSO_4$ (1,15d). 3 h of settling count of 35 mL solution of 50 mL soil
Centrifugation	Sieving and gravity	Soil in water, adding kaolin, centrifuge for 4 min at 1800 g or 2000 g, pellet resuspended in MgSo4 (1.18d) or Ludox solution (1.16 g/ml), centrifuge for 2 min at 900 g or at 2000 g.
Tray	Active movement of nematodes, gravity and sieving	The same as Baermann, but other equipment (tray 38 x27 cm, with letter tray inside) and additionally the resulting solution poured over 3 sieves (53 µm)
Modified Cobb's	Flotation-sieving followed by gravity and active movement of nematodes	Soil in 2 L water, stirring, left 15 sec and decanting (repeat twice), pour through sieve (250 µm) 3 times, pour through sieve (53 µm) 3 times. Debris in Baermann funnel
Modified Baermann	Active movement of nematodes and gravity	The same as Baermann, but other equipement: dishes (Ø10 cm), wire mesh 2 mm

Appendix 3: Arithmetical means per Institute and extraction methods for *Meloidogyne,* other PPN and saprophytes. A=Baermann funnel, A?=unclear Baermann funnel, C1,2,3=Oostenbrink elutriator with 0, 2 or 4 weeks incubation, D= automated zonal centrifugation, E=centrifugation, F=tray method and G=modified Cobb & modified Baermann.

Meloidogyne

Extraction	A	A?	C1	C1+ E	C2	C3	D	E	F	G
Institute										
1	4			144						
2	1									114
3	49		282		3347	3732				
4	411						633			
5	139									
6	1								6	
7	34							33		
8	27									
9	17									
10	43									
12	53									
13	45									62
15	3			16						
17	4								286	
18			108		1473					
19	102		108							
20		33								
22	3							472		
23	7							199		

Other PPN

Extraction	A	A?	C1	C1+ E	C2	C3	D	E	F	G
Institute										
1	25			181						
2	8									264
3	203		448		893	941				
4	128						577			
5	459									
6	17								72	
7	41							123		
8	75									
9	52									
10	260									
12	94									
13	56									193
15	93			86						
17	11								177	
18			361		715					
19	131		614							
20		121								
22	128							428		
23	267							515		

Saprophytes

Extraction	A	A?	C1	C1+ E	C2	C3	D	E	F	G
Institute										
1	833			2159						
2	438									3099
3	2676		6242		7515	7613				
4	921						3526			
5	1384									
6	916								772	
7	450							596		
8	528									
9	798									
10	1127									
12	1151									
13	819									1476
15	1181			905						
17	596								1226	
18			2752		4579					
19	1465		2218							
20		135								
22	1863							3414		
23	1712							1949		

Appendix 4:
Data converted to numbers/100 ml suspension.

	Results						
	A = Baermann			D = Automated zonal centrifugation (AZC)			
	C1 = Elutriation			E = Centrifugation			
	C2 = Elutriation + 2 wk incubation			F = Tray			
	C3 = Elutriation + 4 wk incubation			G = Modified Cobbs + modified Baermann			
Institute	Extraction method	*Meloidogyne*	Other plant parasitic nematodes	Saprophytic nematodes*	Storage temp	Delivery date	Extraction date
1	A	10	40	1140	4	23-12-2010	11/13-01-2011
1	A	0	40	880			
1	A	0	15	720			
1	A	0	20	890			
1	A	5	30	2210			
1	A	10	15	495			13/15-01-2011
1	A	5	10	535			
1	A	0	20	360			
1	A	0	35	770			
1	A	5	20	330			
1	(C1+) E	148	143	1627			13-1-2011
1	(C1+) E	121	183	2380			
1	(C1+) E	198	193	2100			
1	(C1+) E	142	171	3440			
1	(C1+) E	121	170	1520			
1	(C1+) E	146	183	2200			
1	(C1+) E	137	188	2360			
1	(C1+) E	141	211	2220			
1	(C1+) E	155	172	2200			
1	(C1+) E	128	195	1540			
2	A	0	5	180	4	10-1-2011	24-1-2011
2	A	0	5	210			
2	A	0	10	145			
2	A	0	15	195			
2	A	0	0	240			
2	A	0	0	415			
2	A	5	10	330			
2	A	0	10	300			
2	A	0	5	830			
2	A	0	15	1530			
2	G	159,6	256,5	1544,7			
2	G	159,6	142,5	1926,6			
2	G	39,9	205,2	2205,9			
2	G	74,1	233,7	3083,7			
2	G	153,9	438,9	2907			
2	G	68,4	228	1727,1			
2	G	114	228	10790,1			
2	G	119,7	324,9	2348,4			
2	G	153,9	256,5	2194,5			
2	G	96,9	324,9	2262,9			
3	A	45	215	3960	4		14-12-10 and 4-1-2011
3	A	15	205	2690			
3	A	45	145	3285			

3	A	40	240	2750			
3	A	85	180	2335			
3	A	85	245	2085			
3	A	40	245	2105			
3	A	80	185	2785			
3	A	30	200	2725			
3	A	20	170	2040			
3	C1	245	525	6050			
3	C1	375	505	6020			
3	C1	270	430	10550			
3	C1	205	385	6195			
3	C1	235	420	4985			
3	C1	295	335	5200			
3	C1	255	470	6040			
3	C1	340	495	5915			
3	C1	315	455	5810			
3	C1	285	460	5650			
3	C2	3245	1205	7110			
3	C2	4335	940	7505			
3	C2	5585	880	12410			
3	C2	2490	975	7195			
3	C2	2940	1035	6250			
3	C2	740	675	6560			
3	C2	4215	850	7255			
3	C2	3885	890	7350			
3	C2	1955	715	6965			
3	C2	4075	765	6545			
3	C3	3395	1265	7187			
3	C3	4670	960	7570			
3	C3	6815	900	12610			
3	C3	2780	1010	7255			
3	C3	3410	1070	6300			
3	C3	795	735	6685			
3	C3	4640	890	7390			
3	C3	4450	995	7480			
3	C3	2090	815	7080			
3	C3	4275	765	6570			
4	A	405	55	855	20 (<1wk), 10	26/30-12-2010	5-1-2011
4	A	345	130	700			
4	A	495	145	825			
4	A	-	-	-			
4	A	595	225	1240			
4	A	705	175	1125			
4	A	190	70	590			
4	A	470	90	995			
4	A	285	135	1055			
4	A	210	130	900			
4	D	490	540	3126			5/6-1-2011
4	D	712	586	3120			
4	D	454	548	2494			
4	D	592	612	5232			
4	D	714	530	3392			
4	D	508	622	2810			
4	D	640	452	4320			
4	D	870	614	3720			
4	D	720	634	3840			
4	D	628	630	3202			
5	A	375	430	885	4	27-12-2010	19-1 / 4-2-2011
5	A	255	755	1860			

5	A	195	630	1320			
5	A	235	565	2465			
5	A	25	665	1010			
5	A	65	480	1535			
5	A	95	510	1635			
5	A	50	115	705			
5	A	15	200	1415			
5	A	80	240	1010			
6	A	0	15	1065	5 (partially frozen)	23-12-2010	5-1-2011
6	A	0	30	940			
6	A	5	35	1520			
6	A	0	20	765			
6	A	0	5	970			
6	A	0	25	860			
6	A	0	5	1180			
6	A	0	20	455			
6	A	0	15	755			
6	A	0	0	650			
6	F	9	72	765			
6	F	5	86	793			
6	F	0	65	676			
6	F	2	69	817			
6	F	8	97	742			
6	F	5	68	751			
6	F	2	71	858			
6	F	5	39	802			
6	F	18	96	796			
6	F	4	54	717			
7	A	80	35	460	4/6	27-12-2010	4/6-1-2011
7	A	0	50	365			
7	A	5	25	485			
7	A	0	85	820			
7	A	30	70	300			
7	A	60	60	325			
7	A	40	15	360			
7	A	35	10	325			
7	A	30	20	685			
7	A	60	35	370			
7	E	40	120	570			11-1-2011
7	E	25	90	590			
7	E	25	105	505			
7	E	20	170	630			
7	E	20	155	695			
7	E	50	155	575			
7	E	25	90	750			
7	E	50	130	510			
7	E	30	100	455			
7	E	45	110	675			
8	A	35	115	770	8	23-12-2010	10/14/18/21-1-2011
8	A	40	50	365			
8	A	20	35	505			
8	A	35	55	490			
8	A	15	65	550			
8	A	25	110	475			
8	A	20	60	470			
8	A	15	80	540			
8	A	30	40	390			
8	A	35	140	725			
9	A	10	10	520	10	22-12-	5/7-1-2011

						2010	
9	A	0	0	920			
9	A	0	20	840			
9	A	0	60	690			
9	A	40	110	930			
9	A	30	40	620			
9	A	20	30	560			
9	A	40	50	1040			
9	A	0	50	780			
9	A	30	150	1080			
10	A	60	285	1315	4	23-12-2010	11-1-2-11
10	A	55	330	1280			
10	A	30	220	1120			
10	A	35	225	1015			
10	A	40	350	1120			
10	A	60	220	780			
10	A	20	175	910			
10	A	60	280	1280			
10	A	40	275	1285			
10	A	25	240	1165			
12	A	20	60	965	6	27-12-2010	11/13-1-2011
12	A	85	180	1400			
12	A	85	100	1820			
12	A	10	75	605			
12	A	65	55	965			
12	A	-	-	-			
12	A	-	-	-			
12	A	-	-	-			
12	A	-	-	-			
12	A	-	-	-			
13	A	80	30	920	6	5-1-2011	19-1-2011
13	A	70	20	780			
13	A	165	0	850			
13	A	5	10	455			
13	A	5	85	115			
13	A	0	20	385			
13	A	0	285	1165			
13	A	65	0	1535			
13	A	0	10	520			
13	A	55	95	1460			
13	G	100	150	930			24-1-2011
13	G	75	335	2595			
13	G	75	220	1885			
13	G	105	110	1235			
13	G	60	135	750			
13	G	90	175	1350			
13	G	10	255	1250			
13	G	80	215	1130			
13	G	0	175	1630			
13	G	25	160	2000			
15	A	5	95	1440	5	22-12-2010	17/19-1-2011
15	A	0	45	1440			
15	A	0	100	1315			
15	A	5	45	1175			
15	A	5	80	1060			
15	A	5	235	1285			
15	A	5	65	925			
15	A	0	70	1205			

15	A	0	120	855			
15	A	5	70	1105			
15	(C1+) E	4	21	403			13-1-2011
15	(C1+) E	3	12	404			
15	(C1+) E	7	19	798			
15	(C1+) E	44	272	861			
15	(C1+) E	28	54	999			
15	(C1+) E	33	81	1180			
15	(C1+) E	8	88	1063			
15	(C1+) E	1	108	1167			
15	(C1+) E	8	106	976			
15	(C1+) E	26	103	1198			
17	A	0	30	515	8	27-12-2010	8-1-2011
17	A	10	0	550			
17	A	0	0	885			
17	A	5	30	575			
17	A	0	5	540			
17	A	25	0	585			
17	A	0	15	620			
17	A	0	10	550			
17	A	0	10	515			
17	A	0	10	625			
17	F	135	75	510			8-1-2011
17	F	40	80	835			
17	F	170	145	1010			
17	F	490	295	1710			
17	F	215	175	1500			
17	F	140	245	1240			
17	F	135	115	990			
17	F	705	235	2205			
17	F	545	175	1025			
17	F	280	225	1235			
18	C1	80	340	2545	4	22-12-2010	26-1-2011
18	C1	75	350	2345			
18	C1	135	395	2715			
18	C1	120	460	2690			
18	C1	80	330	2305			
18	C1	135	370	2880			
18	C1	130	250	2265			
18	C1	110	350	3555			
18	C1	90	365	2435			
18	C1	120	400	3785			
18	C2	1740	615	4240			
18	C2	2270	695	4080			
18	C2	1135	765	4375			
18	C2	1020	730	4525			
18	C2	1245	865	4690			
18	C2	925	690	4390			
18	C2	2185	640	4095			
18	C2	860	740	5675			
18	C2	975	605	4280			
18	C2	2370	805	5440			
19	A	85	155	1455	5	20-1-2011	25-1-2011
19	A	40	115	1375			
19	A	125	200	1505			
19	A	45	210	1635			
19	A	110	160	1330			
19	A	50	65	935			
19	A	45	70	1375			

19	A	95	195	1680			
19	A	155	90	1555			
19	A	265	50	1805			
19	C1	95	725	2105			
19	C1	85	505	1525			
19	C1	140	450	1955			
19	C1	105	490	2505			
19	C1	115	555	1850			
19	C1	135	405	1520			
19	C1	80	870	1470			
19	C1	50	685	1865			
19	C1	100	590	5585			
19	C1	170	865	1800			
20	A?	90	90	310	4	5-1-2011	januari 2011
20	A?	35	255	230			
20	A?	0	140	50			
20	A?	5	130	65			
20	A?	0	120	90			
20	A?	15	95	160			
20	A?	35	65	115			
20	A?	35	70	75			
20	A?	40	105	110			
20	A?	70	140	140			
22	A	0	75	1435	17 (2w), 6	24-12-2010	11-1-2011
22	A	0	150	1245			
22	A	0	110	1720			
22	A	5	50	1610			
22	A	0	160	2200			
22	A	0	120	1695			
22	A	5	185	2975			
22	A	10	170	2430			
22	A	0	130	1545			
22	A	5	130	1770			
22	E	465	290	2605			17-1-2011
22	E	355	360	2580			
22	E	610	455	3695			
22	E	470	465	3945			
22	E	520	580	4755			
22	E	460	530	3395			
22	E	545	525	3390			
22	E	500	285	4520			
22	E	395	345	2335			
22	E	395	440	2920			
23	A	10	300	1705	8	28-12-2010	31-12-2010
23	A	10	380	1890			
23	A	5	395	2670			
23	A	5	450	1800			
23	A	10	360	2535			
23	A	10	155	1400			
23	A	5	140	1220			
23	A	5	215	1915			
23	A	5	165	845			
23	A	0	110	1135			
23	E	325	465	2080			
23	E	280	810	3280			
23	E	295	630	2290			
23	E	120	440	1775			
23	E	335	350	1600			
23	E	120	465	1880			

23	E	135	630	1955			
23	E	65	385	1845			
23	E	125	460	1525			
23	E	185	515	1255			

EUPHRESCO project *Meloidogyne chitwoodi* and *Meloidogyne fallax*

Topic 2:

Ring test
Detection and identification of *Meloidogyne chitwoodi* and *Meloidogyne fallax* by conventional and real time PCR assays

1. Introduction

This ring test aims at improving the diagnostic process of *M. chitwoodi* and *M. fallax* currently in use, especially by introducing new technology (real time PCR) on a routine basis and for different uses (initial description of ring test provided in appendix 1). The real time PCR tests available are often dedicated for identification, but rarely for detection of the pest in complex matrices (soils, tubers, roots) in a realistic sample size. This topic aims at evaluating real time PCR tests in different contexts of use through a ring tests on nematode suspensions extracted from soil and on isolated nematodes. It also aims at comparing the two different approaches: real time against conventional PCR.

The results of these projects would help in the adoption of an EU consensus approach for *M. chitwoodi* and *M. fallax* detection, identification with real time and conventional PCR tools especially in terms of control, management, and efficiency of eradication or assessment of treatment assays.

2. General organisation of the test

Purposes of the ring test

The purposes of the ring test were to:

- evaluate the performance of conventional and real time PCR assays for detection of *M. chitwoodi* and *M. fallax* in nematode suspensions extracted from soil.
- evaluate the performance of conventional and real time PCR assays for identification on isolated individuals of *M. chitwoodi* and *M. fallax*.

Organising laboratory

The nematology unit of the Anses French Plant Health Laboratory organised the ring test for *Meloidogyne* detection and identification.

Several operators from this laboratory were involved in the conception and management of the test. The following table indicates the staff involved in and their contribution to the proficiency test.

Table 1: Staff involved in the ring test

Staff who contributed to the ring test		
Full name	**Function**	**Contribution**
Géraldine ANTHOINE	Co-ordinator	Organisation, data processing, drafting of the report
Géraldine ANTHOINE Sylvie GAMEL Fabrice OLLIVIER	Technical operator	Technical preparation of the test

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Participating laboratories

Seventeen laboratories registered for the ring test following the Euphresco Meloidogyne's call for applicants.
In the report, the laboratories are not indicated to ensure the confidentiality of results, only reference to sample set number is used.

No prerequisite for participation was required. But as the ring test focused on PCR assays, the participant laboratory needed to be able to perform such assay.

Table 2 : List of participating laboratories

Country	Name Institute	Address Institute	EUPHRESCO Partner
Austria	Institute for Plant Health,	Spargelfeldstr. 191, A-1220 Wien, Österreich/AUSTRIA	AT-AGES
Belgium	ILVO	Burg. Van Gansberghelaan 96, B-9820 Merelbeke, Belgium	BE-ILVO
Bulgaria	Central laboratory for plant quarantine	120,N.Moushanov,1330, Sofia, Bulgaria	BG-NSPP
Bulgaria	Plant Protection Institute	35 P. Volov Str, 2230 Kostinbrod, Bulgaria	BG-NSPP
Czech Republic	State Phytosanitary Administration	Postal address: Ztracena 1099/10, 161 06 Praha 6, Czech Republic	CZ-SPA
Denmark	Frø og Planter, Lab. for Diagnostik i Planter	Ministeriet for Fødevarer, Landbrug og Fiskeri Plantedirektoratet Skovbrynet 20, 2800 Kgs. Lyngby	DK
France	LNPV / LSV	LNPV Domaine de la Motte BP35327 LE RHEU cedex France	FR-DGAL / Anses
France	FNPPPT	FNPPPT-INRA UMR BiO3P Domaine de La Motte - B.P. 35327 -F- 35653 Le Rheu Cedex France	FR-FNPPPT
Germany	Julius Kühn-Institut/Plant Health Institute	Messeweg 11/12, 38104 Braunschweig, Germany	DE-JKI
Netherlands	Plant Protection service	PO Box 9102, 6700 HC Wageningen The Netherlads	NL-PPS
Netherlands	NAK	NAK, Randweg 14, 8304 AS Emmeloord, Netherlands	NL-NAK
Portugal	IMAR-CMA	Dept. Life sciences, University of Coimbra, 3004-517, Coimbra, Portugal	IMAR-CMA
Slovenia	Agricultural Institute of Slovenia	Hacquetova 17, 1000 Ljubljana, Slovenia	SL_KIS
Switzerland	Agroscope Changins-Waedenswil ACW	Schloss, PO Box, 8820 Waedenswil Switzerland	CH-FOAG
Turkey	Plant Protection Research Institute	Gençlik cad. No:6 Bornova-İzmir/TURKEY	TR-GDAR
United Kingdom	The Food & Environment Research Agency	The Food & Environment Research Agency, Sand Hutton, York, YO41 1LZ, UK	UK-Fera
United Kingdom	AFBI	18a Newforge Lane, Belfast BT9 5PX, Northern Ireland UK	UK_AFBI

Instructions to participants

DNA extraction with Promega Wizard Food kit was documented as the recommended DNA extraction procedure. Recommendations for using Kingfisher automate mL were provided.
Initially, the prescribed PCR assays were the real time PCR assays developed by Zijlstra *et al*. (2006) and patented by Blgg AgroXpertus[1]. Technical procedures, such as EPPO diagnostic protocol PM7/41 (2) and BlggAgroXpertus recommendations, were communicated by the organizer to all participants.
For conventional PCR, no specific recommendations were given.

Participants received the sample shipment along with an acknowledgement of receipt. A results form was also sent to participants: to comment and detail the execution of the test within their laboratory; to communicate any information on trouble shooting during the assays; and to report the results.

[1] Blgg AgroXpertus was replaced by Clear Detection after this ring test.

Framework of the ring test

The proficiency test was conducted according to the framework summarised in the following table :

Table 3 : Framework for the ring test.

Steps	Period	Who ?
Call for applicants	September 2010	Organiser
Organisation of the test	September-October 2010	Organiser
Experimental design provided	October 2010	Organiser
Samples dispatched	First parcel : 14/01/11 Second parcel : 28/01/11	Organiser
Deadline for submitting results	End of February 2011, beginning of March 2011	All participants
Final report transmitted to the participants	first draft version May 2011	Organiser

3. Test material

Each participant received one or several sets of coded samples. The individual coding of the samples was randomised and differed for each participant. Coding of the samples was kept confidential by the organiser.

Sample characteristics

Detection purpose
Target samples were obtained after spiking one soil suspension with different numbers of individuals of the target *Meloidogyne* species.
Non-target samples consisted of different soils solutions.

Linearity purpose
Target samples were obtained from bulk DNA extraction of target *Meloidogyne* species.

Specificity purpose
Target and non target samples were obtained from bulk DNA extraction of different *Meloidogyne* species.

Identification purpose
Target samples were obtained after spiking water with different number and stages of target *Meloidogyne* species individuals.
Non-target samples consisted of water spiked with females of *Meloidogyne minor*.

Table 4 : Sample set content and samples description.
All detection samples contain soil suspension, either alone (soil only) or with the addition of target nematodes. Linearity and specificity samples are made of DNA solutions. Identification samples only include water and isolated nematodes.

M. chitwoodi (Mc) assay			*M. fallax (Mf)* assay	
Type	**Tube content**		**Type**	**Tube content**
Go	soil only	Detection	CHR	soil only
5 J2	soil + 5j2 of Mc		**XX J2**	soil + numerous J2 of Mf
xx J2	soil + numerous J2 of Mc		**10J2**	soil + 10j2 of Mf
CHR	soil only		G0	soil only
10 J2	soil + 10j2 of Mc		**5 J2**	soil + 5j2 of Mf
Rh	soil only		Rh	soil only
Go	soil only		Go	soil only
CHR	soil only		CHR	soil only
pur	Mc DNA pure	Linearity	**pur**	Mf DNA pure
1/10	Mc DNA 10 fold diluted		**1/10**	Mf DNA 10 fold diluted
1/100	Mc DNA 100 folds diluted		**1/100**	Mf DNA 100 folds diluted
1/1000	Mc DNA 1000 folds diluted		**1/1000**	Mf DNA 1000 folds diluted
ME	*M. enterolobii* DNA	Specificity	ME	*M. enterolobii* DNA
Mmi	*M. minor* DNA		Mmi	*M. minor* DNA
MJ	*M. javanica* DNA		MJ	*M. javanica* DNA
Mc	*M. chitwoodi* DNA		Mc	*M. chitwoodi* DNA
MJ	*M. javanica* DNA		MJ	*M. javanica* DNA
MF	*M. fallax* DNA		MF	*M. fallax* DNA
Mna	*M. naasi* DNA		Mna	*M. naasi* DNA
MH	*M. hapla* DNA		MH	*M. hapla* DNA
eau	water (no DNA)		eau	water (no DNA)
Hs	*Heterodera schaachtii* DNA		Hs	*Heterodera schaachtii* DNA
xx J2	numerous isolated J2 of Mc	Identification	**XX J2**	numerous isolated J2 of Mf
xx J2	numerous isolated J2 of Mc		**XX J2**	numerous isolated J2 of Mf
5 J2	5 isolated J2 of Mc		**5 J2**	5 isolated J2 of Mf
10 J2	10 isolated J2 of Mc		**10 J2**	10 isolated J2 of Mf
2 fem MC	2 females of Mc		**2 fem MF**	2 females of Mf
2 fem Mmi	2 females of M. minor		1 fem Mmi	1 female of M. minor

Sample validation

During preliminary tests, samples were validated in terms of status (accepted reference value) and stability to ensure that the inter-laboratory study was reliable.

Accepted reference value

The accepted reference value **(ISO-5725-1**) is the value that serves as an agreed-upon reference for comparison, and which is derived as an assigned value based on the experimental work of the organiser.

In our case, as the analysis produces a qualitative result, the accepted reference value could only be: "+" = positive result, "-" = negative result and "?" = undefined result.

The accepted reference value was established according to the preparation of the samples: positive samples were prepared either by spiking soil solution or water with nematodes. For DNA solutions, DNA was extracted from bulk nematodes populations and checked by PCR assay.

Homogeneity

Homogeneity was roughly evaluated for the detection and identification samples. Only one set of samples was randomly chosen and tested, which doesn't allow to conclude about the homogeneity of the samples.

Stability

Sample stability over time, particularly during the course of the ring test, is required for a reliable comparison.

Stability was evaluated for detection and some of the specificity samples as described in table 4. The stability was tested on 5th of January 2011 and on 22nd and 23rd of February 2011 with Blgg PCR assay (period that corresponds approximately to the duration prescribed by the organizer for the ring test's execution, i.e. 40 days). The samples were sent to participants on the 14th of January 2011. An additional dispatch was organized on the 28th of January 2011 for some of the participants.

Detailed results are available in **appendix 2**.

The stability test shows that Mc and Mf samples did not evolve much over time. The Ct value increased less than Ct+3 for detection samples and less than Ct+1 for specificity samples. At the same time, the fusion peak temperature did not evolve more than 0.15 °C.

This observation confirmed that participants should analyse the samples as soon as possible after their arrival. Some of the participants exceeded the total duration tested within the stability assay: until 66 days for DNA extraction and 75 days for total duration of the assay. Nevertheless all the results were included in the ring test analysis.

4. Interpretation of results

All participating laboratories submitted their results, even if sometimes laboratories encountered difficulties in performing some tests and couldn't give results.
Given that the method is qualitative, results were transmitted by each laboratory as follows: "+" (positive), "-" (negative) and "?" (undefined). For real time PCR, complementary data were also sent as Ct values and fusion peak temperature.
Details of equipments, reagents and data analysis method were provided by each participant to the organizer.

Practical implementation of the ring test

The detailed description of the test carried out for each sample set is available in **appendix 3**.

Shipment and receipt of the samples

The ring test samples were dispatched from France on 14th January 2011. Most laboratories received their parcel quickly. Three laboratories didn't receive this shipment and a new one was organized on 28th January 2011.

All laboratories received the samples in good condition, even if for some participants a second dispatch was organized as the first one didn't arrive on time.

Beginning of the test

Seventeen of the nineteen sample sets were treated for DNA extraction within 39 days (duration tested for stability). Fifteen of the nineteen sample sets were treated for PCR within 40 days (duration tested for stability).

Controls

The positive and negative controls used in the assays were those of the participant laboratory.

Critical consumables and equipment

DNA extraction kit : Most of the sample sets (15 out of 19) were extracted with the recommended kit, Promega Wizard Food kit. Several other kits were used for only one sample set each time: MOBIO, Blgg lysis buffer, QIAgen DNA mini kit and QIAgen Dneasy minikit, Roche high pure.

PCR enzymes and mix : For real time PCR assays, the participants used different suppliers for Taq DNA enzyme: Bioline, Roche, Thermo, ABI, ABgene, Biorad, Lonza, Fermentas, Eurogentec, ABI, Sigma. For conventional PCR, the list of suppliers are: ABI, Bioline, Eurobio, Fermentas, MP Biomedicals.

Primers : the suppliers of this type of reagent were very diverse, except for the Blgg assay, for which the primers were provided directly by Blgg.

Probes : the probes were only provided by ABI, but for one laboratory the supplier was Biomers.

PCR machines: for real time PCR assays, different platforms were used: ABI 7900HT, 7500; 7300; Eppendorf realplex 4; Roche LC480; Stratagene MX 3000 and 3005P. For conventional PCR assays, the PCR machines were ABI 2700, 2720, 9700; Biorad MJ mini personal

Analysis of results and interpretation

For real time PCR assays, the definition of the threshold was equally established with either the manual (11 out of 32) or the automatic (14 out of 32) procedure. Only 7 out of 32 assays were analysed with an automatic procedure completed with a manual adjustment.

Transmission of the results

All laboratories used the form provided to submit their results.

Data analysis

Validation of the results by the organiser

The table below summarises the R^2 values obtained for the correlation between Ct values and target DNA solution dilutions.

Table 6 : Correlation between Ct values and concentration of DNA of target species – R^2 values.

Samples set / PCR test / nematode	1	2	3	4	5	6	7	8	9	10	12	14	15	16	17	18	19
Blgg / *M chitwoodi*	0.94	0.99	0.97	0.96	0.96	(a)	0.96	0.97	0.97	0.97	0.96	0.95	0.99	(b)	0.98	0.96	(b)
Blgg / *M. fallax*	0.99	0.90	0.98	0.97	0.97	0.98	0.98	0.95	0.99	0.96	0.94	0.95	0.95	(b)	0.98	0.96	(b)
Zijlstra / *M chitwoodi*	0.96	0.99	(c)	0.90	0.96	(c)	0.94	0.96	0.91	0.96	0.97	(c)	0.95	0.99	0.96	0.95	0.97
Zijlstra / *M. fallax*	0.99	0.95	(c)	(a)	0.98	(c)	(a)	0.97	0.98	0.95	0.96	(c)	0.95	0.98	0.99	0.96	0.96

(a) : Ct value only available for two dilutions points
(b) Not tested
(c) no amplification obtained with the PCR test
x : inconsistent order of Ct values compared to dilution points. R^2 values obtained when respecting the increasing order

Graphs were drawn using the Ct values (see **appendix 4**).
The R^2 values were mostly correct and allowed the use of the results for our statistical analysis. Nevertheless, it must be noted that for some sample sets, a mistake occurred when reporting the code of sample and the associated Ct value (highlighted in table 6).

Evaluation criteria

We interpreted the results for each sample set and PCR test by calculating the number of positive agreements (PA), negative agreements (NA), positive deviations (PD) and negative deviations (ND), according to Table 7. The analysis was conducted for each type of samples: detection samples, specificity samples and identification samples.

Table 7: Definition of the parameters of positive agreement (PA), negative agreement (NA), positive deviation (PD) and negative deviation (ND).

Reference / Laboratory	Accepted reference positive value	Accepted reference negative value
Positive sample set result	PA = positive agreement	PD = positive deviation
Negative sample set result	ND = negative deviation	NA = negative agreement
Undefined sample set result	ND = negative deviation	PD = positive deviation

These parameters were used to calculate the performance criteria (Table 8).

Table 8: Definition and calculation of performance criteria.

Performance criteria	Definition	Calculation
Accuracy	Closeness of agreement between the test result and the accepted reference value (**ISO 5725-1)**. Therefore, the accuracy indicates a laboratory's capacity to obtain the expected result. Criteria of sensitivity and specificity are linked to accuracy. *Comments: the mode of calculation used to determine the accuracy means that a balanced evaluation can be performed even if the sizes of N+ and N- are not equal.*	= [(sum PA/$N^{+)}$ + (sum NA/ N^{-})]/2
Sensitivity (SE)	A laboratory's capacity to obtain a positive result for the samples for which the accepted reference value is	= sum PA /N^{+}

Performance criteria	Definition	Calculation
	positive.	Comment: the result of the calculation (1-SE) gives the number of false negatives obtained by the laboratory.
Specificity (SP)	A laboratory's capacity to obtain a negative result for the samples for which the accepted reference value is negative.	= sum NA/N^- Comment: the result of the calculation (1-SP) gives the number of false positives obtained by the laboratory.

Key: N^+ = number of samples for which the accepted reference value is positive = sum PA+sum ND
N^- = number of samples for which the accepted reference value is negative = sum NA+ sum PD

For the detection samples, the three described performance criteria were evaluated. In this case, the specificity only covered the absence of positive results for samples only including soil suspension (labeled as "soil only" and free from target nematodes), this performance criteria is also called selectivity.
For the specificity samples, the only performance criteria evaluated was the specificity for non target nematodes.
For the identification samples, the three described performance criteria were evaluated.

Results of the data analysis (descriptive statistics and performance statistics)

Real time PCR assays

Blgg real time PCR assays

Many participants were not used to work with this test and were not aware of the reaction obtained (lower Ct and Tm value analysis needed to conclude about the status of the sample).

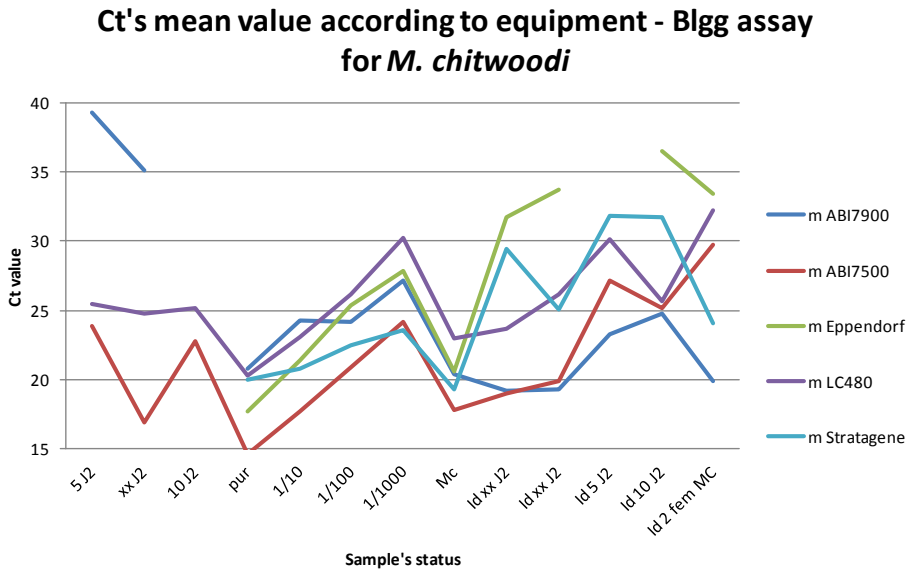
The performance of the tests are summarized in the table below.

Target			Detection Samples	Specificity Samples	Identification Samples
M. chitwoodi	Performance criteria	sensitivity	54.2%		57,5%
		specificity	96.3%	78.5%	62,5%
		accuracy	80,5%		58,3%
Target			**Detection Samples**	**Specificity Samples**	**Identification Samples**
M. fallax	Performance criteria	sensitivity	72.9%		71,3%
		specificity	90.0%	63.9%	68,8%
		accuracy	83.6%		70,8%

For detection purpose, which was one of the aims of this ring test, and from detection samples results, this real time PCR test gives quite good results for both species in terms of selectivity (few false reaction with soil suspensions free from target nematodes) and accuracy. Nevertheless, the result of sensitivity might not be sufficient enough for routine analysis, as described in this evaluation.
The specificity of this test against non target nematodes, from the analysis of specificity samples' results, can be qualified as medium.
For identification purpose and from the results of the identification samples, this test might not have sufficient performance to be used and provide reliable results.

From the obtained Ct values, it can be observed that that standard deviation is generally around 2 when the target is present and that the TM value varies within a 10°C interval.

Furthermore, when analyzing all the data, but only considering the different equipments, there is a drift between equipment, as illustrated in the following figure.



All these results suggest that for a real time PCR assay using SYBRGreen, any cut off value for Ct or TM should be defined in each laboratory, taking into account its specific reagents and equipment. Standardized procedure can be elaborated but internal calibration and reference material would be needed.

Taqman Real Time PCR assay from Zijlstra *et al*. (2006)

Several participants encountered difficulties to get any amplification with this method, even if these laboratories were used to work with real time PCR assays. Individual adjustments according to the PCR machine are probably needed.

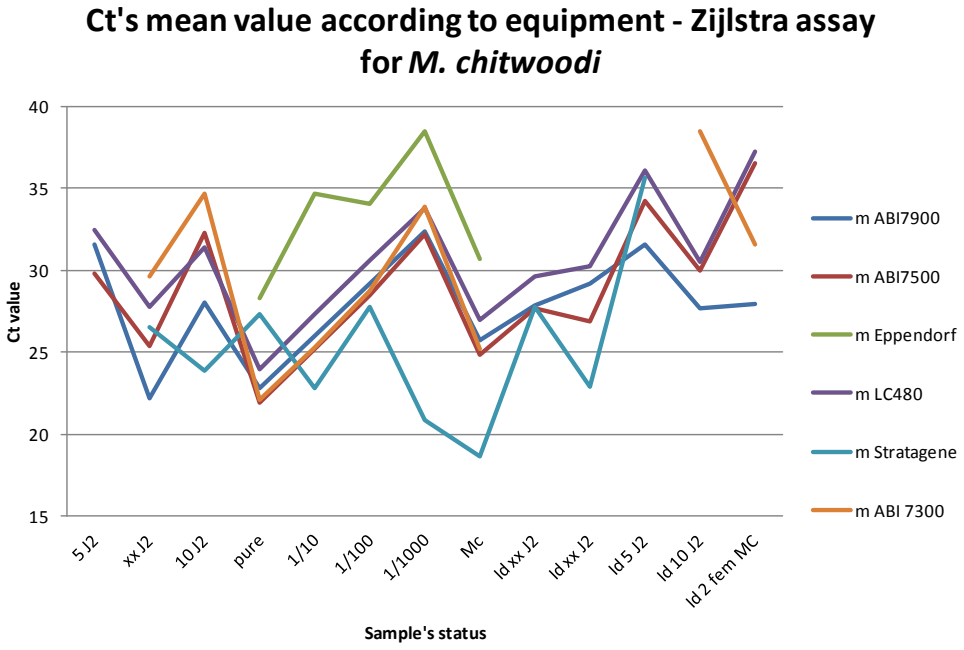
The performance of the tests are summarized in the table below.

Target			Detection samples	Specificity samples	Identification samples
M. chitwoodi	Performance criteria	sensitivity	54,9%		52,9%
		specificity	76,5%	78.4%	64,7%
		accuracy	68,4%		54,9%
Target			**Detection samples**	**Specificity samples**	**Identification samples**
M. fallax	Performance criteria	sensitivity	62,7%		50,6%
		specificity	77,6%	75.2%	64,7%
		accuracy	72,1%		52,9%

For detection purpose, which was the main aim of this ring test, this real time PCR test gives acceptable results for both species, in terms of selectivity (few false reaction with soil suspensions free from target nematodes) and accuracy. Nevertheless, the results of sensitivity might not be sufficient enough for routine analysis as described in this evaluation.
The specificity of this test against non target nematodes, from the analysis of specificity samples' results, can be qualified as medium and is close for both species.

For identification purpose and from the results of the identification samples, this test might not have sufficient performance to be used and provide reliable results.

From the obtained Ct values, it can be observed that that standard deviation varies between 2 and 7. So there is a high variation between laboratories.
This observation is also confirmed when analyzing the data according to the equipment used, as illustrated in the fugure below.



All these results suggest that for a Taqman real time PCR assay, any cut off value for Ct should be defined in each laboratory, taking into account its specific reagents and equipment. Standardized procedure can be elaborated but internal calibration and reference material would be needed.

Conventional PCR assays

Specific PCR from Wishart *et al*. (2000)

Only six sample sets were tested with this method.
The performance of the tests are expressed in percentages and summarized in the table below.

Target			Detection samples	Specificity samples	Identification samples
M. chitwoodi	Performance criteria	sensitivity	66.7%		83,3%
		specificity	82.1%	97.7%	50,0%
		accuracy	76.7%		77,8%
Target			**Detection samples**	**Specificity samples**	**Identification samples**
M. fallax	Performance criteria	sensitivity	33,3%		63,3%
		specificity	96,0%	100.0%	50,0%
		accuracy	72,5%		61,1%

This conventional PCR test is really accurate in terms of specificity for both target species. From the results of identification samples, this test is adequate to identify individuals of the target species. Overall, the results are better for *M. chitwoodi* than for *M. fallax*. Even if this test was not developed for detection, the results obtained for the samples dedicated to this purpose showed

that the test could be used for this purpose, but with quite poor sensitivity, especially for *M. fallax*. So this test is more adequate for identification of individuals.

Specific SCAR PCR from Zijlstra *et al*. (2000)

Only three sample sets were tested with this method.
The performance of the tests are expressed as percentages and summarized in the table below.

Target			Detection samples	Specificity samples	Identification samples
M. chitwoodi	Performance criteria	sensitivity	11,1%		0,0%
		specificity	100,0%	100.0%	100,0%
		accuracy	66,7%		16,7%
Target			**Detection samples**	**Specificity samples**	**Identification samples**
M. fallax	Performance criteria	sensitivity	22,2%		0,0%
		specificity	100,0%	96.3%	100,0%
		accuracy	70,8%		16,7%

This conventional PCR test is really accurate in terms of specificity for both target species whatever the purpose of the analysis (detection, specificity and identification). Nevertheless, its sensitivity is really poor when target species present at low level (detection and identification cases). So this test seems more adequate for a confirmation when individuals or DNA are not limited.
Nevertheless, as only three sample sets were analysed, it is difficult to definitely draw a conclusion about this test.

PCR –RFLP from Zijlstra *et al*. (1997)

Only three sample sets were tested with this method.
The performance of the tests are expressed as percentages and summarized in the table below.

Target			Detection samples	Specificity samples	Identification samples
M. chitwoodi	Performance criteria	sensitivity	33,3%		46,7%
		specificity	100,0%	59.1%	66,7%
		accuracy	73,3%		50,0%
Target			**Detection samples**	**Specificity samples**	**Identification samples**
M. fallax	Performance criteria	sensitivity	50,0%		33,3%
		specificity	100,0%	59.1%	66,7%
		accuracy	81,3%		38,9%

This PCR RFLP test seems to be the least specific test. This test is based on the use of universal primers for DNA amplification, followed by RFLP. This type of test even leads to difficulties in interpretation. Furthermore, this test has been published many years ago and new species arose after that time with possible confusing profiles. For identification purpose, the results from identification samples are worth compared to those from Wishart *et al*. (2000).
Unexpectedly, the results of this test for detection samples are correct, except for the sensitivity.
Nevertheless, as only three sample sets were analysed, it is really difficult to definitely draw a conclusion about this test.

Overall analysis – all tests included

Target species		Test	Detection samples				
			Blgg*	Zijlstra *	Wishart	Zijlstra scar	Zijlstra RFLP
M. chitwoodi	*performance criteria*	sensitivity	54,2	54,9	68,4	11,1	33,3
		specificity	96,3	76,5	84,8	100,0	100,0
		accuracy	80,5	68,4	78,8	66,7	73,3
		Test	Blgg*	Zijlstra *	Wishart	Zijlstra scar	Zijlstra RFLP
M. fallax		sensitivity	72,9	62,7	38,9	22,2	50,0
		specificity	90,0	77,6	96,7	100,0	100,0
		accuracy	83,6	72,1	75,0	70,8	81,3

* real time PCR assay

For detection assay of both target species, according to this ring test, the best accuracy is obtained with the real time PCR assay from **Blgg AgroXpertus**. This test has also the highest sensitivity for *M. fallax*, but not for *M. chitwoodi*. For *M. chitwoodi*, the conventional PCR from Wishart *et al*. (2000) is the most sensitive and its values for accuracy are close to those of the Blgg AgroXpertus test.

Target species		Test	Specificity samples				
			Blgg*	Zijlstra *	Wishart	Zijlstra scar	Zijlstra RFLP
M. chitwoodi	*performance criteria*	specificity	78,5	78,4	98,3	100,0	59,1
		accuracy	79,4	77,6	98,5	96,7	64,0
		Test	Blgg*	Zijlstra *	Wishart	Zijlstra scar	Zijlstra RFLP
M. fallax		specificity	63,9	75,2	100,0	96,3	59,1
		accuracy	66,9	75,9	100,0	93,3	64,0

* real time PCR assay

When facing many different species and genera of nematodes, according to this ring test, the best accuracy is obtained with the conventional PCR assay from **Wishart *et al.* (2000)**. Probably, the results for real time PCR assays could be better after some adjustment in reaction conditions, threshold definition and results analysis.

Target species		Test	Identification samples				
			Blgg*	Zijlstra *	Wishart	Zijlstra scar	Zijlstra RFLP
M. chitwoodi	*performance criteria*	sensitivity	57,5	52,9	77,1	0,0	46,7
		specificity	62,5	64,7	57,1	100,0	66,7
		accuracy	58,3	54,9	73,8	16,7	50,0
		Test	Blgg*	Zijlstra *	Wishart	Zijlstra scar	Zijlstra RFLP
M. fallax		sensitivity	71,3	50,6	62,9	0,0	33,3
		specificity	68,8	64,7	57,1	100,0	66,7
		accuracy	70,8	52,9	61,9	16,7	38,9

* real time PCR assay

For identification purpose of nematodes individuals, according to this ring test, the best accuracy is different according to the target species. For *M. chitwoodi* the most accurate test is **Wishart *et al.* (2000**). For *M. fallax* the real time PCR assay from **Blgg AgroXpertus** is the most accurate**.**

5. Conclusion

A lot of data were produced thanks to the involvement of all participants. It was one of the first ring tests for the evaluation of molecular tools for nematodes detection and identification. The methodology of such ring test should be improved, by recruiting participants that are trained and used to work with the techniques tested, but also by providing more guidance on the implementation of the test (Ct value expected, differences of Ct depending of the equipment, melting temperature expected).

The participants highlighted the duration for performing two separate simplex tests instead of a duplex PCR in the case of Blgg AgroXpertus tools. Participants failed in performing the Taqman real time PCR assay from Zijlstra *et al.* (2006) without explanation.
This study also showed that for detection of the pests with real time assay, in house calibration of the test is needed, especially to define Ct's cut-off values or target melting temperature, but also that reference material must be available especially for determining the limit of detection. Even if the limit of detection was previously defined by the organiser in their laboratory's conditions, this result might not be extrapolated when different parameters (PCR machine, PCR mix) are used.

This ring test finally provides data for selecting the appropriate molecular test (real time or conventional) depending on its use for each target species.

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Appendix 1 : Description of the project

Topic 2 – Assessment of *M. chitwoodi* and *M. fallax* real time PCR tools

Introduction
This topic aims at improving the diagnostic process of *M. chitwoodi* and *M. fallax* currently in use, especially by introducing new technology (real time PCR) on a routine basis and for different uses. The real time PCR tests available are often dedicated for identification, but rarely for detection of the pest in complex matrices (soils, tubers, roots) in a realistic sample size. This topic aims at evaluating real time PCR tests in different contexts of use through a ring tests on nematode suspensions extracted from soil. Ring testing on potato tubers is not included. If deemed useful another ring test in potato tubers could be organized at a later stage. The final scope of the ring test depends also on the availability of materials and procedures.
The results of these projects would help in the adoption of an EU consensus approach for *M. chitwoodi* and *M. fallax* detection, identification with real time PCR tools especially in terms of control, management, and efficiency of eradication or assessment of treatment assays. Some procedures used by companies and that are not published would be introduced in this project. Depending on their availability, the scope of this topic would change.

Aims :
- Assessment of real time PCR assays for the detection and the identification of *Meloidogyne chitwoodi* (Mc) and *M. fallax* (Mf) by ring testing the same tools and samples in different laboratories.
- Indirect assessment of performance of laboratories in using real time PCR tools.

Real Time PCR tests to be ring tested :
Two different protocols would be evaluated (see also EPPO diagnostic protocol):
- Zijlstra *et al*. (2006), modified by PPS (the Dutch Plant Protection Service): Taqman test for *M. chitwoodi* and *M. fallax* (Protocol see annex 1).This test was evaluated within the PORTCHECK project in 2007 with potato peels. Primers are freely available, i.e. not patented.
- Blgg test : SYBRgreen test designed for detection of target species in soil, one primer set for each target species (Protocol see annex 2). This protocol has not been published, primers are patented and to be ordered from Blgg Agro Xpertus.

The participants are strongly encouraged to perform both tests.

Material and methods :

- Assessment for detection purpose:

For detection purpose, the sample's nature would be:

a – nematode suspension extracted from soil and free from Meloidogyne, spiked with different numbers (3 levels) of target nematodes Mc or Mf (6 samples),
b - nematode suspension extracted from soil free from Mc or Mf, 5 samples,
c - DNA solutions (*) of target and non target species, 10 samples approximately
d - DNA dilutions (*) range (4 points) from each target species, 4 samples

Except for the DNA solutions (*), all samples would have to be extracted with Promega Wizard Food kit according to the manufacturer recommendation (ref. FF3750) (guidance in annex 3) for DNA isolation. Warning for the participants not used to working with this kit: start to practice with the kit before the test (theoretical presentation during the start meeting).

Real time PCR execution:
DNA from each sample (a and b) and each DNA solution (c and d) are tested at least twice (replicate) within the same run for each test. At least one run is performed.

- Assessment for identification purpose:

For identification purpose, the sample's nature would be:

e - Isolated nematodes in aqueous solution (2 samples from 3 different levels for each species)

Real time PCR execution:
DNA from each sample is tested at least twice (replicate) within the same run for each test. At least one run is performed.

	Number of		DNA extraction to be completed	Total number of reaction
	samples	PCR replicates		
Detection				
Soils extracts spiked with *M. chitwoodi*	3	2	yes	6
Soils extracts spiked with *M. fallax*	3	2	yes	6
Soils extracts free from target species	5	2	yes	10
DNA solutions for target and non target species	10	2	no	20
DNA dilution range of *M. chitwoodi* when Mc primers used	4	2	no	8
DNA dilution range of *M. fallax* when Mf primers used	4	2	no	8
Identification				
Isolated *M. chitwoodi* in aqueous solution	6	2	yes	12
Isolated *M. fallax* in aqueous solution	6	2	yes	12

Total PCR reaction/test	82

- Other products :

Extraction reagents, primers, probes, real time PCR reagents and materials: each participant is responsible for ordering/ providing adequate products for ring tests purpose.

Time schedule

Protocols and forms, letters of commitment for sending the samples: November 2011
Samples sent: January 2011
Execution of the ring test by all partners: within one month from the dispatch of the samples.
Analysis of the results and writing of report by the organisers: April to June 2011

Contact person for this topic: Géraldine Anthoine

Results of stability study

Nematode content	Tube code	Ct value rep 1	Ct value rep 2	Ct value rep 1	Ct value rep 2	TM value rep 1	TM value rep 2	TM value rep 1	TM value rep 2	Variation **Δ Ct**	Variation **Δ tm**
						M. chitwoodi					
5 J2	soil C274	32,12	32,28	29,5	29,8	82,85	82,77	82,8	82,7	2,585	0,065
xx J2	soil C306	24,18	24,44	26,4	26,1	82,66	82,64	82,7	82,7	1,935	0,075
10 J2	soil C363	29,5	29,32	29,5	29,5	82,73	82,64	82,8	82,8	0,115	0,13
Mc	ADN-n°4	23,93	23,93	24,5	24,7	82,57	82,65	82,7	82,8	0,64	0,125
						M. fallax					
XX J2	soil F474	23,6	24,14	21,1	21,5	83,05	82,98	83	82,9	2,61	0,04
10J2	soil F524	28,04	28,99	30,7	30,7	82,98	82,94	83	83	2,195	0,035
5 J2	soil F580	29,77	31,19	31,1	31,8	83,01	83,03	83,1	83	0,97	0,02
Mf	ADN-n°10	21,9	21,98	20,9	22,1	82,93	82,9	82,8	82,9	0,445	0,095

Xx J2 : numerous J2 individuals spiked in the sample

Initial test

end test (after 40 days from dispatch)

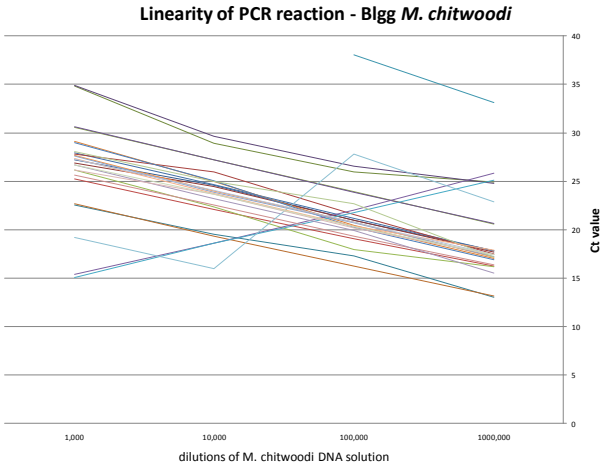
Appendix 3 : Practical implementation of the test for each sample set

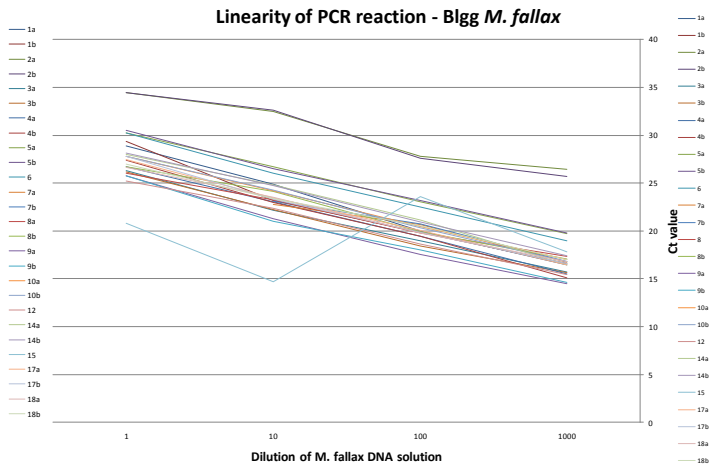
Date of shipment of the samples : 14th January 2011

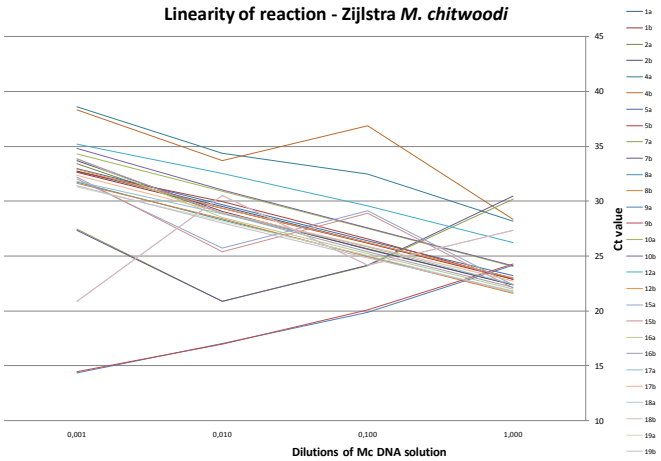
Samples set's code	DNA extraction kit	PCR test				
		Real time PCR		Conventional PCR		
		Blgg	Zijlstra	Wishart	Zijlstra SCAR	Zijlstra PCR RFLP
01	Roche	X	X	X		
02	Promega	X	X	X		
03	Promega	X	X			
04	Promega	X	X			
05	Promega	X	X	X	X	X
06	Promega	X	X			
07	Promega	X	X			
08	Promega	X	X			
09	Promega	X	X			
10	Promega	X	X			
11	Promega + Qiagen			X		X
12	Promega	X	X	X	X	X
13	Qiagen			X		
14	Promega	X	X			
15	n.c	X	X			
16	Promega		X		X	
17	Promega	X	X	X		
18	MOBIO	X	X			
19	Blgg lysis buffer		X			

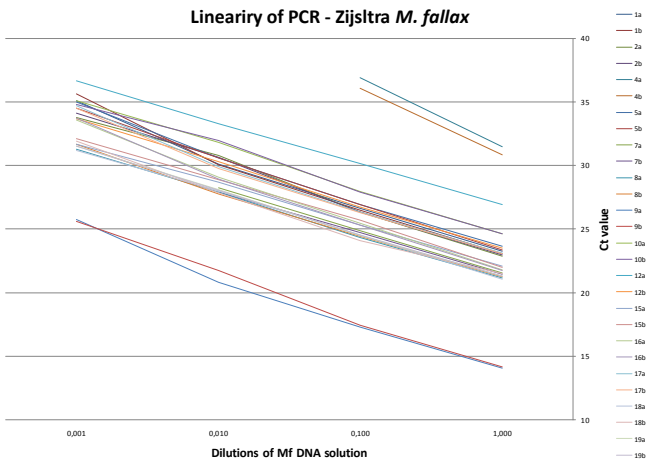
n.c : not communicated

Appendix 4 : Graphs summarising the linearity results









EUPHRESCO project *Meloidogyne chitwoodi* and *Meloidogyne fallax*

Topic 3:

Workshop on detection and management of the quarantine nematodes *Meloidogyne chitwoodi* and *Meloidogyne fallax*

General aim of the project:

- Provision of validated and harmonized sampling, identification and detection tools for use by inspection services and mandated laboratories in the funding countries and wider EU
- Provision of risk management approaches and development of management options in the event of *Meloidogyne* occurrence in plants, soil and plant-related waste materials

Specific for topic 3:

Plant protection agencies, researchers and growers ask many questions about field sampling and detection probabilities of *Meloidogyne chitwoodi* and *M. fallax.* Also the detection of *Meloidogyne* spp. in potato tubers still raises questions: is there no better method than the EU recommended method of visual observation of tubers for the presence of symptoms? What is the best way of analysing roots or bulbs? A workshop with presentations and demonstrations was organized where participants could perform some steps in the processes.

Aims

- To present information (field data, theory, experiences) regarding field sampling so that every participant is aware of all factors involved in sampling and detection (population dynamics, spatial distribution, detection probabilities).
- To present the results of the ring tests described in topics 1 (extraction) and 2 (detection and identification) so that the best choice can be made of reliable extraction from soil and identification methods by each participant.
- To demonstrate extraction of nematodes from tubers, roots and bulbs, so that participants know which possibilities exist and what is involved in each procedure.
- To give participants an opportunity to practice a selection of skills required in detection of *Meloidogyne* spp.

Methods

A schedule for a 2-day workshop was set up by the 3 topic coordinators. People of several organizations (WUR, the Dutch PPO, ANSES, and ILVO) were invited to give presentations and/or demonstrations. Announcements were sent out on February 1, 2011 to the 24 individuals who signed up as being interested during and after the first meeting of this EUPHRESCO Meloidogyne project in Vienna, September 2010. Most countries responded by the due date of March 1, 2011.

The workshop consisted of a mix of presentations and demonstrations, as well as some hands-on exercises, all followed by a discussion at the end of each day. The participants were divided in 3 groups for the practical part of the workshop. A rotation schedule ensured that everybody participated in every exercise and received all the information available. Printed hand-outs of all presentations and some demonstrations were provided.

The program provided information on the following questions:

- Population dynamics in the soil: when to sample and how deep?
- Spatial distribution in a field: what do we learn from field observations and simulation models?
- Detection of *Meloidogyne chitwoodi* and *M. fallax* through visual observation of host plants or through extraction?
- Extraction and detection of *Meloidogyne* spp. from soil: results from the ring test (topic 1)
- Detection and identification of *Meloidogyne chitwoodi* and *M. fallax* using real-time PCR: results from the ring test (topic 2)
- Detection and identification of *Meloidogyne chitwoodi* and *M. fallax*: morphological and alternative molecular methods (different from the ring test methods)

Demonstrations with opportunity to practise:

- Extraction of *Meloidogyne* spp. from soil: a selection of techniques
- Extraction of *Meloidogyne* spp. from potato tubers using centrifugal flotation
- Extraction of *Meloidogyne* spp. from potato tubers using enzymes
- Molecular techniques
- Morphological identification
- Recognizing symptoms in potato tubers
- Simulate sampling and population dynamics using the software program NemaDecide

Results

The workshop took place at the Institute for Agricultural and Fisheries Research (ILVO), Merelbeke, Belgium, on 25 and 26 May 2011. Most participants (those who did not present or demonstrated), paid 150 € to cover costs of the organization (hand-outs, coffee break, lunch). Accommodation, meals and travel costs was at everybody's own budget.

A total of 38 people participated in the workshop. Of these, 26 came from NPPO's, 3 were the topic coordinators, 4 were technical staff of ILVO and 5 participants came from private institutes who deal with sampling, soil analyses and potato growers. Participants came from 14 countries: Belgium, Bulgaria, France, the Netherlands, Portugal, Germany, Serbia, Czech Republic, Austria, Switzerland, United Kingdom, Ireland, Slovenia and Turkey.

The following programme was carried out:

Wednesday 25 May 2011

8h45-9h	**Registration**	
9h-9h10	**Welcome and introduction**	Martine Maes Nicole Viaene
9h10-10h	**Biology** of *Meloidogyne chitwoodi* and *M. fallax:* life cycle, host plants, root exudates, quiescence, ...	Wim Wesemael
10h-10h15	coffee break	
10h15-11h15	**Detection** of *Meloidogyne chitwoodi* and *M. fallax*: extraction techniques and results of the EUPHRESCO ringtest on detection in soil	Loes den Nijs
11h15-12h	**Identification** of *Meloidogyne chitwoodi* and *M. fallax*: morphology (microscopy) and isozymes	Gerrit Karssen
12h-13h	Lunch	
13h-17h **(with 15' break)** Rotation between lab stations, each 45'.	**Demonstrations** visual detection of Meloidogyne in roots and tubers extraction of Meloidogyne from potato tubers: - peeling, mixing - enzymes extraction of Meloidogyne from soil: - zonal centrifuge - elutriator (Oostenbrink) extraction of Meloidogyne from soil ,roots and peels: - Baermann and mistifier	Nicole Damme Anca Bighiu Géraldine Anthoine Anne-Marie Deeren Wim Wesemael Anne-Sophie van BruggenBruggen Nancy de Sutter
17h-17h20	Closure of the day: questions, remarks, small discussion	Nicole Viaene

Thursday 26 May 2011

9h-9h05	**Opening of day 2**	Nicole Viaene
9h05-9h55	**Identification** of *Meloidogyne chitwoodi* and *M. fallax*: molecular methods and results of the EUPHRESCO ringtest on molecular identification	Géraldine Anthoine
9h55-10h45	**Sampling** for *Meloidogyne chitwoodi* and *M. fallax* in fields, lots of potato tubers and other crops.	Thomas Been
10h45-11h	Coffee break	
11h-12h	**Management** of *Meloidogyne chitwoodi* and *M. fallax.*	Gerard Korthals
12h-13h	Lunch + picture	
13h-15h Rotation between stations, each 45'.	**Practical sessions**	
	Molecular identification: presentation/ demonstration	Lieven Waeyenberghe Géraldine Anthoine
	Morphological identification: microscopy	Anca Bighiu Nicole Damme Nancy de Sutter Wim Wesemael
	Exercises on sampling and management practices using computer simulation	Thomas Been
15h15-15h30	Coffee break	
15h30-16h20	**Discussion and closure of the workshop**	Nicole Viaene Loes den Nijs
16h30-17h20	Option to stay longer and continue sampling simulations	Thomas Been

Discussion

At the end of the two days a general discussion was held. Although a lot of very useful information was given during the workshop, some items were still missing. People had the following remarks and wanted more information on:

<u>1) Sampling</u>
-No field sampling was actually demonstrated, only presentations and simulations were given.
- Is a combined sampling scheme for *Globodera* and *Meloidogyne* possible?
- Period of sampling: this is probably different for different countries because of the climate.

The need for fixed regulations for all countries concerning sampling was mentioned. This could be by EU directive or in another way (e.g. EPPO).

<u>2) Extraction</u>
-Should the Baermann funnel technique still be used and recommended? A document with recommendations for the best choice of extraction method is needed. Results of topic 1 (ring test extraction) will be published, but this might not be enough, a general directive might be more practical.
-Is short-time incubation acceptable? Longer incubations for better detection delay the results.

-More details about sub sampling of nematode suspensions could have been provided (see ring test of topic 1)

Which approach is the best: each lab decides or Europe 'tells' which extraction protocol to use?

3) Identification
-Duplex method (Zijlstra) is less costly but possibly less sensitive. What is most important? Costs or detection level?
-EUPHRESCO ring test: Zijlstra real time PCR-method was altered to two simplex PCR-reactions. Why?
-Better recommendations for setting of the baseline (for Ct determination) are needed (automatic, manual…)
-During the ring test little mistakes from the participants could be noticed (wrong labeling,..). Human errors cannot be excluded. Replication of the ring tests needed?

In general, a lot of information was provided during the workshop and interesting discussions were held. People became more exposed to *M. chitwoodi* and *M. fallax* and became aware of the difficulties associated with detection of these nematodes. The participants asked for more guidance on sampling, extraction and identification from a central body, such as the EU or EPPO.

EUPHRESCO project *Meloidogyne chitwoodi* and *Meloidogyne fallax*

Topic 4:

Treatment of waste contaminated by nematodes

Topic coordinator: Nicole Viaene (BE)

Introduction

Soil, plant waste, waste of processing industries and water contaminated with *M. chitwoodi* and *M. fallax* aid in the dissemination of these quarantine organisms. The waste products can be treated in different ways, but exact methods have not been described for all waste products and treatment techniques. Moreover, in many countries, farmers, processors and even laboratory are not fully aware of the risk of spread of nematodes through waste products.

The aims of this topic were:

- To describe methodologies for waste treatment that can be applied in case *M. chitwoodi* and *M. fallax* are present in plant products or soil from fields.
- To identify needs for the development of treatment methodologies.
- To encourage laboratories, processors of potatoes and vegetables and farmers to apply these methods

Methods

The methodologies available for waste treatment, or at least those described as investigated for the disinfestations of waste contaminated with nematodes, were listed in a literature review.
Partners participating in this topic were asked to fill in a questionnaire on waste and waste treatment systems in their country. In the final meeting the results of both the literature review and the questionnaire were shared with the audience (participants of this topic + observers). Results and future actions were discussed.
A final report of the most interesting data will be published in a report (or article) that can be disseminated to the various parties involved.

Results

1) The literature review

A literature review was performed by NVWA (NL) and ILVO (BE), two of the topic coordinators. Authors were Leo van Overbeek (W-UR Plant Research International), Willemien Runia (W-UR Praktijkonderzoek Plant & Omgeving sector Akkerbouw,Groene Ruimte en Vollegrondsgroenten), Wim Wesemael and Nicole Viaene (ILVO, Plant, Crop Protection).

The literature survey revealed that there was little information available on treatment on waste particularly infested with *Meloidogyne chitwoodi* and/or *M. fallax*. The scope of the literature review was broadened to all kinds of plant-parasitic nematodes, and even other pathogens. Information on survival of *Meloidogyne* spp. was also included.
Agricultural waste streams considered were soil, tare soil (adhering to plant parts), crop waste (rejected crops, peels,...), waste water, sludge, compost and digestate. Studies on the survival of nematodes in these waste streams and the influence of several parameters (temperature and time, but also type and stage of the nematode) were summarized.

Methods that have been studied to kill nematodes rely mostly on heat. They include: heating *sensu stricto*, steaming, composting, (co-)fermentation, soil solarisation. Very little

information was available on other techniques such as inundation, UV treatment, chemicals and biological soil disinfestations. This was the case for nematodes in general and even more so for *Meloidogyne chitwoodi* and *M. fallax*. Survival of these nematodes has only been tested for the juvenile stages (not egg masses) in water (not in soil) at different temperatures.

2) The questionnaire (Appendix 1)

A questionnaire was organized by ILVO. It contained 19 questions on 6 subjects, i.e. production of potato and root crops, transportation of these crops, soil tare, plant waste, waste water and sludge. These made up 4 pages, many were multiple choice questions.

The questions were sent by e-mail on 12 March 2012. Participants had to contact their potato and vegetable industry groups to answer the questions, as well as consult national statistics on production, import and export quantities of several crops. All answers were received by 14 May 2012.

The following people from 8 countries participated:

- Jasmina Bačić (Serbia)
- Branimir Njezic (Bosnia-Herzegovina)
- Vladimir Gaar (Czech Republic)
- Géraldine Anthoine and Anne-Claire Le Roux (France)
- Loes den Nijs (the Netherlands)
- Nele Cattoor (Belgium)
- Bilge Misirlioglu (Turkey)
- T. Hristova (Bulgaria)

The 8 participants were a good representation of the diversity between countries in Europe considering the size of the potato industry, the type of industry (e.g. seed potato vs. ware), the trade patterns and the production, but most importantly the treatment of waste products.

The questionnaire revealed the following:

- Number of tons of imported, exported and processed potato tubers varies according to country and so do chances for import and export of *Meloidogyne* spp., also depending on origin of the tubers.

- For carrot, salsify, celery, turnip, parsnip…data were not complete. The available data showed several root crops on which adherent soil can be transported and traded. Some countries have a very large sugar beet processing industry (Turkey, France), mainly with own production (not imported, still transportation between regions)

- Crop products are not transported by third parties in every country (e.g. not Bulgaria, Serbia,). Transportation by the growers themselves reduces the chances for distribution of contaminated soil.

- Soil tare is mostly stored in piles and returned to growers or on agricultural fields. In France, returning soil to a field is not allowed for fields used for potato seed production and for specific crops.

- Most countries return vegetative waste to fields where it is incorporated in the soil. But

many also dispose of plant waste in a proper way (animal feed, compost, even biofuel)

- In some countries there is release of waste water in surface water, which can transport nematodes, but every country uses also water treatment facilities.
- Time of settling of waste in waste water is variable. This time is important for survival of Meloidogyne (the longer the less chance for survival).
- Sludge is mostly returned to fields, sometimes after a specific treatment.

3) The final meeting

The final meeting took place in Adana, Turkey, in September 2012, at the occasion of the ESN meeting (European Society of Nematologists). The results of the literature review and the questionnaire were presented (30 minutes) and discussed.

Most countries did not see the need to take measures to disinfest waste or handle waste in a specific way to avoid survival and spread of *M. chitwoodi* or *M. fallax*. The reason for this is that one is unaware or it is not known whether these nematode species are present in their country, However, what is not currently present, can still come or can be avoided. One person (NL) pointed out that probably all countries will have *M. chitwoodi* and *M. fallax* in 20 years time unless proper management is applied. Moreover, one person (FR) remarked that one should not focus on *M. chitwoodi* and *M. fallax*, but on all *Meloidogyne* spp. and even on all plant-parasitic nematodes. Only Turkey asked for more strict measures: sites where seed potato is produced should be restricted to areas declared free of *M. chitwoodi* or *M. fallax.* Such measures already exist in EU legislation, but their strict application is challenging and differs from one country to another. Something similar to the *Globodera* directive which requires soil sampling prior to seed production might be advisable to be put into place.

4) The report on waste

A report still needs to be written on the different types of waste that are possibly contaminated with *Meloidogyne chitwoodi* and/or *M. fallax*. Based on the questionnaire, the risks for spread of the nematodes will be described and attention will be drawn to the pathways with the highest risks. The literature review on possible waste treatments will be summarized. From all this knowledge, conclusions will be drawn as to what each country can do right now to decrease the chances for spread of nematodes through waste products.

At the same time, subjects that need more research or realisation into practical application will be identified. This report will aid in fundraising for research to fill the knowledge gaps. It will also increase awareness of this aspect of spread of (quarantine) nematodes.

5) Conclusion

Hardly any methodology for the disinfestation of waste contaminated with *M. chitwoodi* and *M. fallax* has been described. Most information on disinfestations of waste and on survival of nematodes is related to treatments based on temperature (heating to lethal temperatures). The knowledge about the survival biology of *M. chitwoodi* and *M.fallax* is limited. Therefore,

more specific knowledge on (new) waste treatment techniques is needed.

More insight in the situation of possible contaminated waste products and their fate in Europe were gathered through the questionnaire. Most nematologists participating in this EUPHRESCO project did not see the need, however, to treat waste or take extra measure to prevent the spread of the two nematode species, at least not during the discussion at the final meeting.

We hope that a written report on the literature review (task 1) and on the questionnaire (task 2) will increase awareness and lead to appropriate actions. These actions need to be initiated by nematologists. Also national plant protection organizations (NPPO's) play a crucial role here. Policy makers, farmers, industry, labs and all involved in this matter should be stimulated by the nematologists, however. A reason for the reluctant attitude of most nematologists present at the final meeting is possibly the fact that most countries have not been confronted with *M. chitwoodi* and *M. fallax* in their fields and thus not with the consequences. They should not wait until they have this experience to come into action. Therefore, the third aim of this topic "To encourage laboratories, processors of potatoes and vegetables and farmers to apply these methods" was not met at the end of this EUPHRESCO project.

Appendix 1:

Topic 4

Treatment of waste contaminated with *Meloidogyne chitwoodi* and *M. fallax*

QUESTIONNAIRE ON WASTE

Aim
The aim of this questionnaire is to have an overview on how waste that is (possibly) contaminated with *Meloidogyne chitwoodi* and *M. fallax* is dealt with in Europe. With this knowledge, it will be possible to prepare appropriate actions to reduce the spread of *Meloidogyne chitwoodi* and *M. fallax* (and other pathogens) within and between countries

What is waste?
Waste in this questionnaire relates to soil, plant products and water that have been in association with the production of potato or root vegetable crops, and because of this, are possible carriers of *Meloidogyne chitwoodi* or *M. fallax*.

Only waste produced by farmers, traders and the vegetable/potato industry is considered, not by individual consumers.

Content of this questionnaire
There are 6 series of questions on the following subjects:

- production
- transportation
- soil tare
- plant waste
- washing water
- sludge

Instructions
You will need to contact people of the potato, beet or vegetable industry and look for statistics on your country. To fill in this questionnaire you will at least need a couple of hours, this is when all information is easily found.

Check all appropriate circles in front of the possible answers in case of multiple choices.

Fill in numbers when asked for. If no exact numbers are known, give an estimation, so that the importance of the subject can be evaluated. An EUROSTAT table about production in the EU is sent with this questionnaire to help answer some questions.

If you have no idea of the situation in the whole country, please provide the limited info you have and indicate it is not referring to the whole country

If you are unable to access information or no information is available on a certain subject, indicate this in the “comments” section. To know that something is not known can call for more investigation (if needed).

Return this form filled in (or a scanned copy in case you fill in by hand) by mail to nicole.viaene@ilvo.vlaanderen.be by May 1, 2012.

Thank you for participating in this topic 4. Your time and contribution is highly appreciated!

COUNTRY: ……..

1. PRODUCTION

1.1. What is the total potato production in your country (in ha)? .....

1.2. Which of the following types of potato production take place in your country? Please indicate percentage of total potato production.

Type	% of total production area
○ ware potato production	.....
○ seed potato production	.....
○ starch potato production	.....
○ other kind of potato production:	.....

1.3. Which of the following activities related to trade in potato take place in your country? Please indicate quantities (in ton), if known.

Trade	ton/year
○ import of ware potato	.....
○ export of ware potato	.....
○ import of seed potato	.....
○ export of seed potato	.....

1.4. Is there a potato processing industry in your country (sorting and packing potato, making chips, French fries, starch...)? yes / no

If yes, how many tons of potatoes are processed per year? .....

1.5 Which of the following activities related to the production of root crops (carrot, salsify, beet,...) take place in your country? Please indicate size (production area, tons).

Type	importance
○ production of root crops	.....ha
○ export of root crops	.....ton
○ import of root crops	..... ton
○ processing industry of root crops	..... ton/year

Comments, questions or additional information on this subject (Production):

---

2. TRANSPORTATION

2.1. How are potatoes or root crops transported from field to trader or processor?

- grower brings his product to trader or processor: transportation vehicle of grower
- transport vehicle of the company
- transportation vehicle of a third party (train, transportation company)
- different: …….

2.2. How are potatoes or root crops packed for transportation from field to trader/processor?

- bulk, in trucks or containers
- in bags
- in boxes
- other: ……..

Comments, questions or additional information on this subject (Transportation):

……..
……...
……..
……...
……..
……...

3. SOIL TARE

Soil tare is soil that comes with the produce from the field; soil left behind in transportation vehicles, soil sticking to tubers or roots that is taken off the produce through shaking or sieving.
It is not the soil that is washed off; this is called washing soil or sludge + water (see further).

3.1. Is soil tare kept at the place of production and only clean product is transported?
yes / no

If yes, how?

- care is taken that soil tare is shaken off produce while harvesting and most adhering soil is left on field of production
- on a pile outside in a central place (e.g. next to farm), not covered
- on a pile, but covered or protected with fences or tarp
- in closed containers or inside a building near the production place

3.2. Is soil tare left at the place of reception (trader, processing plant)? **yes / no**

If yes, how?

- on a pile outside, not covered
- on a pile, but covered or protected with fences or tarp
- in closed containers or inside a building

3.3. Is soil tare returned to the producer (back to the farm)? **yes / no**

If yes,

- it is only soil originating from that same farmer
- it can also be soil originating from other farmers, but from the same region
- it is soil originating from other producers, possibly from other regions
- it can be soil imported from other countries (with imported produce)

3.4. Is soil tare deposited on land that is not used for agriculture (e.g. piece of land next to processing plant, soil used for road work, in construction, waste dump...) **yes / no**

If yes,

- on land that is that is owned and managed by the processor or trader, but not for agriculture
- on public land, freely in nature, where everybody has access
- on land that is not accessible, under official control, where soil tare is considered as waste
- soil is used for other purposes, not agriculture (e. g. road works, construction, ..)

3.5. Is soil tare deposited on agricultural land? **yes / no**

If yes,

- Is it deposited on the original field where the product came from?
- Is it deposited on other fields?

Comments, questions or additional information on this subject (Soil tare):

……
……...
……
……....
……

4. PLANT WASTE: parts of plants that are not used, disqualified tubers or carrots, peels, scraps or smaller parts

4.1. What happens with plant waste?

- dumped as waste
- composted
- used as animal feed
- other:……

4.2. If plant waste is dumped as waste, what happens to it?

- treated by heating
- returned to agricultural field
- ploughed under
- other…….

Additional information:

……..
……...
……..
……...
……..
……...

5. WASHING WATER (still contains pieces of soil) AND PROCESSING WATER (practically without soil)

5.1. What happens with washing water?

- caught in settling tanks so the soil parts can settle, then the water is re-used after soil parts have settled or is treated
- goes into the public sewer system where it is treated together with other sewer water
- drained into the surface water (ditch, canal, river)
- treated in a water treating plant of the company
- other:.....

5.2. If washing water is caught into settling tanks to let soil particles sink, how long is it kept there before the water is taken off?

- < 1 week
- about 1 month
- 1- 4 months
- > 4 months

5.3. If washing water is caught into settling tanks to let soil particles sink, is it treated to be free of nematodes before it is released or re-used?

yes / no

Additional information:
……..
……...
……..

6. SLUDGE

Sludge is what is left after soil and vegetative parts in washing water have settled. It is also what is left after the first steps of water treatment.

8.1. How long is sludge left to sit before it is moved away?

- < 1 week
- about 1 month
- 1- 4 months
- > 4 months

8.2. What happens with the sludge?

- deposited in a field, used as fertilizer, without treating
- deposited in a field, used as fertilizer after composting
- deposited in a field, used as fertilizer after fermentation
- other: ……..

Additional information:
……..
……

EUPHRESCO project *Meloidogyne chitwoodi* and *Meloidogyne fallax*

Topic 5:

A European *Meloidogyne* research agenda

Knowledge about past, present and future research on *Meloidogyne chitwoodi* and *M. fallax* is scattered over scientific publications, reports of research projects and official records, mainly in the USA and in Europe. For countries that have never dealt with *M. chitwoodi* and *M. fallax*, it is difficult to find adequate information quickly. For countries dealing with *M. chitwoodi* and *M. fallax*, it is interesting to exchange information and to present it to each other so that future research efforts could be planned and combined. Identification of current needs for management of these pests would help in elaborating research projects, especially at transnational level.

Aims

1. To increase the awareness of people involved in agriculture (growers, breeders, plant protection agencies,...) of the existence of *M. chitwoodi* and *M. fallax* and their impact, so that they favour research on these organisms and implement control options.
2. To stimulate cooperation and research aimed at reducing the impact of *M. chitwoodi* and *M. fallax* on production and trade.

Output

Put together a European Meloidogyne research agenda by gathering information from all joining countries, identify gaps in the research and make proposals (calls) for filling in those gaps.

Methods

Each participant of this topic was asked to organize a national meeting in his/her country, open to the National Plant Protection Organization, representatives of growers associations, breeders, technical and research institutes and government administration. The aim was to inform this public about *M. chitwoodi* and *M. fallax,* the difficulties in managing them and the solutions available so far. The attending public was asked to pay attention to these organisms and contribute to their control, e.g. in breeding programs, calls for research and funding, collaboration in detection and field experiments. The meetings and discussions with the people and organisations involved with *M. chitwoodi* en *M. fallax* showed which topics need priority for research.

The participants filled in a document summarizing the activities that took place in their country and the results.

Results
The participating countries were Belgium, Bulgaria, France, The Netherlands and Turkey. A summary of the input from the documents:

Country	Number of meetings	Organizations reached	Participants
France	1	Researchers, breeders, farmers, regional official services, potato seed companies, routine laboratories, root crops and beet industries, technical organization	31
Belgium	10	Representatives of grower organizations and potato trade, research organizations, NPPO, seed companies, extension workers, pesticide industry, individual growers	200
Turkey	1	Researchers of institute/stations, breeders, technical staff of provincial and subprovincial Directorates of Agriculture, potato seed companies, growers, chemical companies, government administration (NPPO?)	60
Bulgaria	4	Researchers, national reporters, phytosanitary inspectors, NPPO	70
The Netherlands	22	Representatives of grower organizations and potato trade, extension workers, individual growers, research organizations, breeders, NPPO, seed companies	±600

Techniques used to get the message to the people:
Presentations and/or discussions in various sizes of groups, questionnaires, interviews, field demonstrations, workshops, flyers, posters.

Output
1. Knowledge transfer on biology, symptoms, management of pest in other countries, possible management options with nematicides, use and durability of resistant crops, official national regulations, legislation, detection, advice on sampling in fields and on products, crop rotation, treatment of infested waste.
2. Suggestion to separate seed production area from ware and consumption production areas.
3. The need for tools and knowledge to ensure the sanitary status of the seed production area.
4. In Turkey certified seed is only used by the big commercial companies, many small growers use farm saved seed. Those growers should be made aware of *Meloidogyne*. Enhance the use of certified seed
5. A PhD entitled “Biodiversity and ecology of parasitic nematodes of the genus *Meloidogyne”* has started in Bulgaria.
6. Education of the inspectors who perform the monitoring of vegetables and potatoes.

Proposals for a European research agenda, questions, issues and remarks raised at the above events

- Field experiments for economically sustainable rotation schemes (to eradicate or decrease Meloidogyne below detection level)
- Studies for practical use of chemical compounds isolated from nematicidal plants
- Development, durability and use of resistance to *M. chitwoodi* and *M. fallax* for crops such as potato, carrot, bean.
- Waste soil and waste of plants: information on treatment, practical solutions
- Information via website(s) and other means of communication
- Common national legislation on Meloidogyne in all countries from the EU (trade)
- Transparency of applications of legislation in all countries (trade) (methodology of detection and consequences of positive samples- what happens with the contaminated products, what with contaminated fields?)
- Training of inspection service, but also traders, to recognize symptoms of Meloidogyne infestation, especially in potato tubers
- More sensitive and reliable detection of field infestations through preventive soil sample.
- Availability and development of nematicides
- Research on symptoms in potato varieties (to adapt choice of variety in case of infestation
- Research on risks associated with harvest of root crops (beets, carrots, salsify)
- Surveys at country level
- Alternative crops to replace potato should be found
- Assess the nematode resistence in potato varieties
- Are soils different in supporting Meloidogyne?
- The use of resistance in potato or green manure has been tested in experimental conditions. How does it work in the field?
- Are European *M. chitwoodi* populations genetically identical or should we look into the existence of pathotypes? And does this hold for *M. fallax* as well?
- The intensive sampling of the soil for Meloidogyne is practiced in NL by various laboratories. Comparison between these laboratories shows some methodical problems.
- Is carrying out inspections on the product after harvest better then sampling of the soil before sowing?
- What is the host status of crops and what population levels can be reached on these crops in the different climatic conditions?
- What is the efficacy of chemical and non chemical control measures?

Additionally, a meeting was held during an occasion where nematologists from different countries international were present: the ESN symposium on 23-27 of September 2012 Almost 45 people were attending this workshop. In this meeting the results of the national meetings were presented and attendants were asked for additional input.

Unfortunately, the forthcoming discussion was not very lively and only some few new point were added to the list.

Nematologists from countries where *M. chitwoodi* and *M. fallax* occur were in favour of additional research and outreach. The Netherlands pointed out that these nematodes do not know borders and will move to other countries, if not now at least within 20 years. The need for resistant varieties in all kinds of crops was stressed.

A nematologist mentioned that a survey will probably show that the two nematode species are not present in most countries. This could result in deregulation in the EU. It was also said

that damage does not show in all cases and that finding the nematodes in an *ad random* survey will not reveal much. It was suggested that the NPPOs or even EPPO could make some recommendations on more strict measures than now is written in the EU directive, but that these measure should not be compulsory.

Many attendents were not in favour of a survey, nor of any measures. As they do not have a problem, they thought there is no need for knowledge. To many, the *M. chitwoodi and M. fallax* problem is a local problem for the Netherlands, Belgium and France, even if the extend, the distribution and the control of these nematodes is different between these countries. It is not an EU problem. Even in countries where it has not spread widely yet, it should be considered as a transient problem.

It was clear that the whole discussion was a matter of probability of spread and probability of causing damage, both related to time. However, with the knowledge of what has happened in the Netherlands in the last 10 years, one should be aware that these nematodes do spread and can become a nuisance for many countries.

Nevertheless, some participants expressed an interest to participate in a COST action on *Meloidogyne*. This not only on *M. chitwoodi* and *M. fallax*, but more broadly with concern to the *Meloidogyne* genus including potential tropical and emerging species. Also a new EUPHRESCO project on nematode dynamics in the soil (related to rotation) was suggested.

Final Conclusions

This non-competitive project allowed producing a lot of data and network because of the diverse topics. Several topics in this project allowed an extensive study on the particular subject.

Within topic 1, the ring test on the extraction of *Meloidogyne* juveniles from soil, different extraction methods were compared. The standard Baermann funnel method produced the lowest recovery of *Meloidogyne* juveniles. The incubation of samples as well as the duration of incubation were shown to influence strongly the efficiency of nematode extraction. Also proper attention should be given to the storage conditions of the samples. From the ring test results, a recommendation was formulated which steps of the extraction process to master.

Topic 2 and its ring test on molecular detection and identification of *M. chitwoodi* and *M. fallax* provided objective data on the performance of molecular tests. This allows choosing the relevant test for a specific use, including real-time and conventional PCR tests. This project also highlighted the difficulty to organise such ring test. Enough guidance should be given and limited variation from the participants should be accepted in order to analyse the results obtained.

Topic 3 and its successful workshop including presentations and demonstrations gathered 38 people from 14 countries. This meeting is a starting point of a network dedicated to *M. chitwoodi* and *M. fallax* that could support a harmonized policy and use common procedures. But before this can be realised, more harmonized guidance on sampling, extraction and identification of these nematodes is needed and expected from a central body, such as EPPO or EU.

Topic 4 and its questionnaire underlined the lack of knowledge about nematode survival, especially in waste. This work (report and questionnaire) should increase awareness and lead to appropriate actions. Nematologists should lead national plant protection organisations (NPPO's), policy makers, farmers, industry, labs and all involved in this matter to a better understanding and a better management of waste. The reluctance of some nematologists is possibly caused by the fact that most countries have not been confronted with *M. chitwoodi* and *M. fallax* in their fields and thus not with the consequences. Therefore, major actions are still needed to manage the waste in an appropriate way to avoid dispersion of these nematodes.

Topic 5 provided input from various stake-holders dealing with *M. chitwoodi* and M. fallax in one way or another. It allowed collecting possible topics for further coordinated and transnational research on *M. chitwoodi* and *M. fallax*. More generally, it was suggested to initiate a COST action on *Meloidogyne* (and not only *M. chitwoodi* and *M. fallax*), as tropical species represent a major threat for crops especially with global warming.

To conclude, this transnational project was a unique opportunity to gather people working on specific topics but also planning possible future actions. The fact that this project was developed under the Euphresco umbrella convinced different teams to joint it.