Canada and United States

Guidelines on Surveillance and Phytosanitary Actions for the Potato Cyst Nematodes *Globodera rostochiensis* and *Globodera pallida*

7 May 2014

Table of Contents

1. Introduction

The Canadian Food Inspection Agency (CFIA) and the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) are the National Plant Protection Organizations (NPPO) of their respective countries. These guidelines (hereafter referred to as "the Guidelines") on Surveillance and Phytosanitary Actions for *Globodera rostochiensis* and *Globodera pallida,* also referred to as Potato Cyst Nematodes (PCN), were developed by both organizations in consultation with stakeholders from both countries to:

- Outline the phytosanitary measures to be taken on the detection of PCN.
- Provide guidance to the NPPOs on long term management and/or release of fields in regulated areas.
- Establish the requirements for the movement of seed potatoes and other regulated articles between the two countries.

The Guidelines are intended to ensure predictable and equivalent science-based phytosanitary actions in both countries. The establishment of risk-based regulatory controls and processes for the movement of regulated articles is intended to prevent the spread of PCN.

The Guidelines were developed in accordance with the principles of the International Plant Protection Convention (IPPC) and the World Trade Organization Agreement on the Application of Sanitary and Phytosanitary Measures. The Guidelines also take into account recommendations of an Independent International Science Panel consisting of scientists with direct knowledge and experience with PCN and the recommendations of potato industry stakeholders including the National Potato Council and the Canadian Horticultural Council – Canadian Potato Council, as well as the views of the U.S. National Plant Board. Relevant terms are defined by the IPPC unless otherwise noted in Appendix 1.

2. Rationale for phytosanitary actions

Potato Cyst Nematodes are considered quarantine pests for Canada and the United States. Existing evidence indicates that these pests are not widely distributed in Canada and the United States. Where present in the United States and Canada they are of limited distribution and under official control.

PCNs are long-lived soil-borne pests that are difficult to detect at very low populations with cysts containing viable eggs known to survive decades in the soil. Once PCN is present, there is no quick, economical and effective treatment. Control of PCN is difficult and requires integrated approaches that utilize phytosanitary measures including ongoing surveillance and testing, treatment (nematicides), and the adoption of cultural practices (e.g. crop rotation, use of resistant varieties, trap cropping, and host avoidance).

The movement of seed potatoes and other articles with soil presents the highest risk for the movement of these pests. Appropriate surveillance and regulatory controls are essential to minimize the risk of PCN spread.

3. Soil sampling and laboratory analysis procedures

CFIA and USDA-APHIS officials have agreed on harmonized soil sampling and laboratory analysis procedures in keeping with internationally recognized standards. All soil samples are to be officially collected and submitted to and processed by NPPO-recognized laboratories.

When applicable, the NPPO will provide the procedures used for PCN delimiting and detection surveys to stakeholders and co- operators.

4. Phytosanitary measures

In response to a new PCN detection, the procedures in Appendix 5 must be used to confirm a PCN infestation within a field where cysts were detected. The NPPO will also initiate immediate phytosanitary measures to prevent potential PCN spread to non-infested areas. These actions will include the steps below:

4.1 Fields where PCN infestation is confirmed

1. Restrict movement of regulated articles (Section 5) from the infested field .
2. NPPO and cooperators will investigate any historical movement of the regulated articles that may have been associated with the infested field in order to identify potentially exposed fields.
3. Restrict movement of regulated articles (Section 5) from all fields identified as adjacent or exposed to the infested field.
4. The adjacent, exposed and infested fields will make up the initial regulated area and will be subject to all sampling and regulatory controls outlined in Section 9.
5. If the PCN infested field was used for seed potato production, trace forward information must be collected for the seed lots produced on the infested field. The fields planted with seed potatoes originating from the last potato crop grown on the infested field must be part of the regulated area as exposed fields.
6. Fields used as seed sources for the infested field will be prioritized for surveys but are not necessarily included as part of the regulated area. Seed potato movement from these fields is restricted until the surveys of these individual fields have been completed.
7. Any deregulation of the initial regulated area should occur as outlined in Section 9.

4.2 Fields undergoing confirmation of PCN infestation

In some cases, fields where PCN may have been detected may not immediately be considered as infested with PCN (Appendix 5). Fields undergoing confirmation of PCN infestation are considered suspect fields and should be treated as follows:

1. Restrict movement of regulated articles (Section 5) from the suspect field where the sample was collected.
2. Initiate investigations of any historical movement of the regulated articles that may have been associated with the suspect field in order to identify potentially exposed fields.
3. If a suspect field cannot be confirmed as infested with PCN after following all of the procedures outlined in Appendix 5, all phytosanitary measures are removed.
4. If the suspect field is confirmed as PCN positive, that field as well as adjacent and exposed fields will be regulated and will be subject to all sampling and regulatory controls outlined in Section 9.

While the investigation described above is being conducted, the PCN phytosanitary certification requirements for seed potatoes traded between the two countries (Section 8) will provide the

necessary safeguards to permit the undisrupted trade of seed potatoes from fields outside of the regulated area.

5. Regulated articles

Regulated articles include, but are not limited to:

- Cysts of *Globodera rostochiensis* and *G. pallida*;
- Soil;
- PCN host crops; and
- Any other article that could result in the movement of soil or PCN.

Because the principal pathway of moving PCN is soil associated with equipment, potato tubers, root crops, nursery stock or other articles that move soil, it is important to regulate these articles to mitigate potential PCN spread. Equipment and regulated commodities can move from PCN-regulated areas only after they meet risk mitigation requirements outlined in Table 1, or under compliance agreements, as authorized by the respective NPPO. The NPPO, in collaboration with its respective partners, is responsible for implementing all necessary regulatory controls within a regulated area, for monitoring the effectiveness of such controls, and for ensuring compliance to minimize the possibility of spreading PCN.

To initiate regulatory controls and establish regulated areas, Canada will issue individual notices of restrictions, ministerial orders and/or regulations. The United States will use a combination of emergency action notification, state rules, and federal regulations to create a regulated area(s).

Table 1. Requirements for moving regulated articles from regulated areas, farm units or fields (including infested, exposed, and adjacent fields)

Articles	From a PCN-regulated area
Non-host nursery stock, bulbs, corms, rhizomes, tubers of ornamental plants, grass sod (field grown in soil)	▪ The movement of soil and related matter is prohibited, except as described in section 7. ▪ Must be washed free of soil and originate from a field found free of PCN based on a survey (Method A) conducted within the last 36 months, except as described in section 7. ▪ Plants for planting and propagation may be produced in soil-less growing media in an enclosed facility, or in containers in a PCN pest-free place of production, as described in section 7. ▪ Field-grown plants for planting and propagation may be produced in a PCN pest-free place of production, as described in section 7. ▪ Plants with soil must originate from outside the regulated areas and have been handled and grown in a manner to prevent PCN infestation, as described in section 7. ▪ Other requirements may apply.
Potatoes – not for planting (including processing and table stock)	▪ Potatoes should be grown under an ongoing NPPO-approved PCN management plan. ▪ Processing potatoes (e.g, chipping, dehydration, French fry) must be processed under regulatory control (compliance agreements) at an NPPO-approved processing facility. ▪ Potatoes destined for fresh consumption (i.e., table stock) must be washed, sprout-inhibited and commercially packed under regulatory control (compliance agreements) at an NPPO-approved facility. ▪ Government-issued movement certificates or permits are required to move both table stock and processing potatoes outside of the regulated area.
Potatoes – seed for planting and recertification	▪ Seed potatoes produced in the regulated area should be grown under an ongoing NPPO-approved PCN management plan, and they must not be planted outside of that regulated area.
Soybeans, peas, beans, hay, straw and plant litter	▪ Regulated articles must not be contaminated with soil.
Root crops (other than potatoes)	▪ Root crops should be grown only under an ongoing NPPO-approved PCN management plan. Root crops that are to be used for processing must be processed under regulatory control (compliance agreements) at an NPPO-approved processing facility. ▪ Root crops destined for fresh consumption must be washed and commercially packed under regulatory control (compliance agreements) at an NPPO-approved facility. ▪ Government-issued movement certificates are required to move root crops outside of the regulated area.
Farm equipment, farm tools, used containers and any other equipment or conveyances that may carry soil.	▪ Must be cleaned free of soil or disinfested, as required by the NPPO, and accompanied by a movement certificate prior to leaving the regulated area.

Note: There are no specific requirements for articles from outside of a PCN regulated area, however, other requirements may apply.

6. National PCN detection survey

Canada and the United States will survey a portion of the annual seed potato acreage in each country. Land used for seed potato production, including land owned by universities, government or other research organizations, should be surveyed as a part of any survey efforts. The recommended survey rate is Method B (Appendix 2).

7. Pest-free places of production or pest-free production sites within regulated areas

Pest-free places of production (PFPP) and pest-free production sites (PFPS), as described in ISPM No. 10, are allowed within regulated areas, on a case by case basis, provided that they maintain a compliance agreement with the applicable NPPO.

7.1. Plants for planting and propagation produced in an enclosed facility, or in containers, including mini-tubers and potato plantlets

PFPS in enclosed facilities may be established in the regulated area provided that the criteria below are met:

- PCN detection survey and testing has been conducted and found negative using minimum of Method A (Appendix 2) if soil is present in the facility;
- Production practices prevent entry of soil from the surrounding fields into the facility;
- Soil-less growing media is used;
- Water used is filtered, treated or from a cased and capped well;
- Shipping/receiving, parking and other areas are constructed and maintained in a manner that prevents contact with soil;
- A 4.6 m (5 yd.) perimeter around the site is maintained free of PCN hosts;
- Facility floors are constructed to provide separation from the underlying soil; and
- Equipment is rendered free of soil prior to entry into the facility and when moved outside of the regulated area.

7.2. Field-grown plants for planting and propagation

PFPP producing field-grown plants may be established in the regulated area, except in infested fields, provided the criteria below are met:

- There is no history of host crop production within the last 10 years;
- PCN detection survey & testing has been conducted and found negative (using Method A);
- Ongoing PCN detection survey may be conducted every 36 months (using Method B); and
- Minimum 4.6 m (5 yd.) buffer around the site is maintained free of PCN hosts.

8. Phytosanitary certification of seed potatoes

To be eligible for phytosanitary export certification, fields used to produce seed potatoes traded between Canada and the United States must meet the following conditions:

- The field from which the seed potato shipment originates must be sampled following the production of a potato crop at a minimum rate of Method B and test negative; or

- A field that has been surveyed two times at a minimum rate of Method B and found negative for PCN would be exempt from further PCN surveys for the following three potato crops. Following the exempt period, the field would be subject to an additional survey at a minimum rate of Method B, and if found negative, that field would again be exempt for an additional three potato crops. (Note: Surveys conducted at a minimum rate of Method B since 2009 count toward the two survey requirement.)

These fields must have never had a PCN detection or be under the regulatory controls described in Sections 4 and 9. PCN survey and laboratory testing of the field(s) used to produce the seed potatoes identified for export to the other country must be completed prior to the issuance of any phytosanitary certificate for the seed potato shipments from that field(s).

Shipments of seed potato samples consisting of 500 tubers or less for trials or research purposes are exempt from these requirements, provided the field has been previously surveyed and found negative for PCN.

The following additional declaration will appear on phytosanitary certificates associated with commercial shipments of seed potatoes traded between Canada and the United States:

“Field(s) used to produce the seed potatoes in this shipment were surveyed and tested according to

the current PCN guidelines, and potato cyst nematodes (*Globodera rostochiensis* or *Globodera pallida*) were not detected."

The following additional declaration will appear on phytosanitary certificates associated with shipments of regulated articles produced in a PFPP or PFPS traded between Canada and the United States (Section 7):

"The articles in this shipment were grown in a PCN-free place of production or pest-free production site and in a manner to prevent infestation by potato cyst nematodes (*Globodera rostochiensis* and *Globodera pallida*)."

9. Releasing land from regulatory control

This section describes a step-wise reduction of phytosanitary measures that can lead to the deregulation of all regulated fields. Procedures for sampling and release of suspect fields are described in Appendix 5. Fields under regulatory control must be managed in compliance with the applicable phytosanitary measures as described in these guidelines.

9.1 Regulated non-agricultural land

There are a number of regulated fields in both Canada and the United States that have been converted to non-agricultural uses. Non-agricultural land includes but is not limited to, highways or other paved roads, paved parking lots, industrial parks, other commercial developments (such as shopping malls, apartment housing, and office complexes), residential developments, state or national parks, other recreational areas, racetracks, golf courses etc. All regulated land in this category may be released under these criteria if it meets the following criteria:

1. Records must be available to determine that the land has been out of agricultural production for the last 20 years and will not return to agriculture; or,
2. Construction for non-agricultural purposes has rendered the land non-tillable and is not likely to ever return to agricultural production.

9.2 Regulated agricultural land no longer in host crop production.

There are some fields in Canada and the United States that are regulated and where agriculture does still occur but where all host crop production was prohibited or has ceased for a minimum of 30 years. This could include formerly infested, adjacent or exposed fields. During this time, the fields may have been used for various purposes, including but not limited to hobby farms, fallow fields, forage crops, grain fields, nurseries, pasture, riding academies, sod farms etc. All regulated land in this category may be released if it meets all of the following criteria (except formerly infested fields, which may never be used for seed potato production):

1. Records must be available to determine that land has been out of host crop production for the last 30 years.
2. The field is surveyed at a minimum of Method A.
3. If PCN cysts are found, a viability test must be performed on these cysts.
4. If no PCN cysts are found or no viable larvae or eggs are detected after viability assay, then the field can be released from regulatory control.
5. If host crops are grown after regulatory changes are made, continued surveillance is strongly suggested.

9.3 Adjacent and exposed fields used for host crop production

Adjacent and exposed fields are subject to regulatory measures due to their association with infested fields and the consequent risk they pose for soil-borne spread of PCN. Host crops may be grown in the field as per Section 5. Processing or fresh market potatoes may be grown

on adjacent and exposed fields only for non-seed purposes under regulatory control (i.e., compliance agreements or equivalent). Potatoes may be grown for seed purposes under regulatory control (i.e., compliance agreements or equivalent); however, seed potatoes harvested from adjacent and exposed fields may be used only within that regulated area. Exposed fields are eligible for the lifting of all regulatory controls when conditions 1 and 3, listed below, are met. Adjacent fields, however, are eligible for lifting of all regulatory controls when all of the following conditions are met:

1. **Negative surveys.** In order to proceed to steps 2 and 3, negative test results must be obtained from one survey using Method A or two surveys using Method B following host crop production. Historical survey data can be used if available and the survey method used is at least comparable to 6000 cc (15 lb) per acre and if the surveys were conducted after the original exposure event occurred.
2. **Removal of equipment-cleaning requirement.** Provided the above surveys are negative, and on a case-by-case evaluation, equipment-cleaning requirements may be removed.
3. **Additional surveillance.** Following a susceptible host plant crop, conduct one additional survey using Method A. If this survey is negative then all regulatory controls may be lifted on an exposed field.
4. **Adjacent fields.** The lifting of all regulatory controls on adjacent fields may occur only following negative bioassay results from the corresponding infested field.

9.4 Infested fields used for host crop production

Infested fields to be used for host plant production are subject to the most stringent phytosanitary measures due to the high risk of soil-borne PCN spread. Potatoes may only be grown under an NPPO approved management plan, unless potatoes are being planted as part of a bioassay.

1. **Negative viability assay.** Fields must be surveyed using the Viability Assay Survey (Appendix 2) and viable PCN must not be detected as per the PCN viability assay protocol (Appendix 3).
2. **Negative bioassay**. After a negative viability assay is completed a bioassay must be conducted based on a process identified in Appendix 4.
3. **Release from equipment cleaning requirement**. If the Bioassay is negative, and on a case-by-case evaluation, equipment-cleaning requirements may be removed and host crops may be grown in the field, as per Section 5.
4. **Continued monitoring or in-field bioassay**. Conduct three additional full field surveys using viability assay method. Each survey must be conducted after the harvest of a susceptible host crop.
5. **Further release from regulatory control** If no viable cysts are detected, the field can be released from most regulatory controls except that the field remains restricted for seed potato production.

10. Review and amendment

A review of these guidelines should be undertaken at the request of either NPPO, or considered at regular intervals of two years from the date of endorsement. Such requests for review will be handled without undue delay. Relevant stakeholders, including the National Potato Council and the Canadian Horticultural Council – Canadian Potato Council, as well as the U.S. National Plant Board, will be consulted in this review process.

While the NPPOs may discuss proposed amendments to the guidelines, any such amendments will not be applicable until mutual consent is obtained in writing and signed by authorized representatives of both NPPOs.

Reviews of the programs within Canada and the United States will be conducted jointly between both NPPOs on a rotating basis and a mutually agreed schedule. The results of these reviews will be used to improve these guidelines as needed.

11. Duration of guidelines

These guidelines will be implemented and applied immediately after the date of signature by authorized representatives of both NPPOs and will remain in effect unless they are terminated under one of the following conditions:

- An NPPO has the right to terminate these guidelines at its sole discretion at any time after giving 60 days written notice to the other NPPO.
- The guidelines may be terminated by mutual consent as of a date approved in writing by both NPPOs and as confirmed by the signatures of their authorized representatives.

12. Reporting

To facilitate implementation of these guidelines and ensure timely communication of activities being undertaken, the NPPOs agree to provide reports, including national survey data, on at least an annual basis to stakeholders of both countries. Each individual NPPO is at liberty to communicate with stakeholders regarding issues pertaining to the guidelines within their country.

In addition to reporting on these regulatory guidelines, both CFIA and USDA-APHIS are committed to keeping their respective stakeholders informed about science-based risk mitigation approaches to prevent the spread of PCN.

13. Non-compliance and dispute resolution

In the event of non-compliance with a requirement specified in these guidelines, or regarding the interpretation or implementation, the NPPOs agree to discuss the matter for mutual and prompt resolution. If bilateral discussions between the NPPOs are not able to resolve the dispute the NPPOs will jointly select a facilitator for continued discussions. If the dispute still cannot be resolved, either NPPO may, at its sole discretion, terminate these guidelines immediately or as described above.

14. Acknowledgments & endorsement

The CFIA and APHIS hereby acknowledge that the present version of the Guidelines represented herein by this document is acceptable to their respective NPPOs.

The CFIA and APHIS also hereby acknowledge and accept that the present version of the Guidelines revokes and replaces the previously signed version of the Guidelines, (June 2009). In addition, the respective NPPOs recognize as acceptable all the soil sampling, testing and regulatory activities taken under previous versions of the Guidelines.

The CFIA and APHIS will share information regarding any new PCN detections in their respective countries in a timely manner.

The Guidelines have been executed by the authorized representatives of the NPPOs in duplicate copies.

/ Original Signed Copy on File /

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Greg Wolff
Chief Plant Health Officer
Director—Plant Biosecurity and Forestry Division
Canadian Food Inspection Agency

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Appendix 1 - Definitions

Some of the terms below are defined in the IPPC's *Glossary of Phytosanitary Terms* (ISPM No. 5).

Adjacent field	A field or tract of agricultural land within 13.7 m/15 yards of an infested field.
Certified seed potatoes	Potato tubers officially accepted and classified as propagative material through a seed potato certification program recognized by the Potato Association of America.
Delimiting survey	A survey conducted to establish the boundaries of an area considered to be infested by or free from a pest (IPPC, 2007).
Detection survey	A survey conducted in an area to determine if pests are present (IPPC, 2007)
Field	A plot of land with defined boundaries within a place of production on which a commodity is grown (IPPC, 2007).
Infested field	A field in which *Globodera rostochiensis* or *Globodera pallid*a has been confirmed.
Non-exposed field	A field determined not to be associated with a PCN-infested field.
Exposed field	A field where equipment moved after use in an infested field, or where soil from an infested field was transported, or that received propagative host material from an infested field.
Regulated area	An area into which, within which and/or from which plants, plant products and other regulated articles are subjected to phytosanitary regulations or procedures to prevent the introduction and/or spread of quarantine pests or to limit the economic impact of regulated non-quarantine pests (IPPC, 2007).
Suspect field	A field in which one or more cysts consistent with PCN have been detected but where definitive confirmation of a *Globodera rostochiensis* or *Globodera pallida* infestation has not yet been made as per Appendix 5.
Trace back field	A field that provided seed potatoes to an infested field.
Trace forward field	A field that received seed from an infested field.

Appendix 2 - Potato Cyst Nematodes field soil sampling requirements

Standard Survey Requirements

Notes: 1. All soil samples must be tested in their entirety.
2. 2,000 cc of soil is considered to be one sample of approximately 5 lbs.

Method A:

- Sample the entire field in a fixed grid pattern.
- Minimum of 20,000 cc soil/ha (20 lb or 8,000 cc soil /acre).
- Minimum of 1,000 sampling points/ha (400 points/acre).
- Maximum grid cell size of approximately 18 m^2 (21.5 yd^2).
- For hand sampling, the length of a grid cell should not be greater than 2.5 times the width.
- For rectangular-shaped grid cells, the longest dimension should be parallel to the direction of cultivation.

Method B:

- Sample the entire field in a fixed grid pattern.
- Minimum of 5,000 cc soil/ha (5 lb or 2,000 cc soil /acre).
- Minimum of 400 sampling points/ha (160 points/acre).
- Maximum grid cell size of approximately 30 m^2 (36 yd^2).
- For hand sampling, the length of a grid cell should not be greater than 2.5 times the width.
- For rectangular-shaped grid cells, the longest dimension should be parallel to the direction of cultivation.

Viability Assay Survey:

- Sample the foci of the infested field using a fix grid pattern. If the foci have not been identified, sample the entire field.
- Minimum of 45,000 cc soil/ha (45 lb or 18,000 cc soil/acre).
- Maximum grid cell size of approximately 5 m^2 (6 yd^2).
- Sampling should be accomplished with a soil probe to a minimum depth of 25 cm (10 in.).
- For hand sampling, the length of a grid cell should not be greater than 2.5 times the width.
- For rectangular shaped grid cells the longest dimension should be parallel to the direction of cultivation.

Appendix 3 - Potato Cyst Nematode Viability Assay Protocol

The following viability assay is provided here for guidance. Other viability protocols can also be used if scientifically valid.

Meldola's Blue Viability Assay Protocol

These procedures describe a protocol for evaluating the viability of eggs/juveniles in cysts of *Globodera pallida* (also known as pale cyst nematode or PCN). Cyst(s) are soaked in a stain that is absorbed by dead eggs/juveniles and is not absorbed by viable eggs/juveniles.

I. Cyst Storage

Cysts recovered from field samples should be stored at room temperature in a secure location prior to viability analysis.

II. Cyst hydration

A. Randomly extract 400 cysts from a field sample or composited field sample. Place cysts in 1" square (inside measurement) mesh bag (Figure 1) and seal bag with heat sealer. Place mesh bag containing cysts in a clean 20 ml screw-top vial. If fewer than 400 cysts are available, use as many cysts as possible from a sample or composited sample.
B. Hydrate the cysts by adding distilled water to the shoulder of the 20 ml vial (Figure 2). Replace lid and tighten.
C. Allow cysts to hydrate at room temperature for at least 24 hours, but for no longer than 72 hours.

III. Cyst staining

A. Remove 10 ml of distilled water from the sample vial containing the hydrated cysts.
B. Add 10 ml 0.1% (w/v) Meldola's blue stain solution to the vial containing the remaining 10 ml of distilled water and hydrated cysts to bring the final concentration of Meldola's Blue to 0.05%.
C. Replace vial lid and store at room temperature for at least 48 hours, but for no longer than 7 days.

IV. Reading procedure

A. Stack a 200 mesh sieve on top of a 500 mesh sieve and prime them by running tap water through them. Place the stacked sieves onto a 400 ml tri-corner beaker.
B. Transfer the stained cyst packet onto the top of the 200 mesh sieve
C. Thoroughly rinse stain off of the packet and cysts using a wash bottle filled with distilled water.
D. Crush the cysts by gently pressing the tip of a rubber policeman or a pestle to the cyst packet on the top sieve. Take care to not over-crush the cysts, as this will break open the eggs and pulverize the juveniles.
E. Rinse the cyst packet to ensure that all eggs and juveniles are rinsed through packet and the top (200 mesh) sieve and into the bottom (500 mesh) sieve. Take care to avoid overflowing the tri-corner beaker with rinsate.
    i. After the initial rinse, open the packet and prop a plastic slide cover inside the packet to hold it open and rinse again to ensure all eggs and juveniles are removed from the packet.
    ii. Once all eggs and juveniles are thoroughly rinsed through the top (200 mesh) sieve, remove the top sieve and set aside.
    iii. Debris from the top sieve should be autoclaved and discarded.
F. Rinse eggs/juveniles to the edge of the 500 mesh sieve, and then into a 30 ml graduated conical cylinder. Add distilled water to the conical until a final volume of 5 or 10 ml is reached.
G. Using the fish tank aerator or a volumetric pipette, bubble the sample for at least 30 seconds to suspend eggs/juveniles in the sample.

H. Transfer 1 ml of the suspension from the graduated conical to a 1 ml counting microscope slide. Cover the slide with a cover slip.
I. Use Parafilm to cover the graduated cylinder containing the remaining egg/juvenile suspension. Label the cylinder with the sample ID and date. The remaining suspension may be stored in the refrigerator for up to 7 days.
J. Use the 40x objective of a compound microscope to count the number of stained (purple) and non-stained (pale amber) eggs/juveniles (Figure 3). Use a dual hand counter to record counts in this step.
    i. Do not count empty eggs or juveniles that are less than ½ of a full-size juvenile.
    ii. Count the stained and non-stained eggs/juveniles until a minimum count of 1,000 eggs/juveniles on a single slide is achieved.
    iii. Readers should read a slide in the same direction and from the same starting point to ensure that the same area of a slide is read by each reader.
K. If a count of 1,000 eggs/juveniles is not achieved using the first 1 ml aliquot of egg/juvenile suspension, prepare additional slides as needed from the remaining sample suspension by repeating steps I through K.

V. Controls

A. Each day that field samples are analyzed for viability, both positive and negative control samples should also be read.
    i. Freshly reared *Globodera pallida* or *G. tabacum* cysts are suitable for use as controls.
    ii. Positive and negative controls should be prepared and analyzed separately. At least 100 eggs/juveniles should be counted from each slide preparation of positive and negative controls. To achieve these counts, at least 4 cysts should be used in each preparation of positive and negative controls.
B. Preparation of positive (live) controls:
    i. Positive controls are to be prepared in the same manner as field-collected viability samples.
C. Preparation of negative (killed) controls:
    i. Negative control cysts are heat-killed by autoclaving for one hour at 121^0C.

VI. References for Meldola's Blue viability staining assay

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Twomey, U., Warrior, P., Kerry, B. R., and R. N. Perry. 2000. Effects of the biological nematicide DiTerra®, on hatching of *Globodera rostochiensis* and *G. pallida*. Nematology **2(3):**355-362.

Figure 1. Cyst packet. 1" square (inside measurement) mesh bag made from nylon, woven thermoplastic mesh, micron rating 430 um, rectangular size 0.169"; McMaster-Carr #I 029 M SEFAR NITREX 06-250/3 4

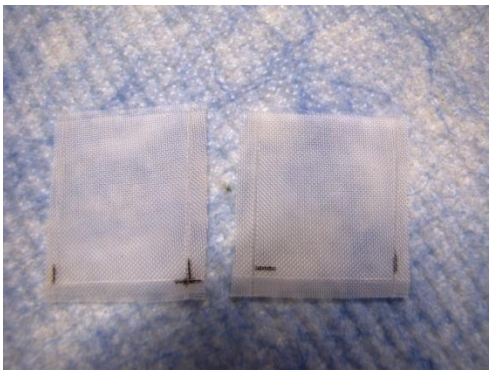
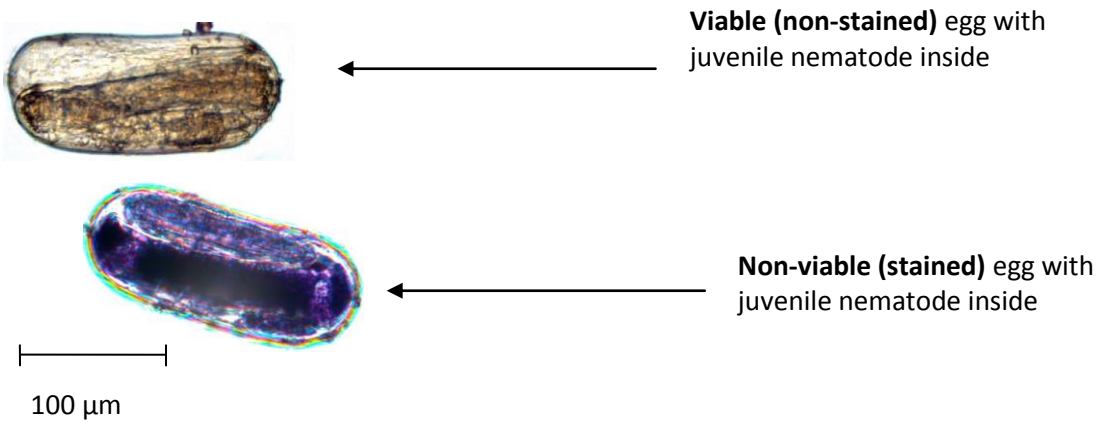


Figure 2. 20 ml vial with water and cysts at sample hydration step

Figure 3. Differential staining of viable and non-viable eggs/juveniles of *Globodera pallida*



Appendix 4 - Potato Cyst Nematode Bioassay

Purpose:

The soil bioassay will be conducted following a negative viability assay (Appendix 3). During the time it takes to complete the bioassay procedure, the normal activities that occur in the regulated fields will continue, including sanitation procedures.

Care must be taken during the bioassay procedure to ensure that the possibility of releasing live PCN cysts or infective juveniles is minimized. Regulatory requirements must be strictly adhered to pertaining to containment, permit and compliance agreement conditions.

Procedure:

Materials required:

- 80 mm by 80 mm nylon mesh muslin "tea" bags
- 1 gallon (min) pots
- 1:3 sterile soil: sterile sand mixture
- Growth facilities with required biocontainment and capable of maintaining the required controlled conditions for the bioassay.

Sample the mapped PCN foci in the field at the viability assay sampling rate (Appendix 2). Extract the organic fraction of the soil using a Fenwick can, USDA cyst extractors or other appropriate device. The appropriate organic fraction or flotsam, which contains the PCN cysts and other associated organic matter, is collected in a 250 ml beaker lined with an 80 mm by 80 mm nylon muslin "tea" bag containing a minimum of 20 cysts per pot, when available. Heat seal each ~20 ml of organic fraction in an individual nylon mesh tea bag.

Place up to 4 muslin bags in an appropriate pot with a mixture of 1 part sterile loam soil and 3 parts sterile sand. This volume should be suitable for growing a potato plant to maturity. Up to 4 bags should be placed in each until all bags have been placed in a pot. A potato tuber should be placed in the center of the pot near the bags.

Grow susceptible potato plant to at least 120 days from planting. Any weeds growing in the pots should be promptly removed. Temperatures in the greenhouse or growth chambers during growth periods must not exceed 24^{o}C. Once the potato plant has grown to maturity, the plant must be carefully removed from the soil sand mixture. Examine all visible roots for the presence of cysts. If necessary, the roots should be washed and the water should be drained into the original pot. If PCN cysts are detected on the roots, the bioassay is considered positive and the fields from which the organic fraction in the muslin bag originated will remain under regulation. If PCN cysts are not detected, the bioassay may immediately continue for up to an additional 2 cycles (no diapause is needed if the fields undergoing bioassay have been out of host production for a minimum of 5 years). Examine the tea bags for deterioration after plant removal and replace as appropriate.

Repeat the bioassay for two more cycles of growth as described above. Once the final potato plant has grown to maturity, the plant must be carefully removed from the soil sand mixture and the roots and soil and sand mixture examined for cysts. If necessary the roots should be washed. The cysts in the muslin bags must also be examined for viability using the methods described in Appendix 3. Additionally, extract the soil and examine the organic fraction for the presence of cysts. If PCN cysts are detected, the bioassay is considered positive.

A bioassay may also be conducted in the infested fields. The fields must have been surveyed at a minimum of the viability assay survey and any and all cysts detected must be tested for viability using either the staining method described in Appendix 3 or viability must be determined by a trained nematologist. If all of the cysts detected are determined to be non-viable, then a susceptible host crop may be planted on the field in each PCN infestation foci and a minimum of 15ft (4.6 m) buffer completely around each focus within a field. If the foci within the field are not known, the full field must be planted with susceptible hosts. After the susceptible host crop is grown to maturity, it may be harvested and moved

under compliance agreement (or other appropriate regulatory tool). After harvest is completed, the field must be surveyed in the areas where the susceptible crops were planted at the viability assay survey rate (45000cc per ha, 18,000cc per acre to a depth of 10 inches/25cm). Any cysts found must be assayed using an appropriate viability testing method.

The planting of a susceptible host must occur each of the next two growing seasons, followed by harvest and viability assay surveys and viability testing each time to any detected cysts. If all viability results are negative, then the bioassay is considered to be negative and the stepwise process outlined in Section 9 may continue.

Appendix 5 - Confirmatory Policy for Suspect Potato Cyst Nematode (PCN) Infestations

Introduction:

This policy is specific to PCN and is based on knowledge about the biology and epidemiology of the organism.

Specimens must be identified and confirmed by an NPPO or NPPO-approved laboratory using definitive morphological/morphometric and molecular identification techniques, including those specimens originating from a non-NPPO or non-NPPO-approved laboratory. If the pest is confirmed, regulatory action may result.

Subsequent samples from a field with at least one confirmed positive sample do not require confirmatory testing. If the suspect sample is not an official sample, the collection of an official sample may be required.

Morphological and Molecular PCN Confirmation:

Complete, definitive identification of *G. pallida* or *G. rostochiensis* is a multi-step process, as follows:

1. Verify that the sample contains suspect *Globodera* spp. or other cyst nematode genera (such as *Cactodera*).
2. Verify that the suspect cysts and/or any juvenile forms have key characters and are morphometrically within the range of the PCN species.
3. Verify that the suspect nematode tissue yields DNA identifiable as a PCN species. (As per PPQ CPHST-Beltsville work instructions posted at www.nahln.org and Skantar et al, 2007 at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2586493/ .)
4. Verify that the morphological and molecular analyses concur.

PCN Infested Field Confirmation:

For a field to be considered infested with PCN, the following criteria should be met:

- at least two cysts from two different soil samples with one of those cysts containing viable PCN eggs or juveniles.

If the above criteria are not met, a survey using Method A must be conducted as soon as possible. If no additional cysts are detected, a Viability Assay Survey must also be conducted following the next susceptible host crop. If no additional cysts are detected after these surveys then the regulatory controls can be removed.

Fields that do not meet the terms of this policy will not be considered as infested, however, continued monitoring of the fields would be prudent after any susceptible host crops.