Final Report

PCN Proficiency Test

AHDB Ref: 100006

Reporting Period: August 2015 – January 2016

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1. SUMMARY

1.1. Aim

The proficiency test was initiated in response to industry concerns raised via the Nematicide Stewardship Programme. The test compares the proficiency of seven UK laboratories to assess the level of soil infestation by potato cyst nematode (PCN). There were three objectives:

1. To test the ability of the laboratory to distinguish PCN cysts from lemon cysts and other material which could be mistakenly identified as PCN.
2. To test the ability of the laboratory to extract PCN cysts from mineral and organic soil and provide a count of cysts/100 g soil and eggs/g soil
3. To test the ability of the laboratory to distinguish between cysts of white PCN (*Globodera pallida*) and yellow PCN (*G. rostochiensis*).

An eighth laboratory prepared the test samples and their results for Objective 2 are provided for information.

1.2. Methodology

For Objective 1, each laboratory was asked to identify five samples of 25 cysts comprising a mixture PCN cysts, lemon-shaped (*Heterodera*) cysts and cysts of grass cyst nematode (*Punctodera punctata*).

For Objective 2, 1 kg samples of mineral or organic soil free of PCN were seeded with five or 20 PCN cysts/100 g soil or left unseeded. There were three replicates of each treatment, nine per soil type. Each laboratory was asked to extract the samples and assess the level of PCN infestation using their own in-house methods.

It should be noted that to comply with Plant Health quarantine requirements for working with PCN, the PCN cysts used in the study had to be heat treated and this meant that it was not to feasible to obtain information on the number of viable eggs/g soil.

For Objective 3, laboratories that use PCR techniques for PCN speciation were sent six tubes containing 50 PCN cysts made up from a mixture of white and yellow PCN. Each laboratory was asked to determine the percentage of each species present in each sample.

As the data for number of cysts extracted (Objective 2) conform to a Poisson distribution it is possible to calculate the probability of recovering a specific number of cysts. The probability of recovering exactly five cysts/100 g soil where only this number were inoculated in the sample is only 18%, but the probability of recovering between three and seven cysts is 75%. The number of cysts recovered by the laboratory was compared against this acceptable threshold on the basis that on 75% of occasions they would be expected to find between three and seven cysts in a 100 g sample of soil. The same logic was applied to samples containing 20 cysts/100 g. In this case it was assumed that

to recover between 15 and 25 cysts is acceptable and the probability of achieving this is 78%. The above assumptions hold true if 100g of soil are extracted. If a larger or smaller volume of soil is extracted the numbers of cysts that are expected to be found will vary in relation to the size of sample.

1.3. Key findings

Objective 1: Five labs overestimated the number of PCN cysts. Two labs were unable to identify grass cyst nematode. Three labs identified PCN and lemon cysts correctly.

Objective 2: When results from all the samples were pooled, laboratories were within the acceptable range of accuracy as follows: Lab 1 88.8%; Lab 4 and Lab 6 77.7%; Lab 2 72.2%; Lab 3 66.7%; Lab 5 61.1%; and Lab 7 44.4%. All laboratories were better at extracting cysts from mineral than from organic soil. Both Lab 5 and Lab 7 recovered PCN eggs where no cysts were inoculated.

Objective 3: Trends in the relative proportions of white and yellow PCN from PCR analysis generally followed those in the original samples but some laboratories detected white PCN when none was present.

1.4. Practical recommendations

Further training should be provided to help improve the accuracy of PCN identification. The proficiency test should be repeated at regular intervals to ensure that the laboratories' ability to undertake PCN extraction and quantification to an acceptable level of accuracy is maintained.

2. INTRODUCTION

The Nematicide Stewardship Programme has been developed by industry and includes partners from stakeholder organisations, agrochemical companies, manufacturers, distributors and research providers. Their overall aim is to address challenges related to the sustainable control of nematodes.
http://nspstewardship.co.uk/

One topic that has been identified for action by NSP members is the concern that there is variation in the accuracy of laboratories offering commercial PCN soil testing services. AHDB has funded the generation of standard test samples for distribution to participating laboratories, to determine if there is variation in the performance of laboratories. Specifically, the project had three main objectives:

1. To test the ability of each laboratory to distinguish PCN cysts (*Globodera pallida* and *G. rostochiensis*) from lemon cysts of *Heterodera* spp. and other material which could be mistakenly identified as PCN.
2. To test the ability of each laboratory to extract PCN cysts from soil (by whatever method) and provide a count of cysts/100 g soil and eggs/g soil in two different soil types (a mineral and an organic soil).

3. To test the ability of laboratories to distinguish between cysts of *G. pallida* and *G. rostochiensis*. This was only applicable to those laboratories that offer PCN speciation.

All results and any other information provided by individual laboratories has been treated in the strictest confidence. The results have been anonymized and each laboratory taking part has been allocated a number, and only that laboratory is aware of its own number.

There were seven laboratories taking part in this study. An eighth laboratory prepared the test samples and did not officially take part in the test. However, a set of randomly labelled samples required to participate in Objective 2 was generated for the eighth lab and their results have been provided for information. A total of four laboratories offer PCN speciation by PCR analysis and were involved with Objective 3.

3. MATERIALS AND METHODS

3.1. Objective 1: to test the ability of each laboratory to distinguish PCN cysts

PCN laboratories must be able to distinguish between the cysts of a number species of cyst nematodes. These include PCN cysts, lemon cysts (*Heterodera* spp.) and those of grass cyst nematode (*Punctodera punctata*). Soils which were known to have a significant population of these different cyst nematodes were collected from the field and extracted using the Fenwick Can.

Five samples were prepared containing 25 cyst nematode cysts comprising PCN cysts, lemon cysts and grass cyst nematode cysts. The total number of cysts from each group varied between samples (Table 1). Samples were provided dry in small snap top Eppendorf tubes.

Table 1. Composition of cyst nematode samples provided to participating laboratories

Treatment	Cyst type	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
1	PCN	25	19	13	9	3
2	Lemon	0	3	8	10	12
3	Grass cyst	0	3	4	6	10
Total		25	25	25	25	25

The participating laboratories were asked to identify the cysts present in each sample and also to collect those for each cyst group in labelled Eppendorf tubes. These labelled tubes were returned to the laboratory which had generated the samples. This was to allow the laboratory staff to verify that the identifications were correct and that the correct cyst count for each replicate sample had not been achieved by chance.

3.2. Objective 2: to test the ability of the laboratory to extract PCN cysts from soil

Laboratories were tested on their ability to extract PCN cysts from both a mineral and organic soil. A sample of mineral soil was collected from a field which had previously grown seed potatoes and had been designated as being free of PCN. Approximately 100 kg of soil was dug up and a sub-sample of 200 g taken and extracted using the Fenwick can to confirm that no cyst nematodes were present.

It was very difficult to find organic soil which was free of cyst nematodes so John Innes No. 1 compost was used. This has an approximate 25% organic matter content. A preliminary analysis of a sample confirmed it contained no cyst nematodes but produced a significant quantity of extracted material. As a result, the John Innes No. 1 was diluted with PCN-free mineral soil. The mix was approximately 50% John Innes No. 1 to 50% mineral soil. Both the mineral soil and the John Innes No. 1 compost was sieved to remove most of the large stones and/or organic matter.

A total of three PCN infestations were created (Table 2).

Table 2. PCN populations created by seeding 1 kg soil samples with PCN cysts

PCN infestation	Source of sample
1.	Soil uninfested with PCN
2.	Soil from infestation 1 inoculated with the equivalent of 5 PCN cysts/100 g soil (50 PCN cysts)
3.	Soil from infestation 1 inoculated with the equivalent of 20 PCN cysts/100 g soil (200 PCN cysts)

There were three replicate 1 kg samples of each population for each soil type giving nine samples per soil type and 18 samples in total for each laboratory. The soil samples were then seeded with PCN cysts, lemon cysts and grass cyst nematode cysts collected from soils used to prepare the cyst samples for Objective 1. An even distribution of all cysts in the soil was achieved by spreading out the soil on trays. The different types of cysts were then placed manually into all areas of the tray to ensure a relatively even distribution throughout the soil sample. This helped to minimise the variability that results from sub-sampling so that on average any sub-sample taken from the original sample will have approximately equal numbers of cysts. The entire sample was then transferred to a bag. The tray was also brushed clean and any residue transferred to the bag so as to minimise the risk of cysts being left behind. Each bag was rotated through 360 degrees five times to help mix the sample. Each bag was labelled with a sample number (Mineral soil1-9 or Organic soil 1-9).

The number of cysts required for each sample are indicated in Table 3.

Laboratories were asked to extract the soil samples by whatever means they usually use and provide an estimate of the number of PCN cysts/100 g soil and eggs/g soil. If a laboratory normally offered a service to quantify the number of PCN on the basis of soil volume they were asked to provide the results on this basis. A set of randomly labelled samples was also processed by the laboratory which had generated all the test samples. Their results have been included in the tables below for information.

Table 3. Number of cysts required to prepare 1 kg samples of mineral and organic soil seeded with PCN, lemon and grass cyst nematode cysts

	Soil type	PCN		Lemon		Grass cyst	
Treatment		No/100g soil	No/kg	No/100g soil	No/kg	No/100g soil	No/kg
1	Mineral	0	0	2.6	26	1	10
2	Mineral	5	50	2.6	26	1	10
3	Mineral	20	200	2.6	26	1	10
4	Mineral	0	0	2.6	26	1	10
5	Mineral	5	50	2.6	26	1	10
6	Mineral	20	200	2.6	26	1	10
7	Mineral	0	0	2.6	26	1	10
8	Mineral	5	50	2.6	26	1	10
9	Mineral	20	200	2.6	26	1	10
1	Organic	0	0	2.6	26	1	10
2	Organic	5	50	2.6	26	1	10
3	Organic	20	200	2.6	26	1	10
4	Organic	0	0	2.6	26	1	10
5	Organic	5	50	2.6	26	1	10
6	Organic	20	200	2.6	26	1	10
7	Organic	0	0	2.6	26	1	10
8	Organic	5	50	2.6	26	1	10
9	Organic	20	200	2.6	26	1	10
Total			1500		468		180

3.3. Objective 3: to test the ability of the laboratory to distinguish between cysts of *G pallida* and *G rostochiensis*.

This involved the preparation of six samples for each laboratory. Only four laboratories that undertake PCN speciation by PCR were involved in this objective. Each sample contained 50 cysts of various proportions of *G pallida* and *G rostochiensis* (Table 4). A total of 50 cysts was chosen as this is the number that laboratories usually prefer to analyse. Each sample was stored dry in a snap top Eppendorf tube.

Table 4. Number and proportion of white and yellow PCN cysts in samples for speciation by PCR analysis.

Treatment	*G. rostochiensis*		*G. pallida*	
	Number of cysts	%	Number of cysts	%
1	50	100	0	0
2	40	80	10	20
3	30	60	20	40
4	20	40	30	60
5	10	20	40	80
6	0	0	50	100

3.4. Statistical analysis

As the data for number of cysts extracted (Objective 2) conform to a Poisson distribution it is possible to calculate the probability of recovering a specific number of cysts. The probability of recovering exactly five cysts/100 g soil where only this number were inoculated in the sample (infestation level 2, Table 2) is only 18%, but the probability of recovering between three and seven cysts is 75%. The number of cysts recovered by the laboratory was compared against this acceptable threshold on the basis that on 75% of occasions they would be expected to find between three and seven cysts in a 100 g sample of soil. The same logic was applied to samples containing 20 cysts/100 g (infestation level 3, Table 2). In this case it was assumed that to recover between 15 and 25 cysts is acceptable and the probability of achieving this is 78%. The above assumptions hold true if 100g of soil are extracted. If a larger or smaller volume of soil is extracted the numbers of cysts that are expected to be found will vary in relation to the size of sample. For example, in a 200 g sample of infestation level 2 (five cysts/100 g soil) it may be expected to recover between six and 14 cysts on 75% of occasions.

There was no detailed statistical analyses done on egg numbers as the number of eggs found within each PCN cyst is very variable. This is often in part due to the age of the cysts with the older cysts containing fewer eggs than fresh cysts. All cysts in this study for Objectives 1 and 2 were taken from the same source so differences in egg numbers are more likely to be due to inherent variation. A table of the mean egg count for each batch of samples and the range +/- the standard error was produced. The consistency of egg counts within infestation levels was reported. An outlier is possible from five replicate samples as the extracted cysts may have low egg counts for whatever reason.

3.5. Additional information and collation of data

All participating laboratories were asked to provide a number of other pieces of additional information which could be used to help with the interpretation of the results. These were the method used for PCN extraction, volume of soil extracted, method used for egg counting and number of samples examined to provide the egg count. All additional information and results for objectives 1-3 were recorded on a data template (Appendix).

4. RESULTS

4.1. Objective 1: to test the ability of the laboratory to distinguish PCN cysts

Each of the five samples provided for each laboratory contained 25 cysts with different proportions of PCN cysts, lemon cysts and cysts of grass cyst nematode. Laboratories were asked to identify and count the number of PCN and lemon cysts and any other material. Grass cyst nematode cysts were classified as the other material. The counts as reported by the individual laboratories are given in Table 5 together with those for the original samples. The total counts for each sample do not always add up to 25 suggesting that some cysts were lost during the identification. However, at least 20 cysts were examined from each sample and this should be sufficient to give an indication of the laboratories ability to identify the cysts correctly.

Lab 1, Lab 2, Lab 4, Lab 5 and Lab 7 overestimated the number of PCN present. In the case of Lab 4 this was by 1 PCN cyst. In the case of the other laboratories, where there were three PCN cysts in the sample, the laboratories identified at least 11 cysts. In contrast where samples contained 19 or 25 PCN cysts these laboratories tended to underestimate the number present in six out of eight samples.

Both Lab 1 and Lab 2 did not distinguish grass cyst nematode cysts and identified them as a mixture of PCN and lemon cysts. Lab 5 underestimated the number of grass cyst nematode cysts and lemon cysts and this accounts for the high numbers of PCN. The high count of PCN for Lab 7 is primarily due to an underestimate of the number of grass cyst nematode cysts. Lab 3 slightly underestimate the number of PCN and this appears mainly due to PCN being identified as other material. Lab 4 wrongly identified one PCN as a lemon cyst and Lab 6 did not record three PCN cysts.

Whilst the data reported by the laboratories give an indication of their ability to identify PCN it is possible that they arrived at the correct counts despite misidentification of cysts. To ensure that the correct cyst count had not been achieved by chance the laboratories were asked to store all those cysts identified as PCN, lemon cysts and other material in labelled tubes and return these to the laboratory generating the samples. The labelled tubes were then examined to assess the accuracy of the identification. In a number of cases the number of cysts returned did not match with the number identified as some cysts were dissected to confirm identification and some were lost during the initial examination. These data are summarised in Table 6. The % accuracy of identification of both PCN and lemon cysts are tabulated but not of other material. This is because neither Lab 1 nor Lab 2 identified any other material. Lab 7 underestimated the amount of other material but no specimens were returned so it was not possible to determine whether those cysts identified as other material (see Table 5) were grass cyst nematode cysts. Where Lab 3, Lab 4, Lab 6 and Lab 5 identified other material it was all correctly identified as cysts of grass cyst nematode.

Labs 3, 4 and 6 identified all PCN and lemon cysts correctly. Lab 3 and Lab 6 identified slightly less than the total number of 125 (117 and 122 cysts, respectively).

Table 5. Numbers of PCN and lemon cysts identified by different labs as part of Objective 1 in comparison with the number of cysts of each type added to each of five samples. (Not all laboratories reported values that add up to 25 per sample. It is assumed some cysts were lost during identification).

	Original test samples			Lab 1			Lab 2			Lab 3		
Sample	PCN	Lemon	Other	PCN	Lemon	Other	PCN	Lemon	Other	PCN	Lemon	Other
1	25	0	0	20	0	0	25	0	0	24	0	1
2	19	3	3	18	7	0	16	8	0	18	3	3
3	13	8	4	15	10	0	15	9	0	12	7	4
4	9	10	6	12	12	0	10	15	0	8	11	2
5	3	12	10	12	12	0	11	14	0	3	12	9
Total	**69**	**33**	**23**	**77**	**41**	**0**	**77**	**46**	**0**	**65**	**33**	**19**

	Lab 4			Lab 5			Lab 6			Lab 7		
Sample	PCN	Lemon	Other	PCN	Lemon	Other	PCN	Lemon	Other	PCN	Lemon	Other
1	25	0	0	23	1	0	24	0	0	22	0	0
2	19	3	3	17	2	4	19	3	3	20	2	1
3	13	8	4	13	8	2	13	8	4	15	8	0
4	10	9	6	12	7	2	7	10	6	13	10	1
5	3	12	10	11	10	3	3	12	10	12	12	3
Total	**70**	**32**	**23**	**76**	**28**	**11**	**66**	**33**	**23**	**82**	**32**	**5**

Table 6. Percentage of PCN cysts and lemon cysts correctly identified by individual labs (based on the cysts returned for verification).

Laboratory	% of cysts identified correctly	
	PCN	Lemon
Lab 1	81.1	86.5
Lab 2	87.7	71.7
Lab 3	100	100
Lab 4	100	100
Lab 5	87.8	86.2
Lab 6	100	100
Lab 7	81.2	91.4

4.2. Objective 2: to test the ability of the laboratory to extract PCN cysts from soil

4.2.1. Cyst numbers

The number of cysts/100 g soil for each laboratory for each batch of samples is given in Table 7. Each laboratory was asked to express the number of cysts recovered as numbers/100 g soil. As the data for number of cysts extracted for each laboratory conforms to a Poisson distribution it is possible to calculate the probability of recovering a specific number of cysts. The number of cysts recovered by each laboratory was compared against this acceptable threshold on the basis that on 75% of occasions they would be expected to find between three and seven cysts in each 100g sample (when 1kg soil was spiked with 50 cysts).

The same logic was applied to samples containing 20 cysts/100 g. In this case it was assumed that to recover between 15 and 25 cysts was acceptable and the probability of achieving this is 78%. If the sample was not seeded with any cysts then it would be expected that none would be recovered by the laboratory. The number of occasions that each laboratory was within the acceptable range for all samples containing five or 20 cysts/100 g soil is given in Table 8.

The laboratory that had generated the samples was within the acceptable range for all samples in both soil types. To an extent this result is to be expected as they were aware of the number of cysts that had been seeded in each sample, although the samples were randomly labelled and therefore the extractions were carried out “blind”. Lab 1 was within the acceptable range for 16 samples (88.8%); Lab 4 and Lab 6 for 14 samples (77.7%); Lab 2 for 13 samples (72.2%); Lab 3 and Lab 5 for 11 samples (61.1%); and Lab 7 for eight samples (44.4%).

The results were then analysed for both mineral and organic soil types (nine samples for each soil type). In the mineral soil Lab 6 was within the acceptable range for all nine samples (100%). Lab 1, Lab 3 and Lab 4 for eight samples (88.8%); Lab 2 for seven samples (77.7%); Lab 5 and Lab 7 for six samples (66.7%).

In the organic soil, Lab 1 was within the acceptable range for eight samples (88.8%); Lab 2 and Lab 4 for six samples (66.7%); Lab 5 and Lab 6 for five samples (55.5%); Lab 3 for 4 samples (44.4%); and Lab 7 for two samples (22.2%).

All laboratories generally recovered cysts best from mineral soil in comparison with organic soil. In mineral soil the number of cysts recovered were within the acceptable range for 52 out of 63 samples (82.5%). In organic soil the number of cysts recovered were within the acceptable range for 36 out of 63 samples (57.1%)

Table 7. Number of PCN cysts/100g soil recovered by each laboratory for each batch of samples (blue shading = outside acceptable range. Acceptable range for 50 PCN cysts/kg soil = 3 to 7 cysts/100g; for 200 PCN cysts/ kg soil = 15 to 25 cysts/100g).

PCN: *Heterdodera*: *Punctodera* cysts added per kg soil	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**	**Lab 7**	**Lab 8**
Mineral soil	Results /100g soil							
0:26:10	0	0	0	0	0	0	7	0
0:26:10	0	1	0	0	0	0	0	0
0:26:10	0	0	0	0	1	0	1	0
50:26:10	5	7	4.6	5.2	3	4.6	6	6
50:26:10	6	8	4.8	4.5	3	4.7	7	6
50:26:10	5	4	2.4	7.8	6	4.7	4	6
200:26:10	19	19	18.2	16.9	15	17.2	17	19
200:26:10	23	19	17.0	15.3	8	18	21	20
200:26:10	6	23	16.6	17.4	10	16.1	11	20
Organic soil								
0:26:10	0	0	0	0	0	0	2	0
0:26:10	0	0	0	3.7	0	0	2	0
0:26:10	0	0	0	0	1	0	0.5	0
50:26:10	6	4	4	4.6	3	4	11	7
50:26:10	5	6	2	2.9	3	3.3	5	5
50:26:10	4	2	7.5	5.3	4	1.3	4	5
200:26:10	15	12	14.5	18.1	11	13.5	10	19
200:26:10	13	8	11	19.3	5	11.9	10	22
200:26:10	17	16	10.5	13.8	14	9.7	11	22
% within acceptable range	**88.8**	**72.2**	**66.7**	**77.7**	**61.1**	**77.7**	**44.4**	

Cysts were recorded as being present when none were seeded on nine occasions out of a possible 42 (21.4%). Lab 7 recorded cysts being present when none were seeded on five occasions, Lab 5 twice and Lab 2 and Lab 4 once each

Table 8. Number of occasions that each laboratory were within the acceptable range for extraction of samples seeded with 0, 5 or 20 PCN cysts/100 g soil (max = 3)

Laboratory	Mineral soil				Organic soil			
PCN cysts/100g soil	*0*	*5*	*20*		*0*	*5*	*20*	
Lab 1	3	3	2		3	3	2	
Lab 2	2	2	3		3	2	1	
Lab 3	3	2	3		3	1	1	
Lab 4	3	2	3		2	2	2	
Lab 5	2	3	1		2	3	0	
Lab 6	3	3	3		3	2	0	
Lab 7	1	3	2		0	1	0	
Lab 8	3	3	3		3	3	3	

4.2.2. Egg numbers

The number of eggs/g soil for each laboratory for each batch of samples is given in Table 9 and the mean count, standard error of the mean and range in Table 10. No detailed analyses were done on egg numbers as the number of eggs found within each PCN cyst is very variable. This is often in part due to the age of the cysts with the older cysts containing fewer eggs than fresher cysts. All cysts in this study were taken from the same source so differences in egg numbers are more likely to be due to inherent variation. This makes it difficult for meaningful comparisons between laboratories.

All laboratories counted more eggs where 20 PCN cysts were seeded/100 g soil than where 5 PCN cysts/100 g soil were seeded. Those laboratories with the lowest standard errors of the mean egg count tended to have the most consistent counts between the three replicate samples. Where there were no PCN cysts seeded PCN eggs were reported in nine samples. These was mainly due to two laboratories: Lab 5 which recorded eggs where no PCN cysts were seeded in three of six samples (one in mineral soil and two in organic soil) and Lab 7 which recorded eggs where no PCN cysts were seeded in five of six samples (83.3%; two in mineral soil and three in organic soil). Lab 4 also recorded 11.7 eggs/g soil in organic soil 4 where no cysts were inoculated although subsequent correspondence suggests that this was a due to a mistake in labelling cysts which were extracted from another sample as coming from organic soil 4. A repeat extraction of organic soil 4 showed that no cysts were present.

Table 9. Number of PCN eggs/g soil counted by each laboratory for each batch of samples. (Blue shading indicates egg counts reported in soil samples not seeded with PCN cysts)

PCN: *Heterdodera*: *Punctodera* cysts added per kg	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**	**Lab 7**	**Lab 8**
Mineral soil	Results /100g soil							
0:26:10	0	0	0	0	0	0	0.7	0
0:26:10	0	0	0	0	0	0	0	0
0:26:10	0	0	0	0	5	0	0.8	0
50:26:10	11	15	6.7	17.3	8	10	11	23
50:26:10	16	18	9.7	10.5	13	13	13.2	21
50:26:10	12	9	5.3	26.5	12	16	6.3	16
200:26:10	48	39	18.1	41	55	56	46.9	70
200:26:10	50	41	24.8	32.6	28	53	36.3	72
200:26:10	13	55	23.9	31.7	39	35	22.4	60
Organic soil								
0:26:10	0	0	0	0	0.5	0	0.1	0
0:26:10	0	0	0	11.7	0	0	0.8	0
0:26:10	0	0	0	0	4	0	0.5	0
50:26:10	22	8	11.3	15.1	8	12	20.6	22
50:26:10	16	10	3.1	11.6	12	8	10.2	16
50:26:10	10	2	9.4	13.8	6	7	14.4	23
200:26:10	39	25	23.6	43.9	31	30	18	63
200:26:10	24	16	16.7	51.2	15	35	16.9	66
200:26:10	49	36	13	37.9	55	31	18.4	74

Table 10. Mean number of PCN eggs/ g soil ± standard error (SE) and range for each laboratory (n=3). Values are provided for the samples which were spiked with (a) 50 or (b) 200 PCN cysts per kg of soil.

	(a)			(b)		
	Mean	+/- SE	Range	Mean	+/- SE	Range
Mineral soil	**PCN eggs/ g soil**			**PCN eggs/g soil**		
Lab 1	13.0	1.53	11- 6	37.0	12.01	13-50
Lab 2	14.0	2.65	9-14	45.0	5.03	39-45
Lab 3	7.2	1.30	5-7	22.3	2.08	18-22
Lab 4	18.1	4.64	11-27	35.1	2.96	32-41
Lab 5	11.0	1.53	8-13	40.7	7.84	28-55
Lab 6	13.0	1.73	10-16	48.0	6.56	35-56
Lab 7	10.2	2.04	6-13	35.2	7.09	22-47
Lab 8	15.1	2.08	16-23	67.3	3.71	60-72
Organic soil	**PCN eggs/ g soil**			**PCN eggs/g soil**		
Lab 1	16.0	3.46	10-16	37.3	7.27	24-49
Lab 2	6.7	2.40	2-10	25.7	5.78	16-36
Lab 3	7.9	2.46	3-11	17.8	3.09	13-24
Lab 4	13.5	1.02	12-15	44.3	3.85	38-51
Lab 5	8.7	1.76	6-12	33.7	11.62	15-55
Lab 6	9.0	1.53	7-12	32.0	1.53	30-35
Lab 7	15.1	3.02	10-21	17.8	0.45	17-18
Lab 8	20.3	2.19	16-23	67.7	3.28	63-74

4.3. Objective 3: to test the ability of the laboratory to distinguish between cysts of *G. pallida* and *G. rostochiensis*.

The results of PCR analysis to distinguish between cysts of *G. pallida* and *G. rostochiensis* are shown in Table 11. Each of six samples sent to each laboratory contained 50 cysts of various proportions of white and yellow PCN. The proportions of each species ranged from 0-100%. Whilst it was straightforward to prepare 50 cyst samples containing different numbers of cysts of each species this did not necessarily correspond to equivalent proportions of DNA as the egg numbers within cysts are variable both within and between species. Consequently it would not be expected that the results of PCR extraction would provide precisely the same proportions of each species as would be predicted from the mix of cysts. The accuracy of PCR analysis should therefore be judged on whether the results follow similar trends to those predicted by the relative proportions of cysts from each PCN species.

Lab 3 did not give the proportions of each species just its presence or absence. They were able to correctly detect the presence/absence of *G. rostochiensis* in all samples but were unable to confirm the absence of *G pallida* in sample 1.

Data from Lab 4 generally followed the trends in the relative proportions of *G. pallida* and *G. rostochiensis* in the original samples and they correctly detected the proportion of *G. rostochiensis* in sample 6 and the correct proportion of *G. pallida* in samples 5 and 6. However, they were unable to confirm that *G pallida* was absent from sample 1. Lab 7 detected the correct proportion of both species in sample 5 and the trends in their data were similar to that in the original samples although they detected levels of both species of PCN when they were not present. Lab 6 were able to detect the presence/absence of *G. rostochiensis* in all samples but also detected *G. pallida* in sample 1.

4.4. Additional information.

The additional information collected from all the labs on cyst extraction and egg counting are summarised in Table 1

Table 11. Results of PCR analysis to distinguish between cysts of *G pallida* and *G rostochiensis.* Proportion of each species is given as a percentage unless otherwise indicated

	Original samples		Lab 3		Lab 4	
	G. rostochiensis	*G. pallida*	*G. rostochiensis*	*G. pallida*	*G. rostochiensis*	*G. pallida*
Sample 1	100	0	Present	Present	60	40
Sample 2	80	20	Present	Present	50	50
Sample 3	60	40	Present	Present	40	60
Sample 4	40	60	Present	Present	30	70
Sample 5	20	80	Present	Present	20	80
Sample 6	0	100	Not present	Present	0	100

Lab 6		Lab 7	
G. rostochiensis	*G. pallida*	*G. rostochiensis*	*G. pallida*
86	14	80	20
74	26	60	40
48	52	50	50
35	65	50	50
17	83	20	80
0	100	10	90

Table 12. Details of extraction and egg counting methods used by each laboratory.

Laboratory	Amount of soil extracted	Extraction method	Egg count method	Number of egg counts made
Lab 1	100g	Fenwick can	Hawksley counting slide	3
Lab 2	100g	Fenwick can	Hawksley counting slide	2
Lab 3	500g	Fenwick can	Gridded slide	3
Lab 4	200ml	Sieving & flotation	Counting slide	20
Lab 5	200g	Fenwick can	Counting slide	3
Lab 6	Entire sample	Elutriation	Counting chamber	5
Lab 7	200g	Fenwick can	Sedgewick slide	3
Lab 8	200g	Fenwick can	Hawksley counting slide	3

The quantity of soil extracted by laboratories varied between 100 g and the entire sample. All laboratories use some form of counting slide to assess the number of eggs. Most (five laboratories) examine three replicate samples to determine a mean egg count although Lab 2 undertake two replicate counts and Lab 4 20 replicate counts.

5. DISCUSSION

5.1. Objective 1: to test the ability of each laboratory to distinguish PCN cysts

Laboratories were provided with five samples each containing 25 cysts. The prepared material contained a mixture of PCN cysts, lemon cysts and grass cyst nematode cysts. The results consisted of tabulated counts provided by the laboratory and labelled material which was checked to ensure that correct identifications had not been made by chance. Tabulated counts did not always total 25 cysts presumably as some were lost during examination. Also as some other material was dissected and examined microscopically to confirm identification of the cysts, not all the specimens were returned for checking. By comparing the tabulated counts and the results of the checked material it was possible to suggest where mistakes had been made in identification.

Where the identified material was returned for checking it showed that only three laboratories were able to identify PCN and lemon cysts with 100% accuracy. These were Labs 3, 4 and 6, respectively. The remaining laboratories were generally at least 80% accurate in their identification of PCN (range 81.1% to 87.8%). Lab 1, Lab 5 and Lab 7 were at least 86% accurate in their identification of lemon cysts but Lab 2 were only 71.7% accurate.

Where other material was returned for checking that from Labs 3, 4, 5 & 6, respectively was all correctly identified as grass cyst nematode. Both Lab 4 and Lab 6 were also 100% accurate in their tabulated counts of grass cyst nematode. Lab 3 failed to identify four grass cyst nematode cysts and Lab 5 misidentified 12 grass cyst nematode cysts. No other material was returned by Lab 1 or Lab 2 and these laboratories were unable to identify grass cyst nematode and considered them to be a mixture of PCN or lemon cysts. Results also suggest that Lab 7 had difficulty picking out grass cyst nematode cysts and appeared to identify them as PCN although no other material was returned for checking.

Under low power magnification (x 10) cysts of grass cyst nematode tend to be smaller than PCN, have a more oval shape, are of a grey/green colour and often have the neck turned down. If they are rolled carefully on a filter paper it is sometimes possible to see two openings one being the anus and the other the fenestra. In contrast PCN has a single opening. If the identification is unclear the cysts would need to be dissected and examined under high power magnification. In practice where grass cyst nematode are identified as PCN it is likely to have limited impact on the PCN egg count. Grass cyst nematode is relatively uncommon and only usually found following permanent grassland. Also eggs of this species are far less persistent than PCN and most tend to hatch each year as permanent grass provides a continual host. Therefore the number of eggs recovered from grass cyst nematode cysts are likely to be low. However, it is still important that any laboratory undertaking soil analysis for PCN should be able to identify anything that could be confused with the pest.

Microcysts can also be potentially confused with PCN but were not included in this proficiency test as a suitable supply of material could not be located. They do not contain any eggs and are very stiff and difficult to break open whereas PCN cysts have a degree of flexibility when prodded with a mounted needle. When microcysts do eventually break they tend to shatter and the wall of the cyst can be seen to be much thicker than in a PCN cyst. Even if picked off microcysts would not contribute to an egg count.

Misidentifying grass cyst nematode cysts or microcysts as PCN is unlikely to have a significant effect on the egg count and so will have no impact on deciding whether nematicide treatment is required. However, it will overestimate the cyst count. The number of eggs per cyst declines as a PCN population ages in the absence of a host crop. If grass cyst nematode or microcysts are identified as PCN it will tend to overestimate the age of the PCN population.

5.2. Objective 2: to test the ability of the laboratory to extract PCN cysts from soil

The ability to extract and identify PCN cysts from soil is crucial in arriving at a final egg count. If cysts are not extracted or not identified as PCN then this will influence the egg count and possibly management decisions. The samples of mineral and organic soil provided for the laboratories were inoculated with PCN

cysts, lemon cysts and grass cyst nematode cysts. Misidentification of grass cyst nematode cysts as PCN had little effect on the final egg count but failing to identify some PCN cysts or misidentifying lemon cysts as PCN could influence egg numbers.

All inoculated soil samples were about 1 kg in weight and all laboratories except Lab 6 sub-sample before extraction. The size of the sub-sample ranged from 100-500 g so in order to compare extraction efficiency across laboratories it was decided to estimate the number of cysts that a laboratory might be expected to recover in a 100 g sub-sample. It is unrealistic to expect that in a 1 kg sample of soil inoculated with 200 cysts that a laboratory would be able to consistently extract 20 cysts from a 100 g sub-sample even though the cysts were distributed as evenly as possible in the original sample. Therefore it was decided to compare laboratories in terms of whether the number of cysts extracted fell within an acceptable range. The acceptable range was determined by assuming that the data for number of cysts extracted (Objective 2) conforms to a Poisson distribution.

Lab 1 was within the acceptable range for 16 samples out of 18 (88.8%); Lab 4 and Lab 6 for 14 samples (77.7%); Lab 2 for 13 samples (72.2%); Lab 3 and Lab 5 for 11 samples (61.1%) and Lab 7 for eight samples (44.4%).

All labs generally recovered cysts best from mineral soil in comparison with organic soil. This is not surprising as extraction of organic samples results in a much greater volume of extracted material which has to be checked for the presence of cysts. Fortunately no more than about 3% of the UK potato crop is grown in organic soils (Denis Buckley, personal communication) and the majority of samples received by laboratories are from mineral soils.

The eight laboratories extracted various volumes of soil ranging from 100 g to the entire sample but there was insufficient data to draw any conclusions on the optimum volume. Also three different extraction methods were used. There was insufficient data to make any comparisons between extraction methods.

It was very difficult to make any meaningful comparisons between laboratories with regards to the egg counts. This is simply due to the inherent variation in egg numbers between PCN cysts. In this proficiency test, the laboratory generating the samples (Lab 8) extracted the most PCN eggs (Objective 2) and this was probably for a combination of reasons. The PCN cysts used to generate the seeded samples had been subjected to a heat killing procedure to comply with the Plant Health quarantine procedures for working with PCN. The technicians generating the samples observed that the method of opening the heat-treated cysts to extract the eggs had an impact on the count. If eggs were crushed with a mounted needle the egg count was lower than if individual cysts were opened with an ocular scalpel. If cysts were crushed a number of the resultant eggs/larvae appeared to be deformed. When cysts were opened with an ocular scalpel the procedure was much less violent than crushing and all eggs/larvae appeared to be viable. It is likely that most laboratories would crush the cysts rather than use a scalpel which would likely result in a lower

egg count. If laboratories failed to extract all the PCN cysts initially this would also result in a lower egg count.

If the egg counts reported by Lab 8 are discounted because of the method used to open the cysts (as discussed above) the mean number of eggs/g soil in mineral soil samples inoculated with five cysts/100 g soil ranged from 7.2-18.1/g soil and in those inoculated with 20 cysts/100 g soil from 22.3-48.0/g soil. In organic soil inoculated with five cysts/100 g soil egg numbers ranged from 6.7-16.0/g soil and where 20 cysts/100 g soil were inoculated the range was 17.8-44.3/g soil.

The Red Tractor Assurance Crop Module: Potatoes 2015 provides guidance on interpretation of PCN soil sampling results. It states that nematicide treatment is usually recommended for moderate or high (see Table 13 below) PCN infestations. For those in the low category nematicide treatment is not advised unless potatoes are grown on a close rotation; or potatoes are grown on very light soils; or a variety very susceptible to PCN attack is to be grown; or *Globodera pallida* is present. On this basis, the variation in egg counts (where 20 cysts /100g soil was inoculated) would not have resulted in different management decisions regarding nematicide use.

Table 13 ADAS categories for interpretation of PCN egg counts (Published 1999)[1]

Category	Advice
Not Found*	Nematicide treatment unnecessary. No action is required.
Very Low*	This is where only non-viable cysts are found. No action is required.
Low (1-10 eggs/g)	Use of a nematicide unlikely to be worthwhile unless cropping a close rotation (i.e. to limit nematode build-up) or cropping on very light soils (which may impose additional stress), or where a variety intolerant of nematode attack will be grown. The use of a PCN resistant variety, where appropriate, will limit nematode multiplication.
Moderate (11-60 eggs/g)	Nematicide treatment recommended. Where infestations exceed 40 eggs/g, growing a PCN-tolerant variety together with the use of a nematicide should be considered. The integration of nematicide and, where appropriate, a PCN-resistant variety will limit nematode multiplication.

[1] Potato Cyst Nematode: A management guide. Parker *at al* 1999.

High (>60 eggs/g)	Cropping with potatoes not advised in most situations. The use of a nematicide with a PCN-tolerant variety may give an acceptable yield, providing growing-season conditions are favourable and nematode levels do not exceed 80 eggs/g.

There were situations where PCN eggs were recorded where no PCN cysts had been inoculated into the soil. These results were primarily due to samples processed by two laboratories Lab 5 and Lab 7. The result from Lab 4 has since been explained as a labelling error as previously discussed (see Section 4.2.2). It seems likely that both Lab 5 and Lab 7 identified lemon cysts as PCN and used these to provide an egg count.

5.3. Objective 3: to test the ability of the laboratory to distinguish between cysts of *G. pallida* and *G. rostochiensis.*

Trends in the relative proportions of *G. pallida* and *G. rostochiensis* tended to follow those in the original samples for Lab 4, Lab 6 and Lab 7. Lab 3 only indicated the presence or absence of each species of PCN rather than their relative proportions. All of the labs detected *G. pallida* in sample 1 when none was present and Lab 7 detected *G. rostochiensis* in sample 6 when none was present. There could be a number of reasons for this. Firstly, it is possible that some cysts of *G. pallida* were present in the samples of *G. rostochiensis* and vice versa. To check this a microscopic examination of 10 dissected cysts from the stock of both species was done. Granek's ratio was estimated, this being the distance from the anus to the nearest edge of the fenestra divided by the length of the fenestra. Results confirmed that the cysts of both PCN species were as originally identified. Secondly, it is possible that the PCR analysis for *G. pallida* and *G. rostochiensis* is not sufficiently specific. It is uncertain whether all laboratories use the same primers/probes and if not this may suggest that assay specificity is not the issue. Thirdly, it is possible that the DNA samples could become contaminated during extraction or that the PCR has been contaminated. Given that three laboratories recorded some *G. pallida* when none was present this explanation seems unlikely.

In general, although results suggest that PCR analysis is able to detect differing proportions of *G. pallida* and *G. rostochiensis* further investigation of the anomalous results (where either species of PCN is detected but was considered to be absent from the original sample) is required. One possible approach would be to compare PCR analysis between laboratories using DNA of both species. These could then be mixed to prepare test samples in much the same way as was done using cysts. This method would take account of the inherent variability of egg numbers within cysts and the potential variability in levels of DNA. This was beyond the scope of the current study.

6. RECOMMENDATIONS

It is suggested that training is provided to address the misidentification of different cyst nematode species which is indicated by some of the laboratories' results.

The PCN proficiency test should be repeated at regular intervals to ensure the ability of laboratories to extract and identify PCN cysts is maintained so that farmers/agronomists can have confidence in the results they receive from all laboratories offering this service. It is understood that in other industries laboratories initiate and jointly fund industry wide proficiency tests and this model should be investigated further.

A more precise comparison of PCR analysis for speciation could be done by preparing a stock of DNA of both species from cyst samples certified as being entirely composed of *G. pallida* or *G. rostochiensis*. This method would take account of the inherent variability of egg numbers within cysts and the potential variability in levels of DNA.

7. APPENDIX. COPIES OF DATA SHEETS AND INFORMATION REQUESTED FROM PARTICIPATING LABORATORIES.

PCN Proficiency Test Results Sheet

Laboratory name: ……

Laboratory contact: Telephone: ……

Email: ……

Method used for PCN extraction: ……

Volume of soil extracted - mineral soil ……

- organic soil ……

Method used for egg counting: ……

Number of samples examined to provide egg counts: ……

Objective 1. Identification of PCN cysts

	Number of PCN cysts	Number of *Heterodera* cysts	Numbers of "other material"		
			Other 1	Other 2	Other 3
Replicate 1					
Replicate 2					
Replicate 3					
Replicate 4					
Replicate 5					

Comments :……

Objective 2. Extraction of PCN cysts from soil

	Number of cysts/100g soil	Number eggs/g soil
Mineral soil 1		
Mineral soil 2		
Mineral soil 3		
Mineral soil 4		
Mineral soil 5		
Mineral soil 6		
Mineral soil 7		
Mineral soil 8		
Mineral soil 9		

	Number of cysts/100 g soil	Number eggs/g soil
Organic soil 1		
Organic soil 2		
Organic soil 3		
Organic soil 4		
Organic soil 5		
Organic soil 6		
Organic soil 7		
Organic soil 8		
Organic soil 9		

Comments:……..

***Objective 2A- reporting of results according to soil volume**

	Number of cysts/litre soil	Number eggs/ml soil
Mineral soil 1		
Mineral soil 2		
Mineral soil 3		
Mineral soil 4		
Mineral soil 5		
Mineral soil 6		
Mineral soil 7		
Mineral soil 8		
Mineral soil 9		

	Number of cysts/litre soil	Number eggs/ml soil
Organic soil 1		
Organic soil 2		
Organic soil 3		
Organic soil 4		
Organic soil 5		
Organic soil 6		
Organic soil 7		
Organic soil 8		
Organic soil 9		

Comments……

Objective 3. PCN speciation

	% *G. pallida*	% *G. rostochiensis*
Spec 1		
Spec 2		
Spec 3		
Spec 4		
Spec 5		
Spec 6		

Comments……..

8. ACKNOWLEDGEMENTS

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